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Weiss Nielsen , Martin ; Sternberg, Claus; Geschke, Oliver; Molin, Søren

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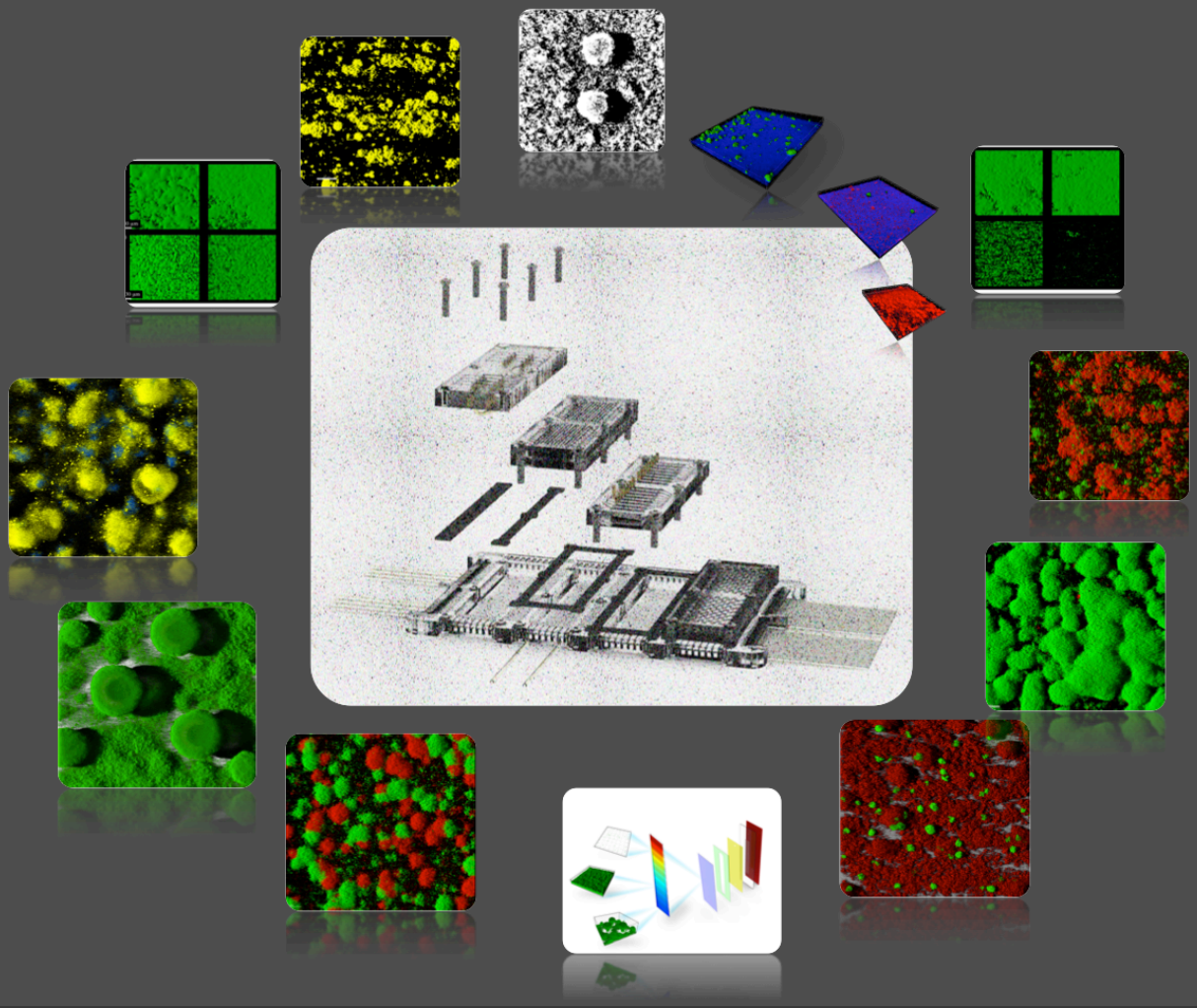

Next generation *in vitro* systems for biofilm studies - a cystic fibrosis patient airway perspective

Ph.D. Thesis

Martin Weiss Nielsen

2012

DTU
Department for Systems Biology
Department of Micro- and Nanotechnology

Next generation *In vitro* systems for biofilm studies – a cystic fibrosis patient perspective

Ph.D Thesis

by

Martin Weiss Nielsen

Department of Systems Biology

Technical University of Denmark

2012

To my family and my unborn daughter "Blobber"……

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Abstract

Bacterial infections have a large impact on the health of the general public, however individuals with cystic fibrosis (CF) are immensely susceptible to acquire pulmonary infections with environmental bacteria, viruses and fungal species. Ultimately pulmonary infections are the major cause of morbidity and mortality in CF patients. The genetic defect of CF patients alters the environment of the airways aiding *P. aeruginosa* to persist and establish chronic infections. Chronic *P. aeruginosa* infections in CF patients are characterized by conversion to a mucoid and intensive biofilm formation. Different microenvironments within the airways of the patients lead to phenotypic and genotypic diversification, sculpting the bacteria to become an efficient colonizer despite the environmental niche in the CF airways. Despite intensive antibiotic treatment and a profound immunologic response, the infections persist. The ongoing polymorphonuclear leukocyte (PMN) immune response contributes to the generation of oxygen changes in the CF airway environments in a major way. *P. aeruginosa* is capable of growing in anoxic environments which makes it capable of adapting to different niches of the airways, while the ongoing immune response gradually leads to pulmonary deterioration. Physiologically different oxygen environments are present in the different compartments of the CF airways and this has a profound impact on bacterial growth and the effect of chemotherapeutics to eradicate the bacteria.

Several *in vivo* and *in vitro* model systems are available to study CF associated bacterial infections. *In vivo* systems like the widely used mouse model primarily lack essential CF related traits as the development of the significant spontaneous lung disease. *In vitro* systems like the flow cell system has proven essential aspects on biofilm formations, however generates highly artificial biofilms that lack several CF airway scenarios.

The driving force and the heart of this project has its origin in the study of the role played by *P. aeruginosa* in the CF airways. One of the aims of this thesis was to develop an accurate tool for growing biofilms that can mimic the cystic fibrosis airways, emulating one of the most important characteristics of the

human airways, the oxygen environments. Microfluidic approaches that allow biofilm formation under controllable oxygen concentrations, and furthermore enable migration between the individual compartments, are proposed in this thesis. An approach to mimic the accumulation of thickened mucus that supports biofilm growth in a 3D matrix within the CF airways is furthermore proposed.

The use of our already set flow cell systems was extended to another biofilm forming *S. cerevisiae*. The potential positive impact that the flow cell system can have on studies with other fugal species as the opportunistic pathogens, *A. fumigatus* or *C. albicans*, is without a doubt highly relevant.

The presented microfluidic systems are in line with the thought of developing *in vitro* systems to obtain optimal conditions for CF research, replace animal models and reduce chemicals used.

Resume

Bakterielle infektioner kan have en stor indvirkning på helbredstilstanden for normalt raske mennesker, men personer med cystisk fibrose (CF) er utroligt modtagelige for at erhverve sig lungeinfektioner med bakterier fra de nære omgivelser, vira og svampearter. Pulmonære infektioner er den væsentligste årsag til morbiditet og dødsfald hos CF patienter. Den genetiske defekt hos CF-patienter medfører et ændret miljø i luftvejene og medvirker til, at *P. aeruginosa* vedbliver og etablerer kroniske infektioner. Kroniske *P. aeruginosa* infektioner hos CF-patienter er kendetegnet ved omdannelse til en mucoid fænotype og intensiv biofilmdannelse. Forskellige mikromiljøer i luftvejene hos patienterne fører til fænotypisk og genotypisk diversificering, modellerer bakterierne til effektivt at kunne kolonisere de forskellige nicher i CF luftvejene. Trods intensiv antibiotika behandling og kraftige immunologiske reaktioner, fortsætter de bakterielle infektioner. Det vedvarende polymorphonucleære neutrophile granulocyt (PMN) immunrespons bidrager til dannelsen af ændringer i luftvejenes oxygen miljøer hos CF patienter. *P. aeruginosa* er i stand til at vokse i iltfattige miljøer, der gør dem i stand til at tilpasse sig forskellige nicher i luftvejene, mens det igangværende immunrespons gradvist fører til pulmonær ødelæggelse. Fysiologiske forskelligheder i oxygen miljøerne er tilstede i forskellige dele af CF luftvejene, og har stor indflydelse på bakteriel vækst og effekt af den kemoterapeutiske behandling af infektionerne.

Adskillige *in vivo* og *in vitro* modelsystemer er tilgængelige for studier af CF relaterede bakterielle infektioner. *In vivo* systemer, som den bredt anvendte musemodel mangler primært essentielle CF relaterede træk, som udviklingen af spontan lungesygdom. *In vitro* systemer, som flowcelle systemet, har givet væsentlige informationer om aspekter af biofilm dannelse, men tillader kun dannelse af meget kunstige biofilm med mangel af adskillige CF luftvejs scenarier.

Hovedpunkterne i dette projekt er inspireret af luftvejene hos CF patienter og den rolle som *P. aeruginosa* har i respiratoriske infektioner. Et af formålene med denne afhandling var at udvikle et præcist redskab til dyrkning af

biofilm, som kan efterligne cystisk fibrose luftvejene og disses ændrede ilt miljøer. Mikrofluide tilgangsvinkler, der tillader biofilmformation under kontrollerbare iltkoncentrationer og endvidere tillader migration mellem de enkelte rum, er foreslået i denne afhandling. En tilgang til at efterligne akkumulering af fortykket slim, der understøtter biofilmvækst i en 3D matrix i CF luftvejene, er ydermere præsenteret.

Brugen af vores allerede etablerede flow cellesystem blev udvidet til biofilmdannelse med *S. cerevisiae*. Den potentielle positive effekt som flowcelle systemet kan have på andre studier med svampearter af opportunistiske patogener som f.eks. *A. fumigatus* eller *C. albicans*, er utvivlsomt af stor relevans.

De præsenterede mikrofluide systemer er i overensstemmelse med tanken om at udvikle *in vitro* systemer til at opnå optimale betingelser for CF forskning, erstatte dyremodeller og reducere mængden af benyttede kemikalier.

Preface

This thesis is written in order to fulfill one of the requirements for obtaining a Ph.D. degree at the Technical University of Denmark.
The work presented in the thesis was carried out at the Department of Systems Biology and Department of Micro- and Nanotechnology, under the supervision of Associate Professor Claus Sternberg and Professor Søren Molin.

I would like to thank Professor Jenny Emnéus, Associate Professor Martin Dufva, Associate Professor Oliver Geschke, Associate Professor Claus Sternberg and Professor Søren Molin for inputs and invaluable help during my Ph.D.

I would like to give my warmest and most profound thanks to Maciej Skolimowski, whom I have had the pleasure of working with during the project. I really cannot believe that we never suffered from dehydration during our hours and hours of testing systems in the 37-degree room. I will “copy-paste” you and thank you for your great friendship, help, and for your overbearance with my many crazy ideas.

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Inna Dashevsky, Maria Zweig, Carolin Stang, Sofia Feliziani, Mariló Macia, Adela Lujan, Anna Koza and not least my always positive laboratory Spanish teacher Cristina Amador Hierro…(Mariquita). A thanks to Lisser (the angel) Clair-Norton, Susanne (Søs) Koefoed and Ann Oxfeldt Olsen – you guys make it all work….

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I am extremely honored that I have had the pleasure of sharing an office with Dr. Anders Folkesson!!!, who has got an amazing drive and an incredible sense of humor. A "thank you" is also sent to the amazing Chinese people, Lei Yang, Yang Lui and the always cheerful Liang Yang, the weekends would have been quite lonely without you guys. I would furthermore like to thank Associate Professor Birgitte Regenberg for all your help and inspiration.

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I would also like to acknowledge Professor Alfred Spormann and all the great people in the Spormann Lab at Stanford University.

Finally I wish to thank my family for their never-ending help and support. Furthermore, a big "thank you" to the Arnaas for all their help.

To my Josefine, thank you for being there by my side. Thank you for your understanding when I once more was struggling with leaking systems and took the last bus…4 hours later than my last "but I just have to … and then I can go". Thank you for being you and for all your love and support.

List of publications

I. Martin Weiss Nielsen[1], Claus Sternberg, Søren Molin, Birgitte Regenberg: *Pseudomonas aeruginosa and Saccharomyces cerevisiae biofilm in flow cells.* J. Vis. Exp. 2011 Jan 15;(47) pii2883. Doi: 10.3791/2383 http://www.jove.com/Details.php?ID=2383

II. Skolimowski, M., Nielsen, M.W., Emnéus, J., Molin, S., Dufva, M., Taboryski, R., Sternberg, C., and Geschke, O., *Microfluidic biochip as a model of the airways of cystic fibrosis patients.* Proceedings of the Thirteenth International Conference on Miniaturized Systems for Chemistry and Life Sciences, p. 1091-1093, 2009.

III. Maciej Skolimowski[1], Martin Weiss Nielsen[1], Jenny Emnéus, Søren Molin, Rafael Taboryski, Martin Dufva, Claus Sternberg, Oliver Geschke: *Microfluidic dissolved oxygen gradient generator biochip as a useful tool in bacterial biofilm studies.* Lab Chip, June 2010. (DOI: 10.1039/c003558k)

IV. Skolimowski, M[1]., Nielsen, M.W[1]., Abeille, F., Skafte-Pedersen, P., Sabourin, D., Fercher, A., Papkovsky, D., Molin, S., Taboryski, R., Sternberg, C., Dufva, M., Geschke, O. and Emnéus, J, *Modular microfluidic system as a model of cystic fibrosis airways*. Submitted to Biomicrofluidics, May 2012

V. Skolimowski, Nielsen, M.W., M., Abeille, F., Lopacinska, J., D., Molin, S., Taboryski, R., Sternberg, C., Dufva, M., Geschke, O. and Emnéus, J., *Microfluidic model of cystic fibrosis bronchi.* Accepted for Proceedings of the Fifteenth International Conference on Miniaturized Systems for Chemistry and Life Sciences, 2011.

VI. Abeille, F., Skolimowski, M., Nielsen, M.W., Lopacinska, J., D., Molin, S., Taboryski, R., Sternberg, C., Dufva, M., Geschke, O. and Emnéus, J., Microfluidic system for *in vitro* study of bacterial infections in cystic fibrosis bronchi. Manuscript in preparation, 2012.

Not included in this thesis:

i. Nielsen, MW[1], Boding, L[1], Bonefeld, CM , von Essen, MR , Nielsen, BL , Lauritsen, JPH , Hansen, AK , Nielsen, MM, Kongsbak, M, Rubin, M , Vennegaard, MT , Ødum, N & Geisler, C. *Polymorphisms of the T cell receptor CD3delta and CD3epsilon chains affect anti-CD3 antibody binding and T cell activation*, Molecular Immunology , vol 47, nr. 15, s. 2450-7, 2010.

ii. Katarzyna Gurzawska, Rikke Svava, Susanne Syberg, Yu Yihua, Kenneth Brian Haugshøj, Martin Weiss Nielsen, Iben Damager, Peter Ulvskov, Leif Højslet Christensen, Klaus Gotfredsen, Niklas Rye Jørgensen: *Effect of nanocoating with modified pectin molecules on titanium surface properties and osteoblast response*, 2012 manuscript in preparation.

iii. Yousef-Coronado,F. Nielsen, M.W., Molin, S. and Espinosa-Urgel, M. *GABL phenotype: growth advantage in biofilm lifestyle*, 2012 manuscript in preparation.

iv. Katarzyna Gurzawska, Rikke Svava, Susanne Syberg, Yu Yihua, Kenneth Brian Haugshøj, Martin Weiss Nielsen, Iben Damager, Peter Ulvskov, Leif Højslet Christensen, Klaus Gotfredsen, Niklas Rye Jørgensen: *Characterization and classification of pectic nanocoating surface for medical devices,* 2012 manuscript in preparation.

v. Kaj S. Andersen, Owen Ryan, Martin W. Nielsen, Rasmus Bojsen, Anders Folkesson, Birgitte Regenberg. *Screening of a complete gene deletion library for identification of genes involved in Saccharomyces cerevisiae biofilm development.* 2012, manuscript in preparation.

Introduction

1 Cystic fibrosis

1.1 Disease characteristics

Cystic fibrosis (CF) is one of the most common recessive genetic disorders in the Caucasian population [8]. CF is a deadly disease that leads to a slow deterioration of the patient's health despite massive efforts to circumvent disease-derived characteristics.

Woe to that child which when kissed on the forehead tastes salty. He is bewitched and soon must die [9].

This old folklore describes one of the implications and ways in which children with possible CF are diagnosed. Sweat tests that are consistently positive for high salt concentrations give indications of a CF diagnosis. Such indications do however need to be correlated with other tests and family history in order to ascertain the diagnosis, as other conditions can lead to elevated sweat electrolytes [10, 11]. The genetic match of two parental malfunctioning genes leads to a 25% risk of a CF child. Approximately 70.000 people are affected by the disease worldwide, with the late 30'es as an estimated predicted age of survival (American cystic fibrosis patients, 2009) (www.cff.org). The disease is established through a deficiency in the cystic fibrosis transmembrane conductance regulator protein (CFTR) [12]. Approximately 1900 mutations have been found in the CFTR gene, which have been classified according to defect (listed at the Cystic Fibrosis Mutation Database [13]). Approximately 70% of the mutations observed in CF patients result from a deletion mutation of a phenylalanine residue at position 508, the ΔF508 mutation [14]. The effect of the deletion is manifested by a misfolding of the maturing proteins in the endoplasmic reticulum, resulting in an ubiquitin mediated degradation of the CFTR proteins [15]. The direct effect is that very few functional proteins reach

the plasma membrane [16]. Several other consequences of CFTR mutations are possible, such as frame shifts, truncation of the protein and instability of the mRNA with outcomes of e.g. incorporation of simply functionally impaired proteins [15, 16].

1.2 The CFTR protein

CFTR is a glycoprotein located on the apical side of predominantly epithelial cells and is ordinarily expressed in gland-rich organs such as the airways, colonic, pancreas, reproductive tract, sweat glands and hepatic bile ducts, where it functions as a cAMP-regulated chloride channel [17]. The 1480 amino acid CFTR protein is a member of the ATP-binding cassette (ABC) superfamily. It is assembled with two membrane-spanning protein domains, two nucleotide-binding domains and a regulatory domain [18, 19].

Phosphorylation of the regulatory domain by the cAMP-dependent protein kinase (PKA) is essential for opening of the chloride channel. It is believed that the importance of the CFTR protein is not only restricted to chloride transport, but also due to its interaction with other transporters, by either obstructing or strengthening their ion transportation [17, 20].

1.3 CF epithelia

Epithelial cells of several organs are affected by CFTR deficiencies, causing a range of clinical problems for the CF patients. The defective CFTR proteins lead to failure in absorption of hypertonic NaCl in the sweat glands [21]. In the lungs of CF patients, there is a dysfunctional regulation of isotonic volume transport [22]. Dysfunction in CFTR leads to a decrease in chloride ion transport and an accelerated uptake of Na^+ on the apical membrane side of the airway epithelial cells [23]. Hyperabsorption of Na^+ results in a dehydration of the airway surface liquid.

1.4 Clinical manifestations of CF

CF related disorders range from the male reproductive tract to multi-organ deficiencies in the gastrointestinal tract, exocrine pancreas, intestine, exocrine

sweat glands, hepatobiliary system and respiratory deficiencies that is the primary course of morbidity and mortality in CF patients [23, 24].

Exocrine pancreatic insufficiency is characterized by deficiencies in production of digestive enzymes leading to malabsorption of fat with manifesting steatorrhea, fat soluble vitamins, nutrients and poor growth capabilities [24]. The main treatment for pancreatic insufficiency include additive pancreatic enzymes, vitamins and a high calorie diet [25]. Approximately 3-10% of CF patients develop severe liver disease characterized by cirrhosis. Fatal liver disease is reported in around 2-3% and is the second most frequent cause of CF related deaths [26]. Intestinal obstruction syndromes are frequent problems in CF patients. Intestinal obstruction can be treated with laxatives, intestinal lavage or intestinal surgery in very complicated cases. Other consequences are male infertility by obstruction of the vas deferens and female patients show fertility problems due to severe obstruction of the cervix by accumulation of viscous mucus [24]. Despite multi organ deficiencies, the primary clinical problems arise with severe chronic infections of the airways.

1.5 Correlated implications in the airways

An array of events leads to maintenance of the airway surface liquid (ASL) within the airways of healthy individuals. Defective CFTR leads to a dehydrating scenario of the airway surfaces due to the defective chloride ion transport [27, 28]. The importance of ASL is connected to the function of the airway clearing mechanism that rely on the production of mucin glycoprotein molecules that constitute the main component of airway mucus [29, 30]. The upper parts of the human airways rely on goblet cells as the principal secretory cells, whereas the Clara cells dominate in the terminal bronchioles with diameters below approximately 2 mm [30]. In this part of the airways the epithelia are ciliated whereas the respiratory zones lack cilia.

The mucus layer of the human airways serves as a mechanical trap for inhaled materials and microbes [23]. Efficient clearing employ a feature in which entrapment of inhaled particles is aided by turbulent movement of the mucus [5]. Sufficient ASL levels are required in order for efficient mucus transport, cilia movement. The periciliary liquid (PCL) fraction of ASL is regulated by Na^+ and Cl^-

transport and is the basis for functional cilia movement, which requires an approximately 7μm of PCL in height at steady-state, figure 1. It has been shown with CF epithelia cultures that PCL dehydration impede the ciliary movement. Optimal functions rely on coordination of Na^+ absorption and Cl^- secretion by epithelial ion transport processes [31].

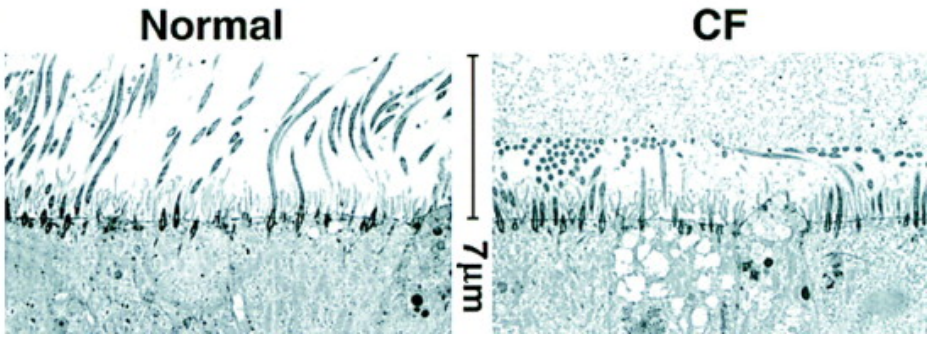

Figure 1. Electron micrograph of human airway epithelia. Left side shows epithelia from normal subjects and right side epithelia from CF subjects. Note the mucus deposited on the CF fallen cilia [31].

The importance of the liquid volume is portrayed by the capability of normal airway epithelia to reach a steady state of volume absorption when subjected to small amounts of excess liquid, whereas CF airway epithelia fail to halt absorption at a physiological appropriate volume [28, 31]. Dehydrating events lead to physiological changes of the airway mucus layer, inflicting the cilia to collapse towards the airway surfaces, Figure 1. The CF airways will in contrast to a normal "sterile" environment of healthy humans represent an environment prone to infections by inhaled microorganisms due to the impaired mucociliary clearing [27, 32, 33].

1.6 Colonization of the CF airways – the usual suspects

Several aspects of CF pathology emphasize the implications generated by the effect of the altered mucosal surfaces in the CF airways. From the early years, CF patients suffer from recurrent infections by an array of microbial pathogens as (i) respiratory viruses (ii) bacterial species from the normal human flora of the skin and respiratory tract and (iii) environmental fungal- and bacterial species.

The most frequently involved bacteria in CF related pulmonary infections are *Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus aureus*, *Haemophilus influenza and Burkholderia cepacia* [34, 35], see figure 2.

The colonization pattern seems to be age related, as *S. aureus* and *H. influenza* have most frequently been found as the dominant species in the youngest patients [3]. *P. aeruginosa* eventually alters the colonization scenario by becoming the dominating pathogen in the older patients. Up to a staggering 80% of young CF adults will become chronically infected with P. aeruginosa by the age of 20 [36-38].

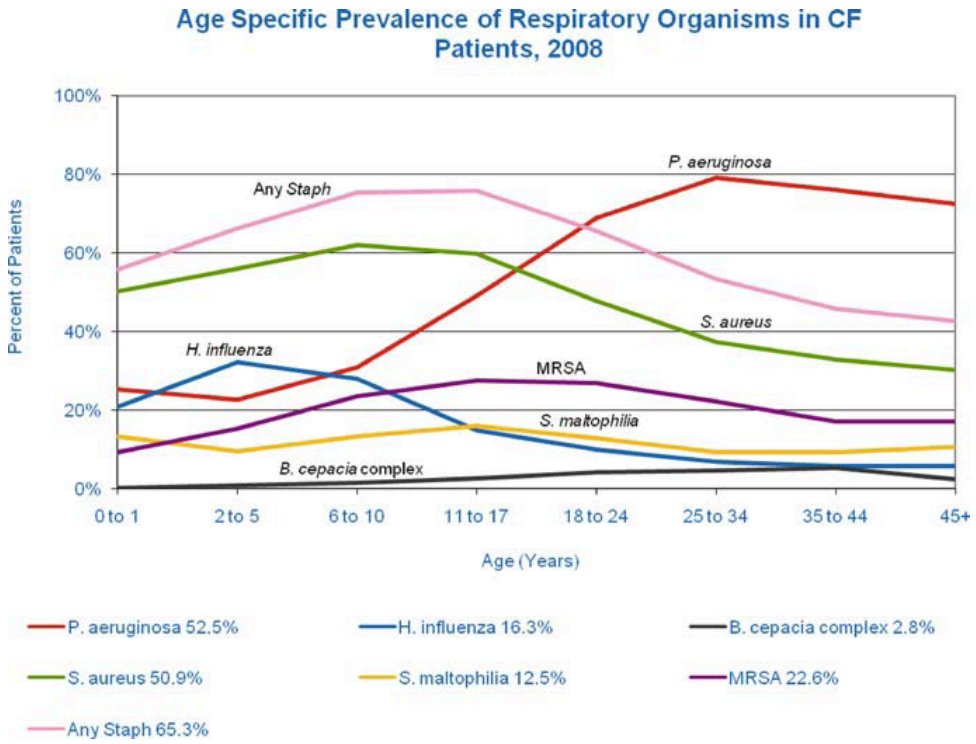

Figure 2. Showing the prevalence of respiratory infections in CF patients by age. Colonization and infection rates follow an age-specific trend with respect to specific bacterial pathogens. Figure adapted from Goldberg[3]

Early infections are typically intermittent but turn chronic if the initial infection remains unresolved, leaving a timespan of approximately 12 months for clearing the infection [39]. Intermittent colonization was defined by Høiby *et al*. as continuous presence of *P. aeruginosa* in the lower respiratory tract secretions for <6 months with normal levels of antibodies against *P. aeruginosa*, whereas a chronic infection was set as >6 months and/or 2 precipitating antibodies against *P. aeruginosa* [40]. Chronic infections predominately coincides with conversion

of *P. aeruginosa* from a nonmucoid to a mucoid phenotype, which is associated with a profound decline in the lung function in CF patients. (section 3.6.1) [41, 42].

2 *Pseudomonas aeruginosa*

The bacteria *P. aeruginosa* is a Gram-negative rod-shaped bacterium that belonging to the family of Pseudomonadaceae and is commonly found in different environments as terrestrial and aquatic [43]. It is a very ubiquitous bacterium capable of colonizing a wide range of kingdoms, from animals to plants and furthermore utilize a wide rage of organic compounds as nutritional sources [43]. *P. aeruginosa* is, unlike most other Pseudomonas spp. capable of growing at temperatures up to 42°C, with an optimal temperature of 37°C. *P. aeruginosa* is an opportunistic pathogen capable of causing severe nosocomial infections [44]. With a large genome of ~ 6.3 Mbp (PAO1 WT strain) its genetic makeup permits flexibility making it a highly versatile settler [45].

P. aeruginosa is considered a notorious pathogen for immunocompromised patients with predisposing conditions as leukemia, diffuse panbronchiolitis, AIDS, burn wounds and in cystic fibrosis patients [46]. Once *P. aeruginosa* infects a patient it elicits ways to evade eradication by administered antibiotic and the immunological defense systems of the host. The protective battery includes e.g. expression and secretion of several virulence factors and overproduction of an exopolysaccharide alginate [41, 47] (see section 3.6.1).

2.1 Pseudomonas aeruginosa in CF

P. aeruginosa has a highly versatile genetic makeup allowing it to adapt and cope with changes in its environments [45]. Many CF patients become colonized with *P. aeruginosa* early in their childhood and carry this infectious strain for the rest of their lifetime [3]. So what makes this bacterium so successful in being the dominant colonizer of the CF environment?

P. aeruginosa produces an intricate variety of extracellular and cellular bound virulence factors that contribute to the initial step of colonization and thereby achieving the foundation for the establishment of enduring infections [48].

2.2 *P. aeruginosa* cell associated virulence factors

Cell-associated virulence factors include adhesion structures as pili and flagella. These motility driving protein appendages have been proven important in

adherence of *P. aeruginosa* to surface moieties. Several flagella genes, as the flagella cap encoding fliD, have been shown to contribute to the adherence to e.g. respiratory mucin [49-51]. This might actually impede bacterial colonization of the healthy airways by aiding to the mucociliary clearing mechanism.

Type IV pili driven twitching motility has been shown to promote attachment to epithelial cells [52, 53]. Twitching motility mediated by type IV pili has been proven important in the formation of distinctive structures of *P. aeruginosa* biofilms [54, 55]. Type IV pili act as a major virulence factor in acute pulmonary infections, seen by the influence of pili structures in causing an inflammatory response in the murine test model [56].

The major cell wall surface component of gram-negative bacteria, lipopolysaccharide (LPS), is a dominating contributor to mediating host responses to *P. aeruginosa* infections [57]. Interestingly environmental isolates express a "smooth" LPS phenotype with long O-polysaccharide side chains while strains that have adapted to the CF lung environment, have lost these side chains (rough phenotype) [57, 58]. Ernst and co-workers have shown that LPS structures from different CF P. aeruginosa isolates have specific lipid A structures, characterized by penta- and hexa-acylation that is associated with increased immunogenicity [58]. Although increasing the sensitivity towards *P. aeruginosa* LPS structures, they do not stimulate as potent macrophage cytokine responses as *E. coli* or *Salmonella* LPS [58]. The characteristics of the negatively charged LPS structures correlate with the use of cationic antimicrobial peptides in the treatment of infected patients. Genetic modifications of the lipid A moieties of the LPS structures has been shown as an underlying mechanism for the adaptive resistance induction towards cationic antimicrobial peptides as the polymyxins (see section 7).

2.3 *P. aeruginosa* extracellular virulence factors

P. aeruginosa produces an array of secreted virulence factors that following initial colonization can cause extensive damage to the host tissue, bloodstream invasion and spreading of the infection.

Exoenzyme S and Exotoxin A are two secreted exotoxins, with Exotoxin A causing local tissue damage and bacterial invasion. This is effectuated by

inhibition of protein synthesis due to inactivation of elongation factor 2 resulting in cell death [59, 60]. Exoenzyme S may be responsible for direct damage and bacterial propagation, as well as disruption of eukaryotic signal transduction [61]. In addition, *P. aeruginosa* produce proteases and hemolysins that furthermore contribute to the invasive strategy. The elastases (LasA and LasB) are members of the zinc metalloprotease family and are responsible for the degradation of host elastin, which is important in the expansion and contraction of the lungs [62]. The elastases interfere with several components of the immune system, as inactivation of human immunoglobulins IgG, IgA as well as components of the complement system [63, 64]. The additional proteases, alkaline protease and protease IV are important contributors to pathogenesis and have been found in sputum samples from CF patients [65-67]. Rhamnolipid and phospholipase C are two hemolysins with the capability of breaking down lipids and lectins structures. Phospholipase C decreases the respiratory burst of neutrophils and induces local production of cytokines as tumor necrosis factor α (TNF-α), macrophage inflammatory protein 1α and 2 (MIP-1α and MIP-2), IL-1β in addition to stimulating neutrophil infiltration, thereby contributing to the inflammatory response in the CF airways [68]. Rhamnolipids have equivalently been found in sputum samples from *P. aeruginosa* infected CF patients where they are found in exceptionally high concentrations [69]. Rhamnolipids have detergent like properties that break down the phospholipid surfactant of the lungs as well as inhibiting ciliary function of the human respiratory epithelium [70, 71]. The direct effects of rhamnolipids on mammalian cells have been shown by the interference of the chemotactic responses by polymorphonuclear leukocytes (PMNs[1]), inhibition of normal macrophage function as well as stimulation of cytokine release from epithelial cells [72, 73]. It has recently been shown that rhamnolipids are important components in causing persistent infections directed by a coordinated intercellular bacterial communication system called quorum sensing (QS) [74]. The QS system upregulate rhamnolipid production and several other virulence factors. The protective properties of

[1] PMNs are granulocytes: neutrophils, eosinophils and basophils,

rhamnolipids produced P. aeruginosa biofilms lead to a direct PMN elimination [74-76].

2.4 Type III secretion system

The *Pseudomonas aeruginosa* type III secretion system (T3SS) is a complex export machinery of effector molecules. The T3SS requires direct contact with the host cells to which effector molecules are actively translocated into the cytosol of the eukaryotic cell [77]. More than 80% of acute *P. aeruginosa* infections express the T3SS machinery, but are expressed to a much lesser degree in chronic CF respiratory infections [78]. A needle apparatus creates a translocation channel that allows molecular transport from the bacterium into the host cell. Four T3SS effector enzymes have been identified until now; the exoenzymes S, T, Y and U (ExoS, ExoT, ExoY and ExoU) [77].

The expression of the T3SS plays an important role in the initial colonization steps by contributing to epithelial cell damage and killing of macrophages and PMNs [79]. Chronic *P. aeruginosa* infections are often correlated with conversion to a mucoid phenotype [41, 42]. It was shown by Wu et al., 2004 that mucoid *P. aeruginosa* down-regulate the T3SS system during up-regulation of genes involved in alginate biosynthesis [80]. The switch indicates a need for the T3SS system during initial colonization however shutting down the secretion system in order to save energy during alginate production.

2.5 *P. aeruginosa* communication – quorum sensing

P. aeruginosa will as mentioned reach high cell densities in the airways of CF patients, which correlate with the induction of the QS communication system. Bacterial communication generates a specific environment of a bacterial group related behavioral pattern. Group behavior can be subscribed to features as the QS communication system, dynamics of biofilm formation, production of virulence factors, extracellular DNA release and iron siderophore production [81, 82]. QS relies on the production and local concentrations of autoinducer molecules. Gram-negative bacteria typically accomplish communication through produced acylated homoserine lactones (AHLs) whereas gram-positive bacteria utilize small peptide molecules [83].

P. aeruginosa employ at least two major interrelated quorum sensing systems [84]. Each of these systems consists of an auto-inducer and its transcriptional regulator protein. As the local accumulation of the auto-inducer molecules reach a threshold concentration, the transcriptional regulators coordinate with the auto-inducers which in turn leads to activation [85].

One system leads to the activation of the transcriptional activator LasR through its autoinducer molecule N-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}HSL$) produced by the signal synthase LasI. The second system responds to and produces N-(buytryl)-L-homoserine-lactone (C4-HSL) with the signal synthase RhlI and transcription activator RhlR [84].

Reaching threshold concentrations will induce transcription of target genes in the respective Rhl and Las regulons. Transcription leads to an enhancement of activating events, as transcription of the AHL synthease genes that push forward a positive feedback loop encompassing an up-regulation of transcription regulators. The overlapping regulation of the system is seen by the ability of the Las systems to activate the Rhl system, figure 3.

Another integrated component in the QS system of *P. aeruginosa* is the *pqs* system[86-88]. The Pseudomonas quinolone signal molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) is under regulation of the *las* and *rhl* systems[86]. PQS is intertwined between the *las* and *rhl* systems as PQS production is positively regulated by the *las* quorum sensing system and PQS strongly induce *rhlI* [87, 88]. The QS systems undertake the induction of a wide range of essential components that style *P. aeruginosa* to become a successful colonizer. QS has been shown to be essential for induction of virulence in animal models. The predominant reduction in virulence was seen with mutant strain defective in both *lasI* and *rhlI* [89]. Several animal studies have shown QS mutants to cause less tissue destruction, reduced pneumonia, disseminated infections and reduced number of mortalities [89-93].

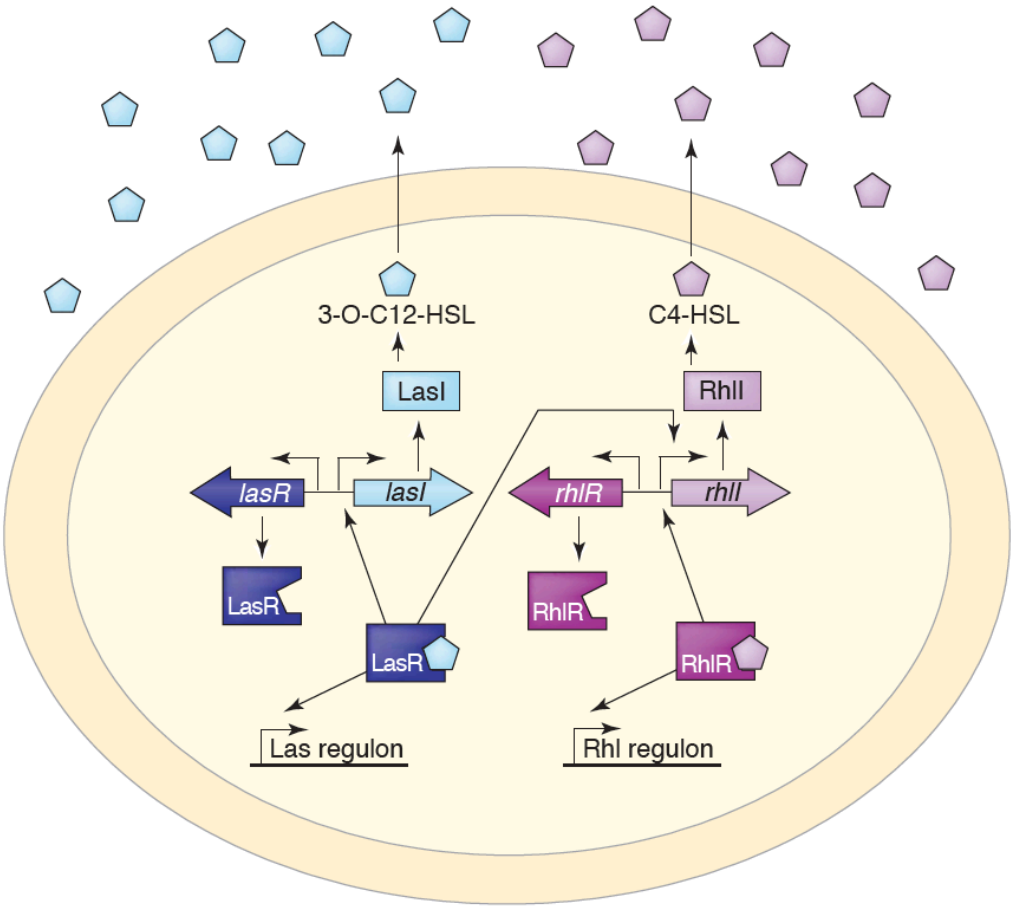

Figure 3. *Pseudomonas aeruginosa* quorum-sensing systems. *P. aeruginosa* uses the two quorum-sensing systems: LasI and LasR (blue), RhlI and RhlR (purple). LasI produces the auto-inducer $3OC_{12}HSL$ (homoserine lactone; blue pentagons), and RhlI makes the auto-inducer C4-HSL (purple pentagons) and RhlR bind to their cognate auto-inducers and induce gene expression. The systems control partially overlapping sets of genes. (Henke and Bassler, 2004[85])

Davies et al. showed that the impact of the rhl-las system was set to the developmental stages, following initial attachment and proliferation, directed by the density of the population [94]. Furthermore they showed that the mutants formed flat and undifferentiated biofilms susceptible to treatment with the detergent sodium dodecyl sulfate (SDS) [94]. QS regulated rhamnolipids have proven important in the development of channel-separated macrocolony biofilm structures as well as aid in the detachment of the biofilms [95]. PQS has been shown to be necessary for extracellular DNA production, which might facilitate cap formation of the mushroom structures[2]. This is due to the fact that type IV pili bind with high affinity to DNA present in higher concentrations on

[2] Mushroom structures, macro colonies that take the shape of mushrooms with stalk and cap formations.

microcolonies in P. aeruginosa biofilms [55]. A recent study has shown the absolute dependency of oxygen in the final step of PQS synthesis from HHQ[3] catalyzed reaction by PqsH. They propose that PqsH could function as regulator in *P. aeruginosa* by controlling PQS controlled traits in response to different environments [96].

[3] 2-heptyl-4-quinolone (HHQ) is a precursor for PQS

3 *P. aeruginosa* and biofilms - CF a biofilm associated infectious disease

3.1 *P. aeruginosa* and biofilms in the CF airways

A major constituent in the cystic fibrosis pathology is the lifelong *P. aeruginosa* infections in the airways of the patients, which has been accepted as a biofilm-associated mode of growth [97]. With increasing age, *P. aeruginosa* infecting CF patients undertake a phenotypic switch to a mucoid alginate overproducing phenotype [41]. The mucoid phenotype corresponds with declining therapeutic possibilities to treat the *P. aeruginosa* infection as the mucoid phenotype corresponds with high resistance to antibiotics [98]. The mucoid phenotype is recognized as a poor prognostic indicator and once a biofilm infection is established, these bacteria are seldom eradicated [41, 99, 100].

3.2 Biofilm definition

The classic way of observing bacteria has been in cultures of planktonic bacteria, however it has become evident that many microbes predominantly live in more sessile communities called biofilms. – Surface associated communities encased in a self-produced extracellular produced matrix – is the general definition of microbial biofilms [101]. Biofilms are involved in a remarkable number of chronic and acute infections, a regime consisting of a single species or assembled by a mixture of bacterial or fungal species [101]. The populations of cells that establish biofilms demonstrate distinct features compared to planktonic cell populations as these communities adapt to a highly construction driven lifestyle.
In nature, the presence of a purely planktonic culture very seldom exists, however the biofilm lifestyle of microbes is seen in more or less all aquatic sediments, and cause extreme problems with costly contamination of industrial production lines, dental plaques and complications with indwelled medical devices [102].

3.3 Biofilm formation

The history of biofilm formations goes back about 3 billion years with similar biofilm formations in modern day environments. The fact that biofilms are dated this many years back shows great adaptation to fluctuating environments and the need to evolve into a complex societies [103].

The development in biofilm formation encompasses a five-stage differentiation process initiated by the attachment of freely swimming (planktonic) cells to a surface (i). The attachment step is a reversible process (ii) followed by an irreversible attachment, where the cells at the surface start to produce EPS[4] (iii) formation of micro-colonies by cell proliferation or further adherence of planktonic bacteria and (iv) the development of the biofilm in construction of macro-colonies and finally (v) seeding dispersal, where detaching cells from macro-colonies exit the biofilm community to occupy new surfaces, figure 4.

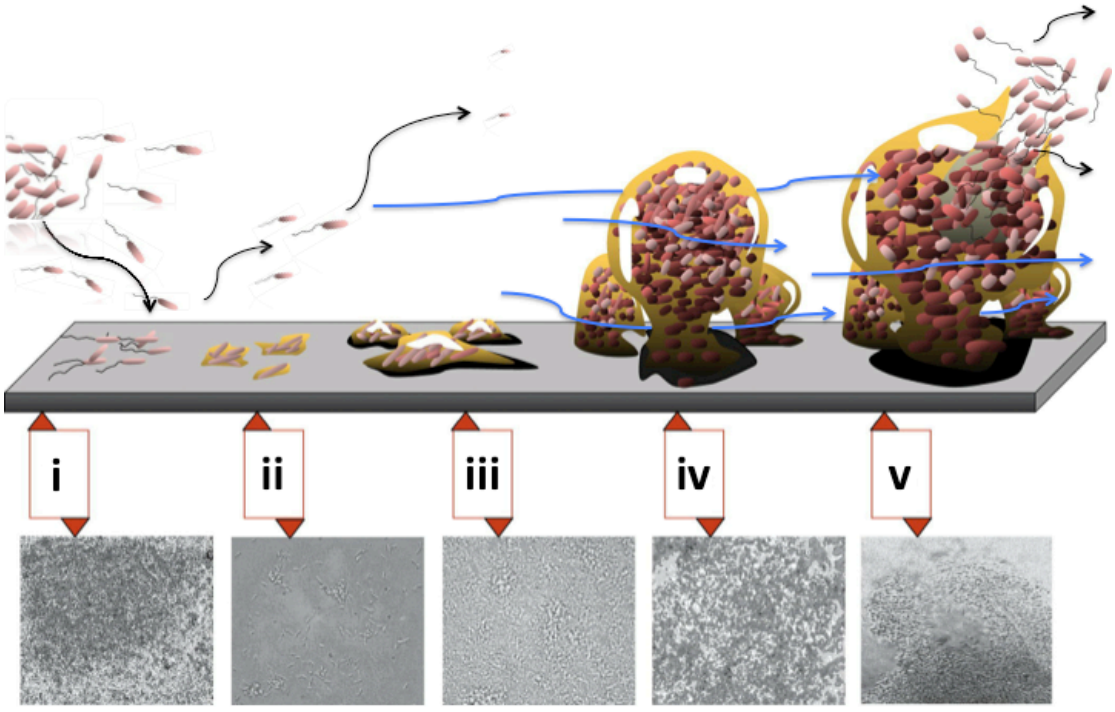

Figure 4. Representation of biofilm development. The process is separated in 5 different scenarios. (i) reversible attachment (ii) irreversible attachment (iii) formation of developing micro-colonies (iv) maturating macro-colonies (v) dispersal. Black lines representing bacterial movement, blue lines illustrating flow of liquids. (modified from, Monroe, 2007[104])

[4] EPS: extracellular polymeric substances

In the process of initial attachment to various surfaces, the cells become irreversibly attached through surface structures as LPS, type IV pili fimbriae and flagella. Type IV pili are important in the development of mushroom shaped macro-colonies, as type IV pili mutants of *P. aeruginosa* show distinctive traits to the stalk formation, whereas the motile population migrate to form the cap of the mushroom [54]. Mutants incapable of producing biosurfactant rhamnolipids (*rhlA* mutants) are deficient in producing biofilms with characteristic architecture of "water channels" and unable to establish strong cell-cell and cell-surface interactions [95, 105]. In an analysis of CF sputum samples, QS system signaling molecules indicate that *P. aeruginosa* convert to a biofilm lifestyle in the CF lung environment [97]. As several factors controlled by QS facilitate biofilm sustainability and protection, biofilm formation must essentially provide beneficial advantages to the community.

3.4 The biofilm lifestyle

Many questions come to mind when considering this community driven lifestyle. One of them being: Why is this a preferable form of community living? There are several aspects that need to be taken into consideration when trying to answer this question.

First of all, going back in time it was not until the late 1970'es that Costerton and co-workers published their work that defines "the biofilm way of life" as a rule more than an exception [106]. So what are the advantages for bacteria to "settle down" in such a structured society? Speculating on the general "purpose of life" for a bacterium is for the species to persist. Settling in a habitat with plenty of nutrients and solid surfaces to attach to, enables bacteria to vividly found a community.

Having neighbors close by can be beneficial in several aspects, as cooperating on the production of common goods and genetic variability through exchange of genetic elements [107, 108]. Within biofilms, cells are tightly packed against each other and share a wide range of secreted molecules as enzymes, iron scavenging siderophores, community enzymatic defense systems as well as extracellular polymers for shielding purposes (section 3.5). Within the proximity of others the evolutionary expansion of cheating phenotypes are also prone to

evolve [107]. This is unambiguously seen in mixed regimes where conflicts prevail over contribution to the shared resources of the group [109]. Competition can be both between relatives or non-relatives in the local environment, where resources may be scarce [109, 110]. It has been shown with siderosphore production, that bacteria living with other highly related bacteria evolve to produce more siderosphores than bacteria living with unrelated individuals [110]. Cooperation for the production of extracellular polymeric substances (EPS) can in the same sense provide a shielding factor for non-producers within the biofilm.

In response to environmental factors as starvation and oxygen depletion, the biofilm community may respond by reorganizing the structures and dissolving the biofilm in a possible search for more nutrient rich environments [111].

3.5 The extracellular polymeric matrix

One of the early observations of biofilm characteristics was the slimy nature of these surface associated communities. A characteristic feature of surface attached micro-organisms is the production of a polymeric matrix that anchors the micro-organisms together and furthermore fosters important shielding properties [112, 113]. The polymeric matrix structurally stabilizes the growing biofilm and provides a shield for antibacterial factors as constituents of the immune system and antimicrobial components [101, 114]. However suggestions that the biofilm matrix bring a low diffusion of antibiotics have been a matter of controversy (see section 7). The extracellular polymeric matrix is composed of several different factors that differ in ratio, but the solids are primarily composed of polysaccharides 40-95%, <1-60% protein, <1-10% nucleic acids, <1-40% lipids (numbers direct from table 1[112]) [112, 114].

As stated, polysaccharides account for a large proportion of the matrix that encases the bacteria of the biofilm. The initial colonization of the CF airways by *P. aeruginosa* typically renders an intermittent colonization, an acute infection that in time turns chronic if the initial acute infection is unsolved [40]. Chronic infections generally correspond with P. aeruginosa converting to the mucoid phenotype, a state that can be measured in the antibody titer towards *P. aeruginosa* produced alginate [42]. However these antibodies do not work in an

opsonizing manner to eradicate the bacteria as they are directed towards this inert material encapsulating the bacteria [42].

3.6 Alginate and essential matrix components by *P. aeruginosa*

3.6.1 Alginate production

A seminal feature of *P. aeruginosa* is that it is frequently found in the airways of CF patients as a mucoid phenotype, which is an infection hallmark that appears over the course of the infection.

Alginates are co-polymers of β-$_D$-mannuronic and α-L-guluronic acids generated from the biosynthesis starting material fructose-6-phosphate. Initially, alginate production is considered one of the virulence factors produced by *P. aeruginosa* in the CF airways [42]. The conversion to an alginate overproducing phenotype is one of the traits going from an intermittent to chronic colonization in the CF airways [42, 115]. This phenomenon takes place in the inner membrane where the anti-sigma factor MucA binds to the sigma factor AlgU (alternatively called AglT or σ^{22}) [41]. Mutations in MucA lead to a strong activation of AlgD transcription, as the alginate operon is under the control of the AlgD promoter. AlgD is essential for alginate synthesis as the AlgD gene product catalyzes the first step in the synthesis process [41, 115]. It has been shown that *P. aeruginosa* in the airways of chronically infected CF patients produce large quantities of alginate, however in wild type *P. aeruginosa* strains PAO1 and PA14 alginate has been shown not to be a be a major constituent of the extra-cellular matrix in the vitro biofilm formation [116].

3.6.2 Psl and Pel polysaccharides matrix components

Two additional polysaccharides are essential components of the EPS matrix produced by *P. aeruginosa*. The *pel* genes (*pel*A-F) encode polysaccharides shown to be essential for the pellicle formation at the air-liquid interface [117]. *Pel* mutants are defective in the formation of solid surface-associated biofilms and clearly show altered colony morphologies [117]. It has been suggested that Pel polysaccharides can have a compensatory role in absence of other adhesins [118]. A resent study has shown that Pel does not play a critical role in PAO1

biofilm development, however describing the Psl as the predominating polysaccharide in the PAO1 biofilm EPS matrix [119]. Psl polysaccharides are encoded by a 15 gene spanning operon and has been shown to be essential for biofilm formation in *P. aeruginosa* strains, PAO1 and ZK2870, however not in PA14 strains that have 3 gene deletion in the *psl* operon [119, 120]. Ma et al. 2006 showed Psl to be required for adherence to mucin-coated surfaces. Using an arabinose inducible *psl* construct, they showed the expression of the *psl* operon to be important in maintenance of the biofilm structure. Furthermore they showed the importance of Psl in cell-to-surface and cell-to-cell interactions as over-expression of *psl* enhanced these events [121].

3.6.3 Extracellular DNA as a matrix component

In addition to the previously mentioned components of the EPS matrix, extracellular DNA (eDNA) is an important matrix component and has been shown to have a stabilizing effect on *P. aeruginosa* biofilms [122, 123]. Larger quantities of eDNA is present in the CF airways, evidently originating from the necrosis of cellular components of the immune system, as the destruction of the abundant neutrophils, recruited by secreted cytokines and chemo-attractants into the airways [124, 125]. The PQS system (section 2.5) plays an important role in DNA release by *P. aeruginosa*. The PQS induced release of DNA could in that sense play an important role in the formation of biofilms in the airways of CF patients, however only in oxygenated regions [96]. Extracellular bacterial DNA has furthermore been shown to contribute as a proinflamatory component of *P. aeruginosa* biofilms [126]. In the flow cell systems (details in section 9.3.3) it has been shown that the early steps of *P. aeruginosa* biofilm formation is disrupted by DNaseI in the biofilm media [127]. The matrix contributes with several important aspects of *P. aeruginosa* persistency during airway infections and furthermore contributes to maintenance of the oxygen environment in the CF airways.

4 The human airways

The humane respiratory system is a complex network of branches and cavities. It can roughly be divided into the upper and lower parts, Figure 5. The airway system can furthermore be subdivided into at least three different compartments; the conductive airways, the respiratory zone and the paranasal

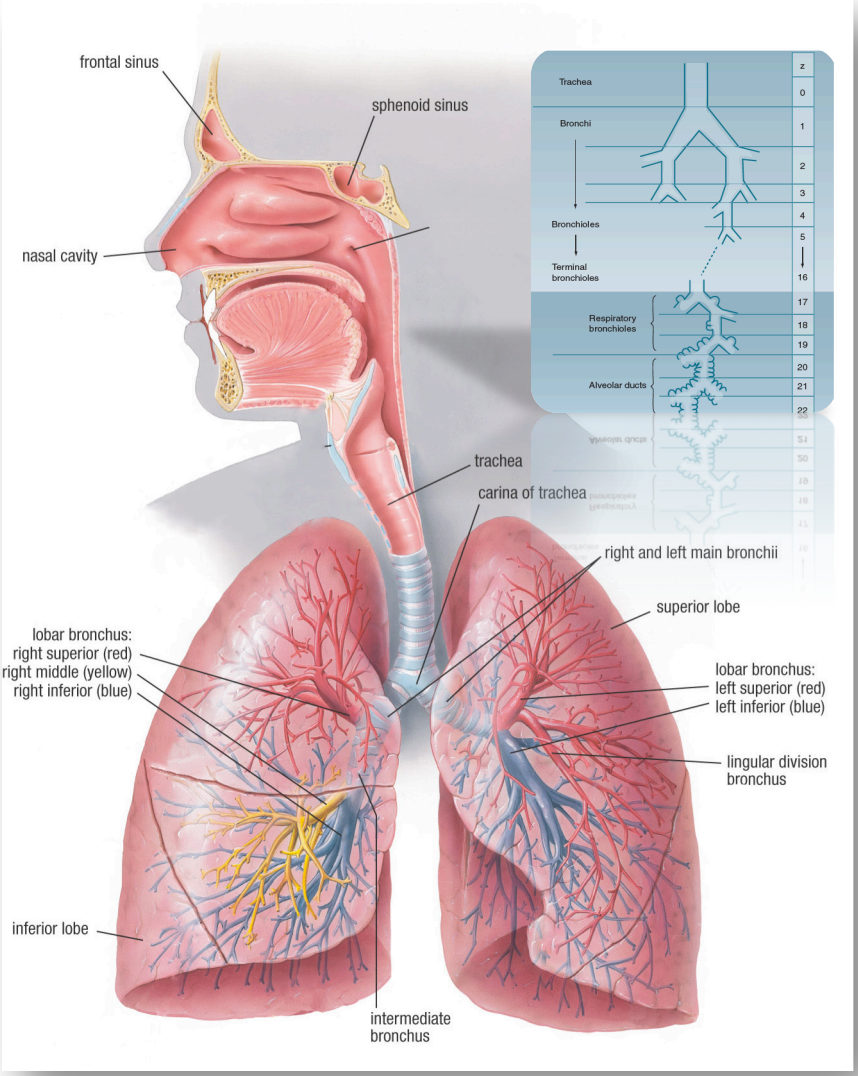

Figure 5. Representation of the human respiratory system. Inset display the conductive and respiratory zones. Pictures modified from [1, 2].

sinuses. The conductive airways branch out from the trachea into the bronchi and bronchioles. The terminal bronchioles branch further out and establish the respiratory zone (insert in figure 5.). The existence of an immense surface area

within the respiratory zone allows the exchange of gasses within the respiratory bronchioles, alveolar ducts and alveolar sacs. The conductive and sinus segments of the airways are broadly considered to be anaerobic or micro-anaerobic, compared to the highly aerated respiratory zone [128].

The branching is the essential groundwork for the functionality of the lungs meaning that obstruction and trauma can lead to collapse and dysfunction in the essential gas exchange. These are essential problems for patients suffering from pneumonia and pulmonary obstructive diseases, as COPD and cystic fibrosis [8, 129].

4.1 The CF airway environments

It has become evident that the airways of CF patients contain different compartments with varying oxygen concentrations, ranging from the highly aerobic compartments to hypoxic and subsections of anaerobic environments. In the CF airways, the thickened mucus deposited in the airways allows bacteria to reach staggering concentrations, as seen with *P. aeruginosa* [130].

4.1.1 The sinus compartments

The nasal cavities are extremely prone to getting in contact with bacteria and function as a first line of defense towards inhaled particles and microbes. The paranasal sinuses function as a humidifier and heat inhaled air and the cavities furthermore assist to exclude unwanted material from the lower respiratory tract [131]. The CF sinus mucosa is an obvious abnormal site in virtually all CF patients that radiographically show opacified sinuses. As the sinus mucosa show the CF signature of reduced Cl^- permeability resulting in thickened mucus, the paranasal sinuses have an immensely reduced airflow [131, 132]. The mucosal linings of the sinuses are ciliated and would ideally transport mucus to the nasopharynx, but CF patients remain impaired in this clearing mechanism [132].

As a direct consequence hereof the paranasal sinuses can act as a reservoir for potential reinoculations of the lower part of the airways [133]. The sinus cavities have a significantly low O_2 content at the mucosa, as shown in the

maxilliary sinuses[5], where several CF patients were identified with anoxic conditions [134]. Treatments for sinusitis remain weakened as antibiotic penetration and realization of sufficient therapeutic levels is less efficient in the sinus cavities than in the lungs, due to fundamental differences in the blood supply to the different compartments. Reduced effects of therapeutics lead to poor eradication of infecting bacteria causing recurrent periods of sinusitis and establishment of chronic infections [134, 135]. The sinuses hold high levels of IgA, compared to the pro-inflammatory IgG, and thereby facilitate low infiltration of PMNs during *P. aeruginosa* infections, which in turn protect the bacteria from a pro-inflammatory response [136].

4.1.2 Conductive zones

The conductive zones of the respiratory system represent approximately 5% of the total lung volume and include the trachea, the bronchi and the terminal bronchioles [100]. Importantly, this section of the airways also sustains the capacity to produce mucus thereby being an important part in the defenses down towards the respiratory part of the airways. In healthy individuals the efficiency of this mucocilliary defense mechanism is practically capable of preventing particle accumulation and pathogenic adhesion and invasion [27, 137]. Respiratory submucosal mucous glands are present in the sinus, trachea and bronchi and produces the high molecular weight gel-forming mucin macromolecules of the airway mucus layer [137]. The beating motion of the epithelium-expressed cilia transports mucus-trapped components out the respiratory tract. As CF patients are impaired in the mucociliary clearance mechanism, due to the consequences of airway surface liquid dehydration (ASL), microbial colonizations occur frequently. In attempt to eradicate infections, patients are set to inhale nebulized antibiotics, which leads to high concentrations of the arranged antibiotics in the conductive zones [100]. During *P. aeruginosa* infections of the CF conductive zones of the airways, the bacteria are primarily located in biofilm clusters within the mucus layer [5, 138]. The consumption of oxygen by epithelial cells and *P. aeruginosa* aid differences in

[5] Maxilliary sinuses; are the largest of the paranasal sinuses, drains into the nose.

oxygen availability in the mucus, however immense respiratory bursts by the PMNs produce anaerobic niches [5, 139]. In contrast to the high IgA levels in the sinuses, the conductive airways are in comparison governed by high IgG levels. [136, 140]. Nebulized antibiotics used to treat CF lung infections reach high concentrations in the conductive zones but only low in respiratory zones[1].

4.1.3 Respiratory zones

The respiratory sections of the lungs constitute the respiratory bronchioles, alveolar ducts and sacs. These specific sections are not lined with cilia expressing epithelial cells nor mucin producing glands [137, 141]. The regions epithelial surfaces are covered by an alveolar lining fluid (ALF) that consist of a plasma ultrafiltrate and secreted components from type II alveolar cells[6]. ALF contain a range of e.g. antimicrobial components, surfactants proteins and immunoglobulins [140]. In contrast to the conductive zones and sinus compartments, the primary immunoglobulin is IgG rather than the IgA that predominate in ASL [140]. Lacking the mucociliary defense mechanism, the respiratory zones rely on secreted defensin molecules, infiltrating alveolar macrophages and PMNs, which are recruited from the blood upon infection (see section 6).

The respiratory zone is immensely oxygen rich as all the venous blood pass through the capillaries of the alveoles [100]. However, if mucus plugs are formed within the respiratory zones of the CF airways, the environment becomes micro-aerobic with highly reduced oxygen levels. Levels drop from approximately 13% O_2 and 5% CO_2 in unobstructed respiratory zones to approximately 5% O_2 and 6% CO_2 if mucus plug hampers the airflow. If abscesses are formed, areas within the respiratory zone turn anaerobic [100], figure 6.

[6] Type II alveolar cells: approximately 5% of the alveolar cells that express microvilli, secrete alveolar fluid and stem cells for type I alveolar cells

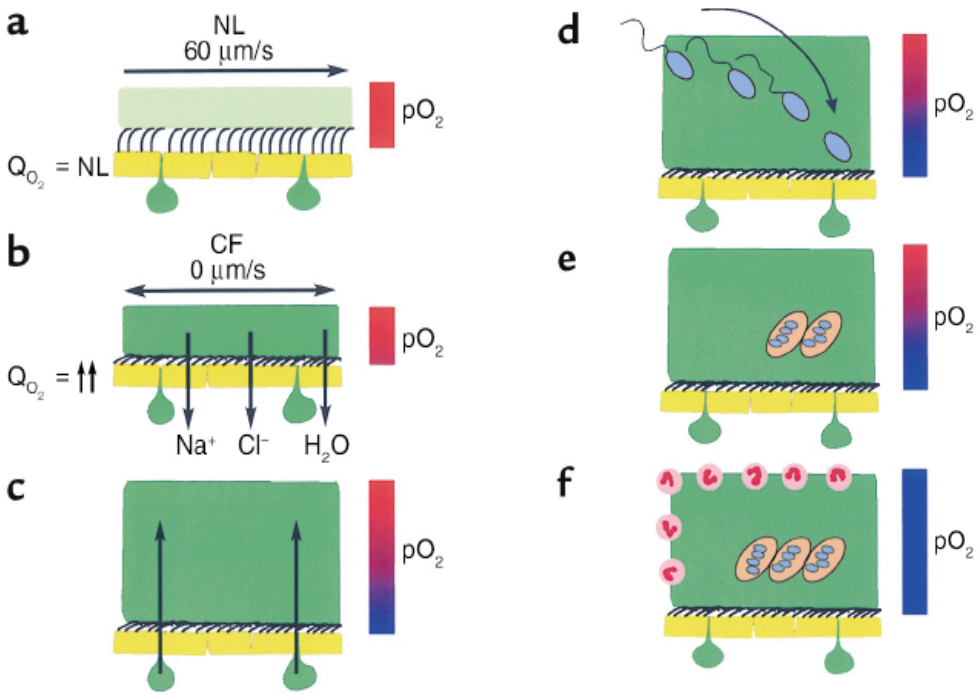

Figure 6. Schematic model of the hypothesized events that leads to chronic infection in airways of CF patients.
(a) Normal airway epithelia with a thin mucus layer (light green) on top of the cilia with normal PCL, (QO_2) normal oxygen consumption, no oxygen gradients within mucus (denoted by red bar). Arrow representing normal mucocilliary movement. (b-f) represent CF airway epithelia. (b) CF airway epithelial leading to a reduced PCL by excessive H_20 depletion, fallen cilia with consequential impaired net-movement of dehydrated mucus (dark green), raised QO_2. (c) Persistent mucus hyper-secretion. Over time the height of the luminal mucus masses increase. Raised QO_2 in the CF epithelia generates steep hypoxic gradients within the thickened mucus (blue coloration in bar, right side) (d) P. aeruginosa deposited on the mucus will penetrate into the hypoxic zones of the mucus either actively or passively (see figure 5) (e) P. aeruginosa adapt to the hypoxic environment. Within the stagnant mucus P. aeruginosa form macro-colonies and produce increased formation of alginate. (f) The macro-colonies that reside in the hypoxic zones of the mucus resist secondary defense mechanisms, including attracted neutrophils, setting the stage for progression of the infection into a chronic phase[5].

5 *P. aeruginosa* and the CF airways as habitat

As stated, most CF patients will eventually get colonized with *P. aeruginosa* that will eventually establish long term infection if intermittent infections are not resolved. Colonization is facilitated by the rich supply of components in the mucus, with cell debris from both host and bacterial derived factors as mucins, lipids, nucleotides and proteins providing an array of amino acids, nitrates and carbon sources to sustain long term infections [142]. Whereas several bacterial species only transiently colonizes the CF mucus, or are only present in low concentrations, *P. aeruginosa* commences to establish a chronic infection with high cell densities [130]. Generating energy for demanding processes as alginate production and biofilm formation, the versatility of *P. aeruginosa* to utilize various energy sources and nitrogenous oxides as electron acceptors, enables the organism to switch to anaerobic respiration thereby being able to cope with the micro-aerobic and anaerobic environments [143-145].

In a study by Worlitzsch *et al*. 2002, the properties of *P. aeruginosa* and its capabilities to penetrate into hypoxic zones of epithelial mucus layers was investigated. It showed that if deposited on a mucus layer, *P. aeruginosa* penetrates into the low oxygen zones, figure 7.

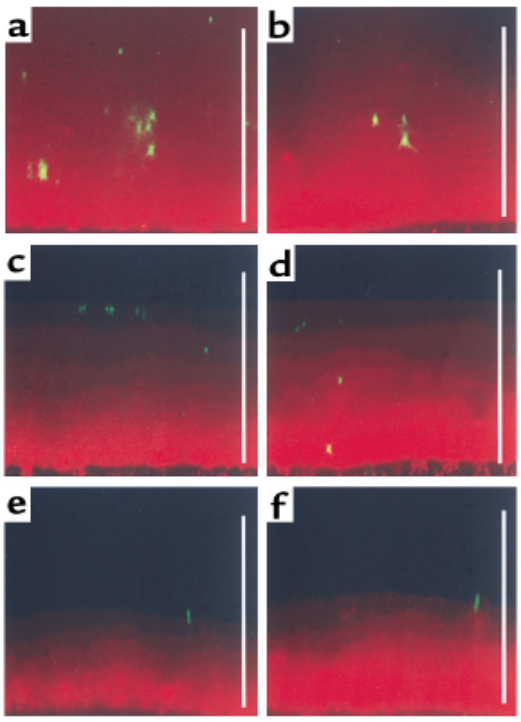

Figure 7. Localization of *P. aeruginosa* and flourescent beads in transported and stationary ASL (mucus) produced by planar CF cultures. Representative confocal images of ASL (red) fluorescent *P. aeruginosa* (green) or green fluorescent beads. *P. aeruginosa* or beads were added to the air-liquid interface. (a) X-Z confocal image of *P. aeruginosa* or (b) beads, 3 minutes after addition to the surface of ASL (mucus) exhibiting rotational transport. *P. aeruginosa* 3 minutes (c) and 15 minutes (d) after addition to stationary mucus. Beads at 3 minutes (e) and 15 minutes later (f) after addition to stationary mucus. Scale bars, 100 μm.[5]

In the absence of oxygen *P. aeruginosa* can utilize alternative electron acceptors. The ability of *P. aeruginosa* to utilize inorganic nitrogenous oxides; nitrate, nitrite and nitrous oxide as the terminal electron acceptors enables *P. aeruginosa* to sustain growth under anaerobic conditions [143]. Under anaerobic conditions *P. aeruginosa* is furthermore able to use arginine in a deiminase pathway for energy production although only able to sustain limited growth [146]. Analysis of the CF sputum composition has revealed abundant amino acids as well as ample nitrate levels [145]. Palmer *et al*. 2007, have shown the dependency of *P. aeruginosa* membrane-bound nitrate reductase for growth in an *in vitro* CF medium [145]. Antibodies raised against the nitrate reductase narG have been located in sera from *P. aeruginosa* infected CF patients, indicating that narG is expressed *in vivo* [147].

6 Immunological responses to *P. aeruginosa* infections

During infections of the respiratory system, microbes are challenged with several defense mechanisms as physical barriers and the mucociliary clearing mechanism [27, 137]. The mucus form the physical protective layers that furthermore contain a range of antimicrobial defense components [148].
Several scenarios take place in the attempt to eradicate commencing colonizing bacteria from the airways of CF patients. Antimicrobial treatments are administered to the patients in the clinic and the body activates the innate and adaptive immune systems to fight of the bacteria [98, 149].

One of the hallmarks in the lung infections with *P. aeruginosa* is a strong PMN response. This division of the cellular innate immune weaponry relies on germ line encoded pattern recognition receptors (PRRs) that are expressed on cell as macrophages and neutrophils, in order to battle off the intruding microbes [150]. As initial microbial recognitions take place, an inflammatory response that drives the recruitment of effector cells to the inflammation site is initiated. Neutrophils are recruited in large numbers to the site of infection [151]. Of the PRRs are members of the Toll-like receptor family (TLR) that recognizes conserved microbial structural moieties as lipopolysaccharides, pili and flagella. TLRs are expressed on the surface of inflammatory cells as well on the apical side of epithelial cells, figure 8 [152, 153]. Cells are recruited to the infectionsite, where they engulf bacteria or release their antimicrobial weaponry. Recruited dendritic cells transport foreign bacterial antigens to the lymphoid organs to activate an adaptive immune response, figure 8 [149].

Activated neutrophils generate a range of microbicidal molecules as reactive oxygen species (ROS) and defensins [151, 154]. Epithelial TLRs recognize bacterial surface components as lipopolysaccharides and flagella. One epithelial TLR, TLR-4 bind through a recognition pathway of LPS bound by LSP-binding protein (LBP), LPS:LBP complex bind to membrane protein CD14 which then coordinate with TLR-4. Successful TLR stimulation leads to induction of transcription factor NF-κB, which drives IL-8 production and thereby recruitment of further neutrophils to the site of infection [153, 155]. Planktonic

bacteria are efficiently phagocytized by the neutrophils, however if the infecting bacteria are not cleared, an ongoing vigorous inflammatory response will lead to

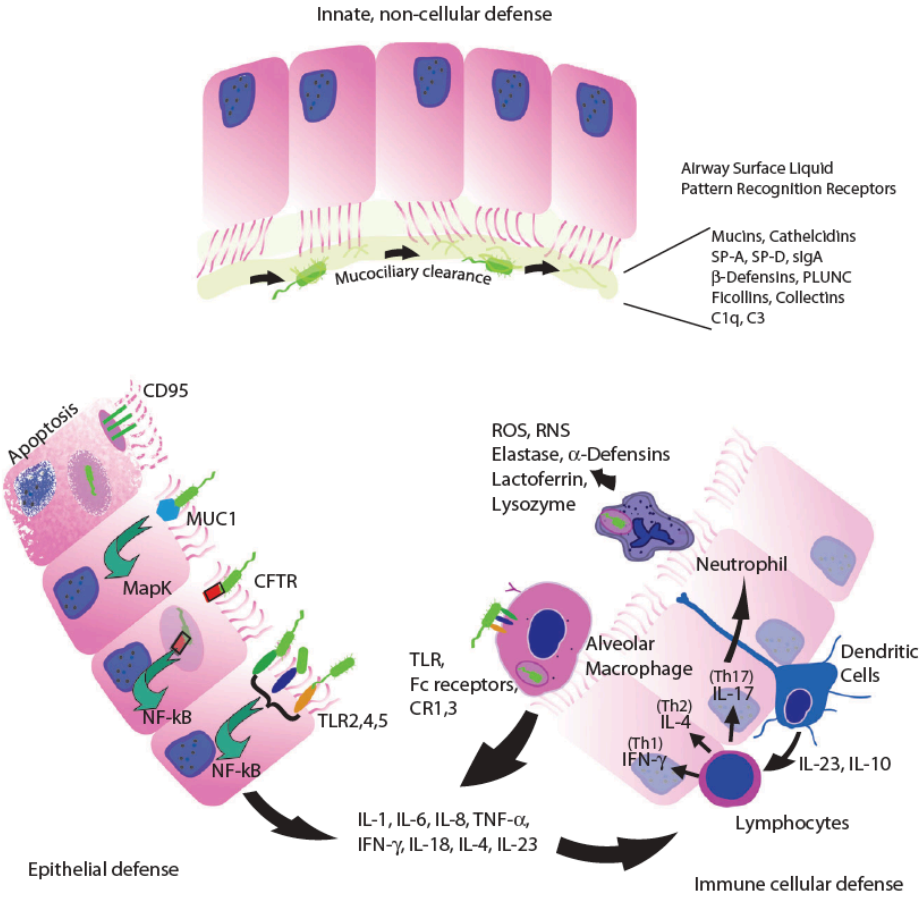

Figure 8. Respiratory defence against *Pseudomonas aeruginosa*. The respiratory defence against *P. aeruginosa* requires many mechanical, innate and adaptive defence mechanisms. First line of defence upon inhalation of *P. aeruginosa* is mechanical clearance via binding to mucins in the airway surface liquid (ASL) and clearance by the mucociliary transport mechanism. The ASL contains a myriad of anti-pseudomonal molecules originating from e.g. the epithelium. The epithelium may also apoptose upon endocytosis to prevent further spreading. The epithelium also presents transmembrane PRRs that can signal immune cells through multiple cytokine networks. Several immune cells are implicated in the battle against *P. aeruginosa* with neutrophils as probably the most critical component in bacterial clearance and resolution of infection. Lymphocytes with helper T-cells in three major subtypes have all been implicated in host defence [153].

substantial tissue damage [37]. This scenario constantly takes place in the CF airways, where the viscous dehydrated mucus contributes to bacterial colonization due to the impaired mucus clearing mechanism. *P. aeruginosa* can reside within the mucus surrounded by an immense number of PMNs that are practically incapable of removing the bacteria, as seen in the lower airway

compartments [138]. The sinuses however, do not present a comparable PMN dominated inflammation [136]. The short-lived PMNs effectuate protection through phagocytosis and by release of inflammatory mediators, antimicrobial proteins and ROS [156].

ROS exocytosed by the PMNs may induce mutations in the MucA gene of *P. aeruginosa,* leading to overproduction of the exopolysaccharide alginate, see section 3.6.1. This switch from a non-mucoid to a mucoid phenotype is correlated with the formation of alginate encapsulated biofilms leading to a rise in serum circulating antibodies and PMNs that are totally incapable of destroying the bacteria [138]. Mucoid bacteria are now capable of profoundly evading immune responses and can persist even under increasing antibiotic challenges[1, 98, 157].

7 Antibiotic resistance

The frequencies of the generally acquired resistance to antimicrobials vary according to continents and the wide uses of antimicrobials in medicine, crop production, production of food animals and as disinfectants [158-161]. An ever growing concern, is the trend of emerging multi-drug resistant strains that emerge worldwide [162]. This has critical implications for treatments in the future and on the efficiency of the current therapies available for combating bacterial infections. Emerging multi resistant bacteria have devastating outcomes for patients with infections spanning from severe urinary tract infections and pneumonia to surgical site infections, many of which are caused by Gram-negative bacilli [44].

7.1 General resistance and tolerance mechanisms of *P. aeruginosa* to antibiotics

Treatment of *P. aeruginosa* infections with antimicrobial agents can be hindered by several, both inherent and acquired, resistance mechanisms. *P. aeruginosa* is resistant to a wide range of antibiotics that are normally used to treat Gram-negative infections. Innately *P. aeruginosa* has the ability to produce several multi-drug efflux pump systems, including the major MexAB-OprM system, which removes a wide range of antimicrobial compounds as B-lactams, chloramphenicol, flouroquiniolones, macrolides, novobicin, sulfonamides, tetracycline, gentamycin, ciprofloxacin and trimethoprim [163, 164]. The system is composed by the MexB broad-spectrum pump that together with the MexA linker protein connect to the outer membrane porin forming OprM [164]. The efficiency of these pump systems together with an overall membrane associated decreased influx, are the key parameters to the multi drug resistant bacterial phenotype [165-167]. In its natural environment it has been proposed that efflux pumps play a role in removing toxic components from the cytoplasm of the bacteria. It has been shown that the expression of several *P. aeruginosa* efflux pump systems are not significant when commencing to biofilm growth, as the expression of the systems decrease with maturation of the biofilm [168]. It was also shown, that the expression was most profound at the bottom of the biofilm.

Deletion mutant biofilms showed that none of the multidrug efflux pumps enhanced an overall increase in resistance to antibiotics [168].

Bacterial biofilms are capable of being extensively more resistant to antibiotics and disinfectants than their isogenic planktonic equivalent with differences of approximately 100-1000 fold [154]. As stated, *P. aeruginosa* can reach high concentrations in CF sputum that entail mutations and exchange of genetic material, which in turn intensify the base for overall biofilm resistance [169, 170].

P. aeruginosa carries resistance to β-lactam antibiotics encoded on the chromosome, expressed through the AmpC β-lactamases [171]. The target for the β-lactam antibiotics is the penicillin binding protein (PBP), which is localized in the cytoplasmic membrane and is essential for cell wall synthesis by peptidoglycan crosslinking. Several genes have been shown to influence the expression of AmpC with systematic effects on mutations in regulatory genes, leading to a hyper production of β-lactamases [172-174]. Secretion of β-lactamases into the extracellular matrix embedding the biofilm forming bacteria might hamper the penetration of the antibiotics due to β-lactamase activity[175-177], figure 9.

P. aeruginosa employ a mechanistic change in the structure of the membrane in order to reduce the permeability, thereby increasing the resistance to antimicrobial compounds. *P. aeruginosa* incorporate an outer membrane protein OprD, a porin structure, that allows passive uptake of amino acids as well as allowing carpenems into the cell. Mutations leading to loss of OprD expression raise the resistance towards carpenems. Loss of OprD e.g. raises the MIC values for imipenem substantially to clinically relevant levels, however leaving no change in MICs of non-carpenems [178].

Resistance towards cationic antimicrobial peptides (CAMPs) are among the most widely distributed resistance mechanisms, as the production of CAMPs has been reported in essentially all groups of organisms [179]. *P. aeruginosa* acquires resistance against CAMPs as colistin and polymyxin B, by outer membrane modifications of the LPS moieties. Polymyxins, like all CAMPs bind to negative charges on phosphates of the LPS component lipid A [179]. Perpetrating the outer membrane organization, by CAMP binding to the lipid A, results in a

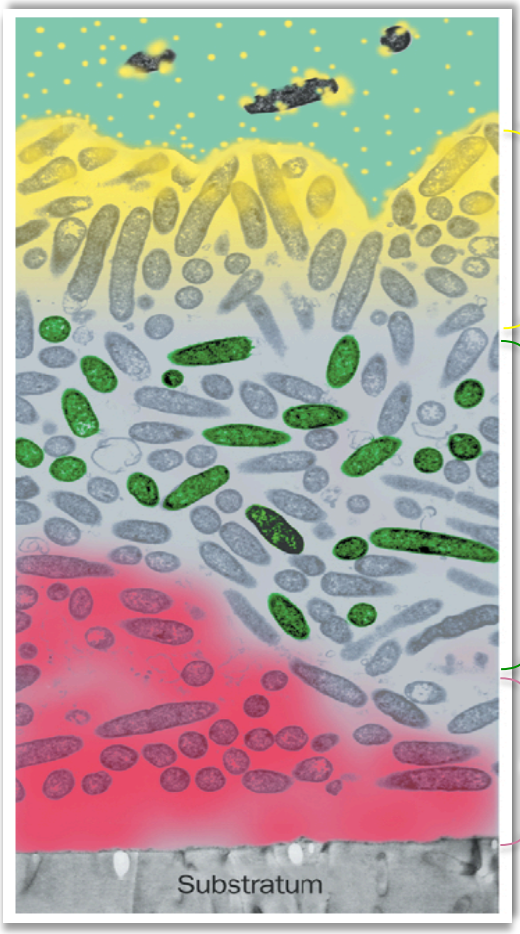

Figure 9. Hypotheses for mechanisms of antibiotic resistance in biofilms

Slow penetration
Antibiotic (yellow) fail to penetrate into the deep layers of the biofilm thereby only affecting the outer layer of the biofilm.

Resistant phenotype
Some bacteria may differentiate into a phenotypic resistant state of dormancy (green).

Microenvironment changes
Micro scale gradients of waste - e.g. changes in pH and available nutrients, changes in oxygen availability – anaerobic niches (red).

destabilization and disruption of both the inner and outer membrane. LPS modifications are accomplished by aminoarabinose incorporation into lipid A, which changes the overall negative charge of the outer membrane, thereby lowering the affinity of the positively charged polymyxins [180, 181]. Colistin is one of the very important CAMPs used to treat *P. aeruginosa* infections in CF patients [40]. Combinatory antibiotic therapy is efficient due to the fact that selected antibiotics can be directed towards metabolically diverse subpopulations of *P. aeruginosa* biofilms [182, 183].

7.1.1 Biofilm and its role in resistance

The overall resistance level or shielding properties displayed by biofilms towards antibiotics have been proposed by three hypotheses (i) that the biofilm serves as a diffusion barrier to the antibiotics enforced by the produced matrix that slow down the diffusion of the antibiotics into the biofilm, (ii) that few of the biofilm forming bacteria will differentiate into a protected phenotype, a dormant phenotype, which are highly protected to antibiotics (iii) that the biofilm

microenvironment, such as oxygen depletion, waste product accumulation and changes in nutrient availability, causes changes in the individual cells, figure 9.
The hypothesis of the diffusion barrier formed by the biofilm EPS matrix has been proposed to hold ground for the fact that some positively charged antibiotics, e.g. aminoglycosides (tobramycin) can bind to the negatively charged EPS, preventing the antibiotics to reach the cells encased in the depth of the biofilm but this has also been contradicted by presentation of readily penetration without killing of the cells [7, 184]. However it is highly improbable that this will occur to comparatively uncharged antibiotics as β-lactams [185]. It has been suggested that cells residing in biofilms grow slower in response to nutrient and oxygen deprivation and that this could account for the specific tolerance of biofilms. Yet other studies have suggested that the full magnitude of resistance cannot be subscribed to such mechanisms [184, 186].
The described efflux pump systems have not been considered an essential part of the biofilm resistance regime, as four of the best studied efflux pump systems in *P. aeruginosa* had no impact on the biofilm specific resistance in mature biofilms [168]. But it has later been suggested that the MexAB-OprM and MexCD-OprJ efflux pump systems are involved in biofilm resistance to azithromycin, showing the MexCD-OprJ essential for azithromycin resistance in developing biofilms [187].

The cells located at the biofilm surfaces are readily supplied with adequate amounts of nutrients and are metabolically active, while deeply embedded cells are more prone to have a slower metabolization due to reduced levels of nutrients and oxygen. Local oxygen limitations have been shown to diminish the effect of antibiotic *in vitro* with additive effects due to the presence of nitrate, a rich component in CF sputum[142, 188]. The metabolic rate and growth are highly controlled by the environmental factors, leaving the biofilm subdivided according to various metabolic stages. Several antibiotics have a more profound effect on the most metabolically active cells e.g. ciprofloxacin, tobramycin and tetracycline and this can be exploited when choosing antibiotics at the clinic.

7.1.2 Persister phenotype

Within the evolving biofilms a small fraction of cells is believed to display an exceptional form of multidrug tolerance. Cells in biofilms are slow growing and many are possibly in stationary state. A small fraction of these cells are thought to practically shut down cell division and go into a probable state of dormancy. This small subpopulation remain living irrespective of the antibiotic concentrations used [189]. These so called persister cells are not considered mutants as they overtly revert to reproduce the original wild type population upon reinoculation in an antibiotic free environment [190]. In that sense, this division of cells would be considered tolerant rather than resistant to antibiotic treatment [190, 191]. One hypothesis of the principal mechanism for persister formation is that they are phenotypic variants and are results of stochastic fluctuations of toxic proteins [192]. However, several genes appear to be involved in the persistence of *P. aeruginosa* [193, 194]. Quorum sensing has formerly been excluded as having a role in this phenomena, however a recent study showed that cell-free spent medium induces persistence in *P. aeruginosa* strains, but not in *E. coli* or *S. aureus* [190, 192, 195]. Both the phenazine pyocyanin and the acyl-homoserine lactone 3-OC12-HSL, which are normally secreted in the later stages of growth, significantly increased the number of persisters as well as on overexpression of *P. aeruginosa* LasR, which leads to increased levels of 3-OC12-HSL which resulted in increased tolerance to the fluoroquinolone ofloxacin [195, 196].

Mechanically, the *in vivo* situation of persister cells in CF would also be challenged by the deep embedment within the biofilm EPS. Phagocytes remain impaired in reaching the EPS embedded surviving persister cells after antibiotic therapy, setting the stage for recurrent *P. aeruginosa* infections.

8 Central problems in *P. aeruginosa* infection routes

Understanding disease transmission is fundamental for our possibility to predict and control epidemics as well as understanding virulence and routes of transmission between and within patients. In a regime of colonizing bacteria the race for nutrients and habitats is a constant battle for acclimatization to the surroundings. Infecting pathogens need to cope with changing environments and adapt to each specific niche and its specific challenges in order to persist. A central dogma in bacterial pathogenesis can be considered as micro-evolutionary adaptations to the fluctuations in the growth environments [197, 198]. There are many aspects that need to be taken into account when trying to pinpoint the central mechanisms for manifestation of diversity and maintenance of phenotypic diversity in infectious bacterial populations. Adaptive genetic traits as a response to local environmental changes take place as a result of natural selection [199], building the foundation for the establishment of a dominant population adapted to a specific niche [200, 201].

The *P. aeruginosa* infections in CF patients is a paradigm of how an environmental bacterium can become a successful pathogen able to persist in an atypical hostile environment, under selective pressures from therapeutics and immune defense mechanisms [40, 151, 201].

Importantly, *P. aeruginosa* has been shown as a successful biofilm former in the airways of CF patients adding structural complexity and nutrient patches to the environment [32, 141, 142]. Genetic variation and adaptive diversification readily emerge from populations growing in highly structural environments. Spatial complexity, as the various multifaceted biofilm structures, can be seen as a cradle for genetic variations and adaptive diversifications, in which the maintenance of the diversifying population can be foreseen due to a balance in fitness effects [200, 202].

Bacteria living in biofilm communities are subjected to a stressful environment of changing nutrient and oxygen concentrations [5, 7]. Stressful environments lead to stress-induced mutations by which the mutated bacteria prominently enhance the chance of adapting to challenges in the environment. Structured

environments give rise to different niches, which facilitate organization and a battle for survival of the fittest[203].

Apart from adaptation through regulatory processes, phenotypic variants arise when different niches are present in a heterogeneous environment. Rainey et al. 1998 have shown that when *P. fluorescens* was introduced into a spatially structured microcosm, the founding genotype rapidly diversified and gave rise to a collection of morphological diverse niche specific strains [200]. In an identical non-structured environment, no diversification had arisen in the incubation period. The impact on diversification through the biofilm mode of growth in flow cells, where heterogeneous distribution of cells leads to local changes in nutrients, leads to a rapid production of morphological different variants, see figure 10. Fast niche adaptation within the environment may generate populations of specialists that have adapted to and are populationwise maintained, however niche-specialists often suffer loss in overall fitness [204].

The acquisition of *P. aeruginosa* by CF patients is predominately accomplished from an environmental source [205]. The switch to the CF airways challenges the environmental bacterium by a fundamental alteration in growth conditions and environment. The CF airways are a heterogeneous environment and present several niches with different oxygen concentrations and a variety of endogenous stress factors as well as administered chemotherapeutics [141, 152]. Comparable to the microevolution studies in test tubes, the airways give rise to profound adaptation to the various niches with fluctuating environmental changes putting pressure on metabolic and physiological adaptation. Moreover, the impact of the immune defenses in the airways, through the production of e.g. oxygen radicals, have been associated with DNA damage in CF *P. aeruginosa* isolates and mutations in the genes responsible for DNA repair systems lead to the mutator phenotype [169]. Mutations arising during an infection of the CF airways create a foundation for arising variants that are fit to cope with the changing and hostile environment.

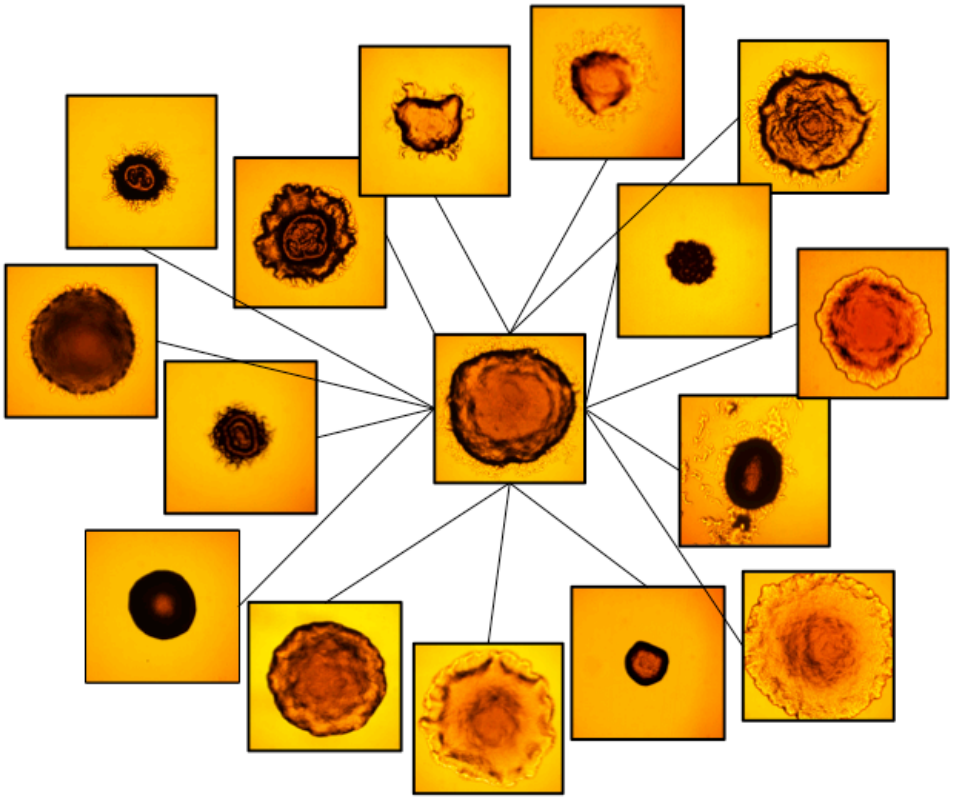

Figure 10. Morphotypic diversification in PAO1 flow cell biofilms. 4 day old PAO1 biofilms grown in flow cells (center coloni, from PAO1 original inoculum culture). The flow cell systems were set up as shown in[206] with a 10% LB media supply. At day 4, each channel was harvested with glass beads in 0.9% NaCl. The harvested biofilms were plated on LB agar plates and left overnight at 37°C before colony morphologies were screened with a Zeiss axioplan microscope with a x2.5 plan objective. Here showing 4 day old harvested biofilm however morphotypes were already found at day 2 (not shown), unpublished data Martin W. Nielsen and Fatima Y. Coronado, 2011.

Importantly, *P. aeruginosa* has proven itself highly capable of coping with the CF environments underlined by the fact that virtually all CF patients end up with a chronic *P. aeruginosa* infection, often with the same lineage persisting for decades [207]. Furthermore it has been shown, that CF patients, who receive a lung transplant are very often recolonized by the identical strain [208]. This gives strong indications on a scenario of the upper airways as a reservoir for reinoculations of the lower airways [133].

8.1 Bacterial epidemiology in the CF patient

The Copenhagen CF Center has since 1973 been storing *P. aeruginosa* isolate samples from CF patients [209]. This impressive collection of isolates has opened an opportunity for exploring the evolutionary path that has taken place since the start of the collection [4, 210]. Furthermore, a sinus surgery program has been initiated at the Copenhagen CF Center opening up a possibility to gain further insight into the foundation of the inescapable switch from intermittent to chronic infections. During intermittent colonization, and at the early stages of chronic infection, the genotypic diversity is high [207]. Early aggressive antibiotic treatments have been successful in temporarily eradicating the bacteria from intermittently colonized CF patients, however showing a pattern of recolonization by identical clones or environmental strains [211]. Intermittent colonization can range from several months to years with repeated cycles of treatment and recolonization [133]. The antibiotic penetration in the sinus compartments is less efficient and might allow for adaptive mutations that prime the bacteria to cope with the far more harsh environment of the lower airways [98, 210].

Interestingly, long-term chronic infections show a very low genotypic diversity and within the group of CF patients at the CF center, two dominant clones DK1 and DK2 (earlier denoted r and b) have been identified [207]. The two dominant clones have spread among the patients probably through exchange by patient-to-patient contact in the Copenhagen CF clinic. However, as case studies they are highly interesting. The strains must be considered as highly adapted to the CF airways as they are shown to be capable of outcompeting other strains or as co-infecting the patient as seen for patient 11,[207]. A large fraction of the chronically infected patients have furthermore been identified with the DK1 and/or DK2 lineages, with 43 patients out of 162 identified with the DK2 linage since 1973.

The DK2 is a highly successful and transmissible *P. aeruginosa* linage that has persisted for more than 200.000 generations in CF patients from the same cohort [4]. Over the many years the DK2 lineage has persisted in the CF community, it must initially have had to adapt to the stressful conditions of the

CF airways, having a high impact on the evolutionary progress of the bacterial population. In many ways one can parallelize the evolutionary events with the long-term study conducted by Richard Lenski and colleagues, although their experimental setup lack the environmental complexity of the host [212]. The DK2 linage has undergone an array of adaptive events to become a successful colonizer of the CF airways. Data analysis of genotypic and metabolic traits show that the early isolates genotypically show more similarity to the wild type PAO1 strain, see figure 11. The data indicate that an evolutionary path leading to major adaptive events in the DK2 lineage occur before 1979. The following period of 29 years were only characterized by few phenotypic changes, with early regulatory mutations seen in the *mucA, lasR and rpoN* affecting; expression of alginate production, quorum sensing and nitrogen metabolism and motility [4, 41, 85], respectively. The DK2 linage was shown to undergo an initial period of rapid adaptation. Interestingly, it was found that the adaptive processes leading to the

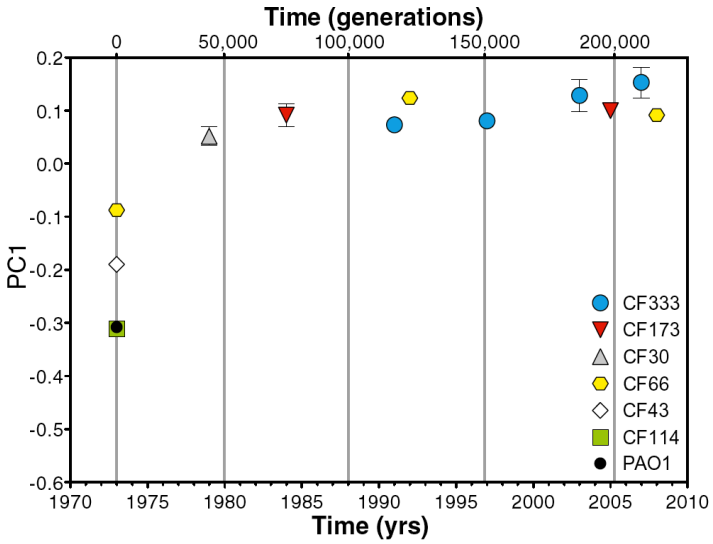

Figure 11. Phenotypic relationships and dynamics among DK2 isolates. Principal component analyses of the microarray expression data. Show a similar pattern for phenotypic biolog data (not shown). Data suggest that major changes occurred before 1979. First principal component (PC1) loadings for each isolate are graphed as a function of time (and estimated generations). PC1 represents 42% of the total variation among isolates. Error bars indicate standard deviations of biological triplicates of the same isolate, Yang et al, 2011[4].

chronic stage and persistence in the DK2 linage did not lead to high diversity in the individual patients, judged from phenotypic/genotypic comparisons in

sputum samples or from comparisons between patients infected with the DK2 clone [4]. Evolutionary trails furthermore leads to the foundation of specialists that are more fit to a discriminating environment but have a reduced fitness to other environments.

The CF lung environment is a heterogeneous habitat with several niches where microorganisms can grow in structured communities [138]. Several events as coughing, the use of the "airway clearing vest[7]", flushing of the airways etc., leads to geographic fluctuations of the bacterial distributions, accelerating interactions and competition. Adaptive mutations have the potential to endow evolving strains with the capacity to replace strains with lower fitness in a specific environment. However, if the genetic makeup leads to a reduced fitness relative to the ancestral or opposing strain, it will not be capable to outcompete the opposing strains though still being a specialist to the environment as it originally adapted to.

Traditionally competition experiments have been conducted in simple selective setups with growth rate as the measured parameter, however the complexity of the CF airways with the wide range of selective forces as nutrient levels, oxygen concentrations, antibiotics and ROS needs to be taken into consideration, described in the previous sections. This scenario is shown in Figure 12 with a fairly simple setup, that show the importance of one of such factors to evaluate how fit the adapted strain is relative to the ancestor. If two isolates are mixed in a 1:1 ratio without an environmental challenge, then the fastest growing isolate takes over, see table 1. At the 0μg/ml of colistin, the ancestral CF333-1991 strain with a doubling time of 56 min will take over the population by outcompeting the slower growing adapted strain CF333-2007 with a doubling time of 78 min. Between 24-48h CF333-1991 takes over (not shown data). However sub-MIC concentrations lead to a shift in fitness and hence dominating strain, Figure 12 C and D. CF333-2007 is more fit to the conditions that correlate with long adaptation in the CF environment.

[7] An air-inflated vest that pulsate to shake loose the accumulated mucus

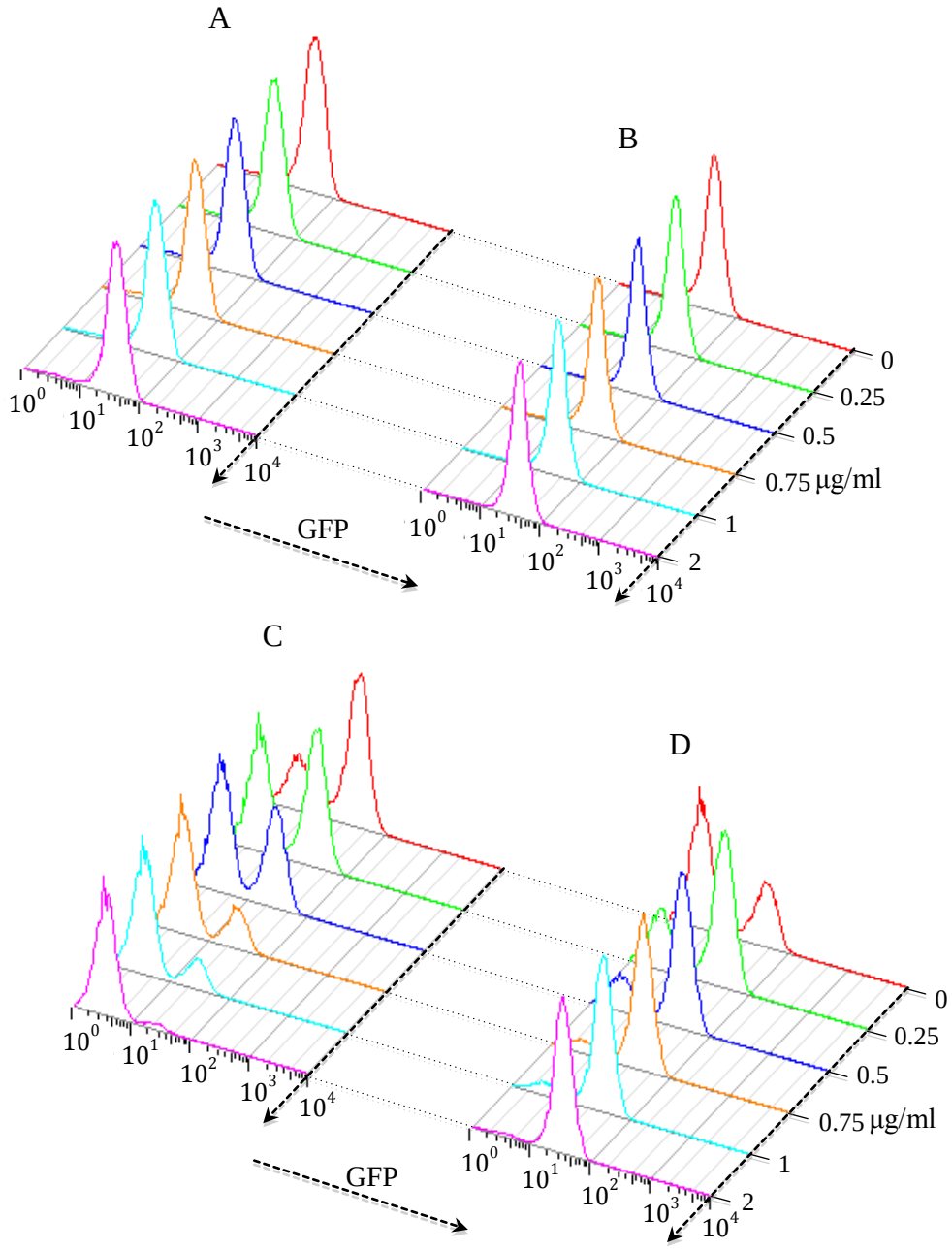

Figure 12 Competition experiment. Two DK2 *P. aeruginosa* isolates form CF patient CF333 competition in test tubes under different colistin concentrations. Competitions are set up in a 1:1 inoculum of each isolate, and allowed to grow together in a LB media with individual concentrations of colistin sulfate (Sigma Aldrich, Glostrup, Denmark) in 5 ml shaken test tubes for 24 hours before the samples were fixated in 2% paraformaldehyde and analyzed by Flow Cytometry (BD FACSCanto™II, BD Biosciences, San Jose, CA, USA). Histograms represent GFP- or non-expressing populations. Patient CF333 isolates CF333-1991 and CF333-2007.
A and B represent the individual isolates grown for 24h in LB media under the specific colistin concentrations, A) CF333-1991-GFP and B) CF333-2007-GFP.
C and D represent identical isolates as in A and B +/- GFP expression of the strains, inoculated 1:1. C) CF333-1991-GFP vs. CF333-2007 and D) CF333-1991 vs. CF333-2007-GFP. Unpublished data, Martin W. Nielsen et al. 2011.

Table 1

Strain	*in vitro* doubling time (min)	MIC Colistin	*mucA*	*algU*	*rpoN*	*lasR*
CF333-1991	56	7μg/ml	ΔG430	A55G	T1256C	deletion
CF333-1997	59	>256μg/ml	ΔG430	A55G	T1256C	deletion
CF333-2003	74	>256μg/ml	ΔG430	A55G	T1256C	deletion
CF333-2007	78	>256μg/ml	ΔG430	A55G	T1256C	deletion

Table adapted from Yang et al. 2011, supplementary data table S1[4]. Inserted MIC values from Lei Yang unpublished data, [213]

Conducting experiments to measure the adaptive traits and changes in the relative fitness in order to gain insight into the evolutionary trajectory in microbial populations, sets the requirement for integration of selective and community shaping forces. The impact of the information will provide significant contribution to understanding aspects of evolutionary biology. Recognizing these aspects, will contribute to sculpting directed treatment strategies suited for both generalists and niche specialists that might be dominating the different stages of infection.

For obvious reasons is not possible to conduct infection studies with CF patients. Therefore, an immense amount of work has gone into developing both *in vitro* and *in vivo* model systems to investigate *P. aeruginosa* infections and the individual aspects thereof. Conducting long-term experiments to study the evolutionary traits of *P. aeruginosa* in the CF airway environments, is highly problematic though as the available models do not embrace the complexity of airways as *in vitro* systems or animal models are either practically inaccessible or do not recapitulate the human disease, see section 9. This is the foundation for the Ph.D. project; to construct *in vitro* models for future investigations of e.g. fitness measures, inter-airway transmissions, adaptability to applied stresses and competition with oxygen as the key component.

9 Model systems for CF investigations

9.1 Experimental systems – patients

Culturing bacterial specimens from CF patients remains important both diagnostically and for *in vitro* studies and as a resource for subsequent genotypic analyses. *P. aeruginosa* samples from CF patients have made the foundation for the unique collection for the long term evolution study conducted on isolates from the Copenhagen CF center [4, 207]. Samples from patients are accessible through different sampling processes. Bronchoalveolar lavage fluid (BALF) is considered a gold standard for detection of pathogens in the lower airways of CF patients and might be the solution in young children, who have difficulties in producing coughed up sputum samples [214]. However, as BALF samples require the use of a bronchoscope and is time consuming, sputum samples are far more accessible. Lungs from transplanted or succumbed patients, give direct access to the most distal generations in the lungs [5, 138]. Such direct access to aggregates from both the conductive and respiratory zones of the lungs, have given profound insight to the vast biofilm formation with heavy PMN infiltration [138].

Performing infection studies on CF patients are obviously not possible, therefore the use of *in vitro* and *in vivo* model systems is essential for accomplishing such studies. However such systems might not perform to a sense that allows direct extrapolation to the human CF pathology.

9.2 *In vivo* models for cystic fibrosis

For many years the use of animal models as substitutions for the human organism has been a central tool for scientists in order to investigate human diseases. The production of transgenic animals has been the central turning point for investigations of specific cellular functions. Knocking out or replacing genes for resulting nonfunctional or altered phenotypes, has accomplished this. Several animal models have been produced to investigate traits in chronic *P. aeruginosa* infection studies. Animal studies enable repeated experiments, which is a valuable advantage when performing infection studies. Model species include several rodent models as rat [161], mice [215-217] and guinea pigs [218,

219] as well as other species like ferrets[220], monkeys [221] and the recently produced CF pig [222, 223]. The ongoing effort to produce alternative animal models has had the purpose of generating models capable to recapitulating the development in CF pathology. CF airway and pancreatic disease progression show scarce resemblance between mice and humans [224]. For many years mice have been the primary in vivo models for CF research. This is primarily due to the obvious reasons as low costs, ease of production and handling. It only took three years after the identification and cloning of the CFTR gene until the first CF mouse model was reported [215]. However mice do not acquire spontaneous and chronic infections as seen with human CF patients [225]. $Cftr^{-/-}$ mice do not show the same upregulation of ENaC as seen in the lower airways of CF patients [226, 227]. Attempts to reproduce the effect of mucus accumulation in the airways of mice have been accomplished by overexpression of ENaC as well as induction of mucus overproduction [228, 229]. Several groups have attempted different methods to inoculate the lungs of CF mice with bacteria in order to reproduce more chronic traits as evident in the human infections. Methods have generally been focused on mechanical confinement of the bacteria in the lungs of the mice by embedment of the inoculating bacteria in agar, agarose or alginate beads, resulting in a more successful defect of bacterial clearance [216, 230-232]. Successful studies with non-embedded bacteria have been conducted with mucoid *P. aeruginosa* strains [233, 234].

CFTR knockout pigs have been shown to develop cystic fibrosis disease in multiple organs comparable to human CF patients. The piglets did not show any signs of lung disease or signs of infection or inflammation in the first days of life and both $CFTR^{-/-}$ and $CFTR^{F508del/F508del}$ developed lung disease within the first months of their life [235]. Stoltz *et al.* 2010, showed that the CF pigs are impaired in clearing bacteria just after birth in the absence of inflammation. They furthermore state that over time, the CF pigs show several CF hallmark features of inflammation, mucus accumulation and infection as seen in the human CF patients[235, 236].

However complications with the models are severe as CRTR-deficient piglets develop meconium ileus[8] and surgery is required in order for the piglets to survive [235]. Production of the CF pigs is indisputably an important research tool for CF research, that will answer many questions, but it is also a model accompanied with high financial costs. For this reason it is restricted to a small fraction of the CF research community.

In conclusion, differences in disease traits in various organs do not make the majority of CF animal models ideal for especially long-term infectious studies as anatomic, physiologic and differences in intrinsic defense mechanisms gives rise to limitations of the models [230].

9.3 *In vitro* models for cystic fibrosis investigations

9.3.1 Universal Test tubes

Test tubes have been used for hundreds of years to grow microbial cultures and still remain an essential tool in research. Immense amounts of research have been conducted with planktonic cultures as they are easily manipulated in laboratory settings and grow in e.g. microplates, flasks, test tubes, chemostats and membrane diffusion chambers. Such experimental setups are substantial tools for a wide range studies of e.g. culture developments, growth rates, antibiotic susceptibility tests and long term evolutionary experiments [237]. Easy system manipulations provide suitable environmental conditions for investigating desired microorganisms and culture combinations. The simple test tubes are the obvious choice for initial tests, fast visualizations of e.g. aggregations, biofilm capabilities, antibiotic tests etc. However test tubes are highly limited in the slightest comparison to the natural habitat of microbes. One such limitation, is how to extrapolate data to the microorganism's natural habitat, as the culture systems might be even too artificial, with e.g. less than 1% of the 10^9 bacterial cells/gram soil are estimated to be culturable in laboratory

[8] condition that leads to obstruction of the intestine

media [238].

Furthermore, as biofilms are considered the major general natural lifestyle of bacteria, (see section 3) and as transitioning from planktonic cultures to the biofilm mode of growth highly differ in gene expression and regulation, the comparison to a natural habitat becomes even less distinct [239]. However, screenings in microplates for phenotypic profiles (Biolog), biofilm formation, competition experiments among others are highly beneficial in fast screenings for selection of interesting candidates from e.g. recovered *P. aeruginosa* isolates from CF sputum samples.

9.3.2 Cell lines

In addition to available animal models, the development of immortalized cell lines has had a substantial positive impact on biochemical, cytotoxic, genetic, physiological studies for many years. In CF research immortalized cell lines have contributed to the understanding of the underlying mechanisms of CF due to the scarcity of tissue and primary cell material for such studies. Airway epithelium has been the main focus of immortalized cell lines, leading to the availability of a variety of WT and $CFTR^{-/-}$ cell lines [240]. *In vitro* cultures of immortalized epithelial cells from the airways of both CF and non-CF individuals, have made important contributions to characterization between the CFTR genotype and disease phenotype [241]. Such cell lines have furthermore been indispensable in high through put screening for the development of new therapeutics for CF treatment [240].

The airway epithelium is an essential environmental interface and epithelial cells have been cultured from both animal and human airways. Both primary and immortalized cell cultures can present the specific cellular properties but lack the structural complexity of the organs. Most immortalized cell lines do not maintain proper cellular differentiation and the specific cell properties [240]. Ongoing work is carried out to establish new and improved cell lines for CF research [242].

9.3.3 Biofilm investigations – methodology

Due to the notion that microbes primarily live in biofilm communities, an immense amount of work has gone into refining ways to obtain detailed information on bacterial biofilms.

One crude high throughput biofilm screening method is firstly based on the adhesive properties of microorganisms to microtiter plates followed by biomass detection by a staining with crystal violet. This method is a highly useful tool for screening purposes in the identification process of genetic evaluations for important biofilm promoting genes, however it is a very crude and error prone method [243, 244].

Aspects of biofilm formation at the air-liquid interface can be addressed by angling the culture chamber, allowing the biofilm to establish and inspect by phase-contrast microscopy [245]. This type of static assay utilizes visualization aspects compared to the pellicle formation assay that relies on properties as intercellular adhesion of surface proteins in order to sustain a floating phenotype [117, 246]. Two tremendously high resolution visualization methods are: Atomic Force Microscopy [247], a member of the scanning probe microscopy family; and electron microscopy [248]. These methods rely on an altering of the specimens for visualizing measures, making each sample time point measurements with no possibility for continuous biofilm investigations. A big leap forward arose with the Confocal Laser Scanning Microscope (CLSM) which was developed towards the 1980s. This specific type of microscope has the possibility of obtaining high-resolution optical images at selective depths. The advances in different staining techniques have allowed researchers to gain insight into *in vitro* biofilms from patient samples by the utilization of various fluorescent staining techniques or the by expression of various reporter proteins.

The flow cell system is one of the most important contributors to studies on microbial biofilms, figure 13.

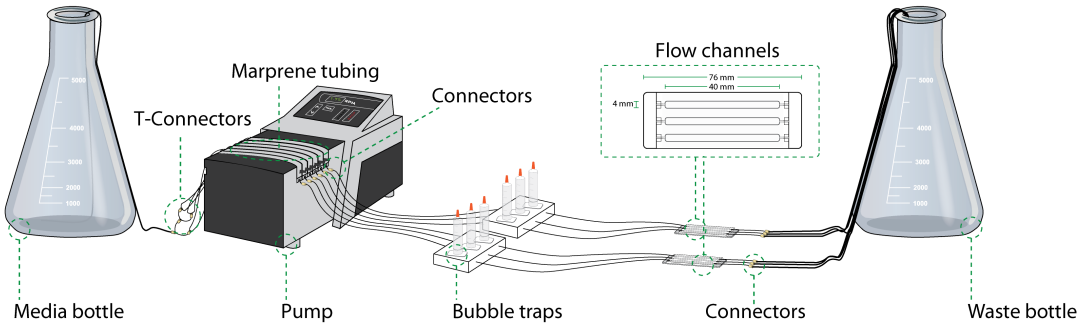

Figure 13. Graphic representation of the assembled flow cell system[6]

In combination with the confocal microscope and various staining techniques, the flow cell system becomes an impressive tool for niche specific investigations of microbial biofilms, figure 13. Furthermore it is an efficient tool for analyzing the distributive effects of antimicrobial agents and other environmental factors [98, 184, 188, 191]. Confocal images are acquired at section by section and collectively assembled in computer programs as the Imaris software (Bitplane AG) to generate 3D representative images [249]. Additionally the biofilm data can be quantified with computer programs such as COMSTAT [250, 251]. However, biofilm formed in these systems is an "artificial biofilm" with biofilm propagation on a glass substratum compared to the *P. aeruginosa* biofilms in the airways of CF patients [138]. Furthermore, the flow cell systems are mono-compartmental and lack the dynamics of the CF airways.

10 Microfluidic systems – organ on a chip

To imagine possible microfluidic applications one could look at how nature through millions of years of evolution has sculptured many parts of the mammalian physiology into an amazing network of microfluidic systems. These systems are essential for us to function as humans. Examples of such structurally specialized systems are the lymphatic- and cardiovascular systems as well as the fundamental nephrons in the kidneys. Essential to these systems is the fact that minimizing distances between compartments allow specific laws of physics to dominate. Diffusive properties are the ruling forces that drive gas exchange between the blood capillaries and the respiratory segments of the lungs (alveolar sacs, duct, bronchioles), principles and ideas that I have taken into consideration in my works.

The uses of microfluidic systems have expansively progressed in the world of biology during the last decades. Many beneficial aspects to microfluidic systems have proven their worth in several aspects due to the small volumes and benefits of the large surface area to volume ratios [252]. Only small volumes are needed in such systems thereby reducing cost for reagents, expensive drugs in approval trials and reducing amounts of chemical waste produced.

The most pronounced effort has primarily gone into the development of systems for evaluation of cellular behavior to soluble components. The world of microfluidics has many application possibilities that will gain further ground within the next decades, as the advances in micro- and nano-fabrications are constantly progressing.

Researches are equipped with several options for studying isolated proteins and cultured cells, however whole animals or newly dissected body parts are needed for tissue experiments and do raise a series of ethical issues. The concept of “organ on a chip” is still a work in progress, however single compartments and functionalities of organs have already been constructed in microfluidic systems. Conducting animal studies are very costly and in some instances not even good enough models for representing the human disease in question, as discussed in section 9.2.

Several projects have gone into the fabrication of micro-devices that can generate identical physiological patterns of the organs in question. One of the important applications for microfluidic systems is the refinement for drug testing. *In vitro* cultures of liver cells have been extensively studied as many drugs fail in clinical studies because they have damaging effects on the liver or because of the toxicity of the liver metabolites [253, 254]. Preclinical drug trials on animals have proven to be insufficient for toxicity evaluations due to species-specific differences between humans and animal hepatocytes [254, 255]. Several groups have looked into fabrication of microfluidic systems for specific organ representative devices. Among these devices are microfluidic systems for bone [256], muscle [257], gut [258], kidney [259] and the gastrointestinal tract[260, 261]. Sung *et al*. 2010 used a microfluidic system to investigate the toxicity of an anticancer drug on cultures of liver, lung and bone marrow cells. This was achieved in a device with interconnections between the cell chambers, mimicking organ-organ interactions [262]. Günther *et al*. 2010 combined microfluidics with surgically removed arteries to study the small vessels ability to prevent the blood from flushing into and damaging the capillaries [263]. Such combinations can bring down the number of animals used over time as it allows more accurate setups and have even improved reproducibility and imaging [263]. Studies on imitating lung functionalities and gas exchange have been attempted by microfluidic approaches. Potkay *et al*. 2011, recently published their work on a device that will efficiently ventilate the blood without pumps or pure oxygen supplies, unfortunately the system remain far from being clinically relevant [264]. Huh *et al*. 2007 have performed experiments to investigate induced cellular injury upon small airway epithelial cells inside a microfluidic device [265]. A functional study of an *in vitro* lung model was produced by the same group years later, where they constructed a multi-compartment microfluidic device that enabled vacuum-induced stretching of a PDMS membrane seeded with endo- and epithelial cells [266]. Several aspects of a normal functional lung were investigated and suggested synergetic effects of physiological mechanical stresses due to breathing could act in synergy with

nanoparticles accelerating toxic effects in the lung. They also conducted TNF-α stimulation for ICAM-1[9] expression on the endothelium facilitating adhesion to the activated endothelium of neutrophils. Adherent neutrophils were shown to squeeze through the cell layers and pores in the chamber separating PDMS membrane. The system was used to mimic the innate immune response to a bacterial pulmonary infection by migrating neutrophils' engulfment of live E. coli deposited on the epithelial side in the device [266].

[9] ICAM-1: Intercellular adhesion molecule-1 is a surface expressed adhesion molecule that is important attachment anchors for e.g. circulating immune cells.

11 Fabrication and oxygen sensing

11.1 Materials for fabrication

Many work aspects with microfluidic systems are defined by the way of fabrication and choice of materials. Several fabrication methods can be used in order to make the microstructures needed for the desired designs.

Silicon is one of the best known and purest materials known with a high demand in fabrication of electronics as microprocessors, memory circuits, microstructures and was initially also used for fabrication of microfluidic devices [252]. However, due to inflexibility, cost of production, need for special fabrication facilities and lack of optical transparency, thermoplastics and silicone rubber materials have become the primary materials for microfluidic fabrications. The poly(dimethylsiloxane) (PDMS) elastomer have become a preferred material due to ease of rapid prototype fabrication and ease of bonding to other materials. Besides being a very biocompatible material PDMS is furthermore impermeable to water but very permeable to gasses [267]. These properties have been used in the studies of constructing the human airway model biochips (paper II-VI).

11.2 Methods for fabrication of microstructures

Soft lithography techniques allow replication of PDMS microfluidic devices by the use of fabricated mold systems. Several techniques as e.g. photolithography or micro-milling can be used for fabrication of the mold [268-270]. The mold holds a template of the designed structures to be formed where to a PDMS two-component pre-polymer mixed with a cross-linking agent is poured and allowed to cure at moderately elevated temperature[269] (paper II-V). To obtain a functional micro-channel device, the PDMS model must furthermore be bonded to enclose the fabricated micro-structures. The highly gas permeable properties of the cured PDMS polymer can be abolished by conformally shielding it with low permeable materials (paper IV).

Microstructure fabrication can furthermore be accomplished by laser ablation. This method requires a high-powered pulsed laser to remove material from a surface. The lasers used for ablation rely on light absorption in the material as well as the pulse rate produced by the laser. The pulsed excimer UV laser is one of the highly used lasers as most plastics absorb UV light (193nm, ArF), (248 nm, KrF) and is for this reason used for ablation in materials as PS, PC, PVC and PMMA [271, 272] whereas CO_2 lasers function in the infrared region (10.6 μm) and are typically used for micro-fabrication in PMMA. An advantage of the laser ablation method is its use for precision cutting in different materials such as membranes and foils [273-275].

Milling is a very flexible method for structure fabrication in a wide range of different materials. Even though the tools for precession milling are scaled down to the range of 5μm it is a method that produces a high roughness in the materials. The milling method is not capable of achieving very small features in the polymers but will be able to produce reproducible structures down to 100μm [252]. Several parameters need to be taken into consideration for successful fabrication such as the right size tool, coolant and setups that eliminate vibrations in the polymer. The method has limitations regarding the choice of material as soft polymers are problematic due to their elastic properties, however it has been accomplished by applying croygenic cooling to the polymer during the milling process[276].

11.3 Assembly of microfluidic devices

Many fabrication methods are possible for producing microfluidic systems in a wide range of different polymeric materials, however no matter which fabrication method is employed, sealing of the open channels is necessary in order to finalize the fabricated fluidic paths. There are different considerations to take into account when selecting the method of bonding, especially if integration of materials of other chemical and functional properties into the device is desired. The strength of the bonding is a critical consideration for generating a functional seal between the two substrates. Important considerations for the interface between the substrates are surface chemistry,

optical considerations and material compatibility. Thermoplastics are comprised of a wide range of polymers that, as elastomers, change their physical properties near the glass transition temperature (T_g). The T_g temperature is specific for each polymer and is the specific temperature at which the polymer reversibly changes in viscosity [277]. The transition point in the material viscosity can be utilized to bond different kinds of thermoplastics with compatible T_g to each other. The process of thermal fusion bonding requires that the materials are heated to temperatures near T_g. When the materials are assembled pressure is applied to increase the mating contact forces [277]. Exposures to either plasma or UV light are successful ways to lower the T_g of different materials, which can improve the bonding strength [277-279].

Enclosing the microfluidic channels between two polymers, can furthermore be accomplished by solvent bonding [280]. The solvent bonding method utilizes the thermoplastic solubility in specific solvents to attain entanglement of the polymer chains at the interface between the substrates [277](papers IV-VI). Rapid prototyping as well as bonding materials of different polymer groups can be accomplished by the use of pressure sensitive adhesive tapes (PSA) [281]. The ease of pattern cutting in PSA enables fast and easy variations in shapes and sizes. This method has been used in papers (II-IV).

11.4 Oxygen sensing and control of oxygen concentrations

In cell-based applications, the potential to generate micro-environments with controllable oxygen concentrations will enable investigations in environments that have substantial influence on cell development and function. With the talks of monitoring and controlling the oxygen concentrations, the permeability of the selected materials is of crucial importance. The choice of material needs to satisfy criteria as biocompatibility and low oxygen permeability. Table 2 shows the oxygen permeability of different polymers, which are highly used in fabrication of microfluidic devices. Furthermore each polymer realizes a range of different properties with respect to e.g. stiffness, transparency, T_g, hydrophobicity as well as the gas permeability.

Polymer	Oxygen permeability			
	Barrer	$cm^3 \cdot mil \cdot 100inch^{-2} \cdot 24h^{-1} \cdot atm^{-1}$	$cm^3 \cdot mm \cdot m^{-2} \cdot 24h^{-1} \cdot atm^{-1}$	$m^3 \cdot m \cdot m^{-2} \cdot s^{-1} \cdot Pa^{-1}$
PDMS	800.0	133 333	2 067 183	$6.00 \cdot 10^{-15}$
VITON®	14.0	2 333	36 176	$1.05 \cdot 10^{-16}$
FEP	4.5	748	11 600	$3.37 \cdot 10^{-17}$
COC	2.6	428	6 641	$1.93 \cdot 10^{-17}$
PC	1.8	302	4 677	$1.36 \cdot 10^{-17}$
PMMA	1.4	225	3 488	$1.01 \cdot 10^{-17}$
ETFE	0.6	100	1 550	$4.50 \cdot 10^{-18}$
PEEK	0.1	20	309	$8.97 \cdot 10^{-19}$

Table 2. Oxygen permeability of different polymers: PDMS- polydimethylsiloxane, FEP - fluorinated ethylene propylene, COC - cyclic olefin copolymer, PC – polycarbonate, PMMA - poly(methyl methacrylate), ETFE - poly(ethylene-co-tetrafluoroethylene), PEEK - poly(ether ether ketone) (table from Skolimowski, M.[282])

Several different functional microfluidic devices produced in PDMS have been applied to biological applications [283]. PDMS is a chemically inert, thermally stable, permeable to gasses and biocompatible material, properties that are all favorable in cell culture applications [267]. The material's permeability to vapor and gasses makes it ideal for separation of liquids of different composition with possibilities of desired uniform gas concentrations. Fabrication of microfluidic devices, where PDMS is implemented as a separation between liquid media channels and gas channels, will allow control of e.g. dissolved oxygen by the composition of the gas mixture [284, 285]. The desire for conducting experiments with oxygen concentrations of above air saturation will require pure oxygen supply. When desired the use of oxygen scavenging liquids, to control oxygen levels in microfluidic devices, reduces the complexity of the setup.

Incorporating PDMS into microfluidic devices enables the vast diffusion of gasses across the PDMS polymer. As seen in Table 2, several of the polymeric materials have a considerably lower gas permeability compared to PDMS. PEEK has the lowest permeability of the polymers listed. The PEEK polymer is a non-

transparent polymer that is a very resilient and biocompatible material however is has difficulty in bonding [286-289]. However constructing devices in low gas permeable materials is desirable in transparent and cheaper polymers such as polymethyl methacrylate (PMMA) or polycarbonate (PC) [290, 291]. PC is a very low cost material with high robustness and a high Tg temperature, however it has very poor resilience to certain organic solvents and high absorbance in UV [290], (paper IV-VI). The slightly less gas permeable PMMA is often used for injection molding purposes of mass productions, however most thermoplastics can be used for injection molding. It is a chemical and biological compatible material with good bonding capabilities and with its low cost one the most preferred thermoplastics for microfluidic fabrications [291](paper II-III).

11.4.1 Oxygen scavengers

Implementing oxygen into experimental setups often rely on the use of connected gas containers. Besides being a safety precaution, gas tanks set restrictions to flexibility and maneuverability. The use of scavenging liquids have successfully been used to prevent oxygen corrosion of metal surfaces as well as preservatives in food [292]. The direct use of oxygen scavenging chemicals in the media of biological systems will alter the chemical environment for the cells and could furthermore have toxic effects [292, 293]. But the use of scavenging solutions can though eliminate the use of gasses to control oxygen environments, however separation between the cell culture environments and scavenger is required. Materials with high oxygen permeability as silicone rubbers (PDMS) can be utilized as separating membrane material [294] (paper II, III and IV)

11.4.2 Oxygen sensing

Integration of oxygen sensors within microfluidic devices allows quantification of oxygen concentrations in integrated cell culture environments, being a powerful tool for evaluating the oxygenic effects on cell viability, behavior and consequences for drug efficiency.

Early work with oxygen sensors has been focused on the Clark-type electrode sensors that record a current flow that is caused by the reduction of oxygen

[295]. The integration of such electrodes does however require miniaturization of the sensors to microscale. Miniaturized sensors have been developed and integrated into microfluidic devices where they have been used in measuring oxygen consumption by *E. coli* [296]. For long-term measurements however the electrode will easily be contaminated besides adding complexity to the setup by requiring electrical connections and furthermore it consumes oxygen in itself. To circumvent such disadvantages for measurements in microfluidic devices there is a lot of interest in integration of optical detection methods.

Many of the optical oxygen sensors function by the principle of reversible luminescence quenching by the presence of oxygen. Oxygen absorbs the energy that would have been emitted as a luminescence photon [297]. Various oxygen sensitive indicators are available and mainly fall into two main categories, the ruthenium complexes and metalloporphyrin type molecules. The most commonly used oxygen sensing compounds include the ruthenium-based Ruthenium-tris-4,7-diphenyl-l,l0-phenanthroline and Ruthenium(II)-tris(l,l0-phenanthroline)[298, 299]. Ruthenium complexes have a very short lifetime in the state of excitation (100ns - 1μs), which means a lower sensitivity to oxygen. In low-oxygen environments, as anaerobic bacterial cultures, it is necessary to use highly sensitive oxygen sensors as metalloporphyrin based types that have a longer excited lifetime state (10μs - 1ms) and thereby a higher sensitivity to oxygen [300, 301]. The metalloporphyrin based probes are primarily based on the platinium(II)- and palladium(II) complexes of actaethyl-porphyrin (Pt- and Pd-OEP) (paper III) [302, 303]. They exhibit long luminescence lifetime but have somewhat modest photostabilities which is why other complex Pt- and Pd-derivatives of octaethyl-porphyrin ketone (Pt- and Pd-OEPK) have been developed with higher photostability [304]. Intracellular sensing complexes have furthermore been proposed for the probe that comprise the Pt(II)-meso-tetrakis-(pentafluorophenyl)porphyrin with a nanoparticle of a cationic polymer [305, 306] (paper IV). The metalloporphyrin compounds are predominantly embedded in a polymer or sol-gel matrix, as certain kinds of metalloporphyrins are not soluble in water [300, 307].

Embedment of the probe in a polymer or sol-gel matrix is very often carried out

in order to immobilize the sensor to a substrate and shield off the oxygen sensor to the cell culture media for longer durability of the sensor and shielding of unwanted side effects in the cultured organism [300, 307, 308]. The polymer matrix must be biocompatible to be used with cell culture systems as the organic modified silica (ormosil) and sol-gels that have been optimized for optical properties as well as good oxygen permeability [309].

12 The microfluidic systems of the thesis

The extent of the work presented in this thesis has primarily been focused on the development of new systems for investigating bacterial biofilms. Ideas for the systems were inspired by the knowledge of the physiological events in the CF airways. An extensive amount of work has gone into optimizing the emerging systems in order to adapt and improve the developed prototypes.

The first task pursued was to translate essential parts of the established flow cell system (section 9.3.3) into a microfluidic setup, which quickly generated a range of challenges. Leaking systems were the most profound and problematic part of the optimizing steps as small leakages can terminate entire systems. Gas bubbles generate massive inconsistencies in flow systems, especially in microfluidic channels, where they obstruct channels and lead to change or bring the liquid flow stream to a halt. Many tests have been terminated due to gas bubble formations and have lead to the development of systems to avoid bubble formations in the microfluidic devices. A vast numbers of biological trials have been conducted in order to optimize the systems to the versions presented in this thesis. Many prototype versions are not presented in the thesis, but have been an extensive part of the work of this thesis and furthermore the foundation for the final presented versions. The systems have been revised and redesigned in order to cope with material inconsistencies, requirements of sterility, detection methods and formation of bubbles in the systems.

The presented systems are finalized designs and functionalized microfluidic systems to mimic physiological important scenarios of the bacterial infections taking place in micro-environments of CF airways. The systems have the capability of controlling and monitoring the oxygen environment within the culture chambers. Moreover, bacterial migration between the different oxygen environments is established in one system. A compartmental modeling of the human airways and CF bronchi in a microfluidic approach that has previously not been reported is presented here. Ideas from the microfluidic systems have furthermore been transferred to the established flow cell system in order to circumvent bubble formation in the flow chambers (not presented in the thesis).

On of the most important achievements with the microfluidic devices is the incorporation of a gas permeable membrane, which is an essential component that allows control of dissolved oxygen concentrations in the growth chambers (paper II-III). Within the growth chambers oxygen gradients are steered throughout the length of the channel, facilitated by an oxygen scavenging liquid. A successful “easy to fabricate” and “easy to handle” system was produced and allows easy oxygen control of the oxygen environment, within the culture chamber. *P. aeruginosa* PAO1 was tested for its capability of initial attachment to the substratum, and for several days of cultivation, in a system with lowered oxygen concentrations.

A development was made to advance the oxygen controlling systems to produce a more physiological relevant approach in order to mimic the previously subdivisions of the airways, the micro-aerobic/anaerobic niche of the conductive airways, the oxygen rich respiratory areas and the essentially anaerobic sinuses. These different compartments gave the inspiration for the initiative toward making a microfluidic system of the airways, the Microfluidic Airways Model – MAM. The micro-chambers in the presented model allow cultivation in oxygen environments set to mimic the three airway compartments. The assembled modular system is highly versatile, with modules that can easily be exchanged for a wide range of alternative setups. The extraordinary complexity of the system increased the amount of trials that has gone into validating the material’s capabilities for sterilization, pressure endurance and cultivation of bacteria became one of the most time consuming missions before finalizing the presented system (paper IV).

The module for mimicking the three compartments was finally achieved by interconnecting media of different oxygen saturation levels, figure 14. The system allows connections to be made between the chambers, created in a manner that allows opening and closing between the chambers.

For bacterial cultivation, this allows free movement between the connected compartments mimic-king the rather free movement in the human airways.

One of the most fundamental occurrences in the CF airways is the impaired mucociliary clearing system and accumulation of mucus in the airways. To mimic this, a system was developed that would be able to imitate the accumulated mucus in the airways, as a CF bronchi (paper V and VI). The idea of the chip was to induce a solid support, which was realized by the introduction of an alginate hydrogel. The separate channels are mimicking the air lumen and the arteries of the bronchi. The membrane furthermore supports epithelial cells that are cultured on the bottom side of the membrane. The cells are supplied with nutrients from the channel that can diffuse across the membrane-associated barriers and diffuse into the hydrogel, figure 15.

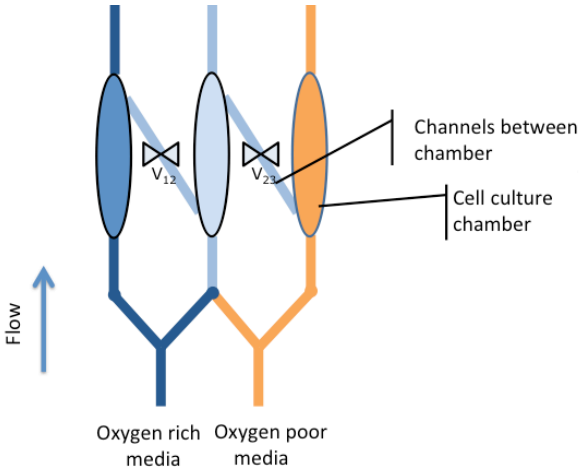

Figure 14 Media interconnections MAM model (paper IV)

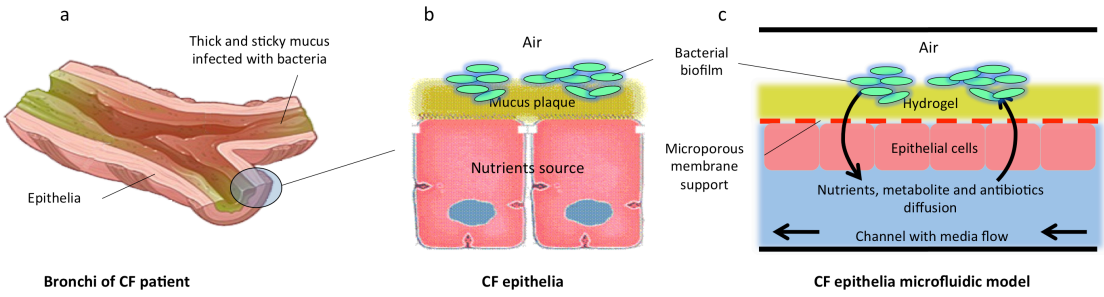

Figure 15 Presentation of the founding idea for the microfluidic device to mimic the CF bronchi. a) Representation of the mucus accumulation in the CF bronchi, b) schematic visualization of the circled section in a., c) illustration of the model for the microfluidic interpretation of the mucus covered CF epithelia.

It present a ready system set for bacterial inoculation and subsequent confocal microscopy visualization of the forming bacterial micro-colonies.

The introduced systems are presented in the following publication section, which include a DVD (backcover) with the video file from the paper I.

13 Publications

13.1 Paper I

Martin Weiss Nielsen[1], Claus Sternberg, Søren Molin, Birgitte Regenberg: *Pseudomonas aeruginosa and Saccharomyces cerevisiae biofilm in flow cells.* J. Vis. Exp. 2011 Jan 15;(47) pii2883. Doi: 10.3791/2383 (video included on DVD)
http://www.jove.com/Details.php?ID=2383

Video Article

Pseudomonas aeruginosa and *Saccharomyces cerevisiae* Biofilm in Flow Cells

Martin Weiss Nielsen[1], Claus Sternberg[1], Søren Molin[1], Birgitte Regenberg[2]

[1]Department of Systems Biology, Danish Technical University
[2]Department of Biology, University of Copenhagen

Correspondence to: Martin Weiss Nielsen at Mawen@bio.dtu.dk, Claus Sternberg at cst@bio.dtu.dk

URL: http://www.jove.com/video/2383/
DOI: 10.3791/2383

Keywords: Immunology, Issue 47, Biofilm, Pseudomonas aeruginosa, Bacteria, Yeast, Saccharomyces cerevisiae, Flow cell system, Confocal Lases Scanning Microscopy, Microbiology, FLO11, Systems biology,

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Citation: Weiss Nielsen, M., Sternberg, C., Molin, S., Regenberg, B. Pseudomonas aeruginosa and Saccharomyces cerevisiae Biofilm in Flow Cells. J. Vis. Exp. (47), e2383, DOI : 10.3791/2383 (2011).

Abstract

Many microbial cells have the ability to form sessile microbial communities defined as biofilms that have altered physiological and pathological properties compared to free living microorganisms. Biofilms in nature are often difficult to investigate and reside under poorly defined conditions[1]. Using a transparent substratum it is possible to device a system where simple biofilms can be examined in a non-destructive way in real-time: here we demonstrate the assembly and operation of a flow cell model system, for *in vitro* 3D studies of microbial biofilms generating high reproducibility under well-defined conditions[2,3].

The system consists of a flow cell that serves as growth chamber for the biofilm. The flow cell is supplied with nutrients and oxygen from a medium flask via a peristaltic pump and spent medium is collected in a waste container. This construction of the flow system allows a continuous supply of nutrients and administration of e.g. antibiotics with minimal disturbance of the cells grown in the flow chamber. Moreover, the flow conditions within the flow cell allow studies of biofilm exposed to shear stress. A bubble trapping device confines air bubbles from the tubing which otherwise could disrupt the biofilm structure in the flow cell.

The flow cell system is compatible with Confocal Laser Scanning Microscopy (CLSM) and can thereby provide highly detailed 3D information about developing microbial biofilms. Cells in the biofilm can be labeled with fluorescent probes or proteins compatible with CLSM analysis. This enables online visualization and allows investigation of niches in the developing biofilm. Microbial interrelationship, investigation of antimicrobial agents or the expression of specific genes, are of the many experimental setups that can be investigated in the flow cell system.

Video Link

The video component of this article can be found at http://www.jove.com/video/2383/

Protocol

1. Assembly of the Flow Cell System with All Components

The assembled flow system includes: autoclavable tubing, bubble traps, medium/waste bottle and flow cells as shown in Figure 1. All these parts can be reused between experiments.

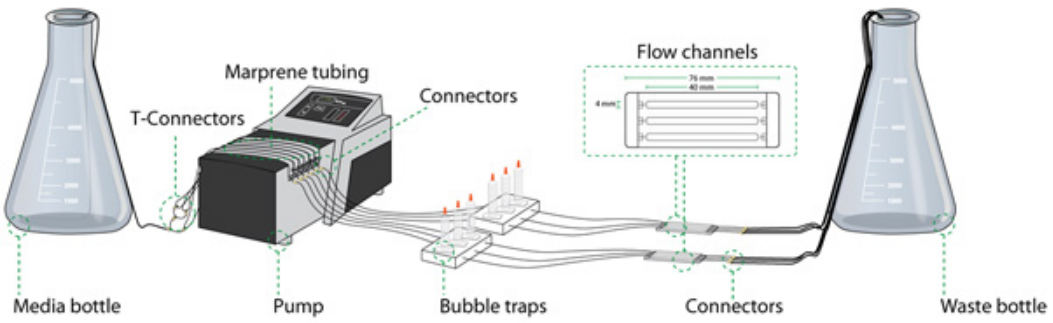

Figure 1. The flow cell system setup (essential components of the setup). The flow cell system consists of several components: a medium bottle, a peristaltic pump, bubble traps, the flow cell, a waste bottle, and diverse sections of tubing interconnected by various connectors. Figure kindly provided by Rune Lyngklip.

2. Assembly of the Flow Cell

1. The flow cell (Figure 2a) is treated with thin lanes of silicone glue, using a syringe (Figure 3).
2. Place a cover slip on top of the silicone lines (Figure 3). Glass cover slips are used as substratum for *P. aeruginosa* while PVC cover slips are applied for *S. cerevisiae* biofilm.

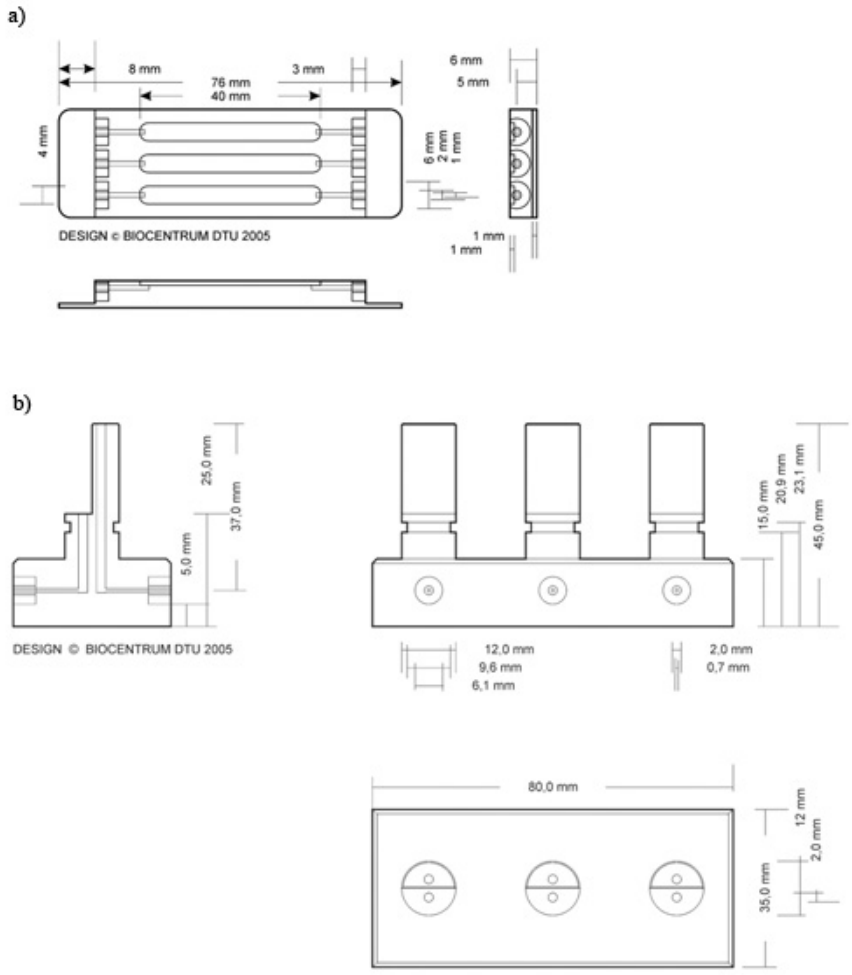

Figure 2. Schematic drawing of flow cell and bubble trap[2]. Detailed description of the dimensions used for the production of a) flow cell b) bubble trap, DTU Systems Biology. Reprinted with permission of John Wiley & Sons, Inc. (DTU Systems Biology was formerly entitled Biocentrum, as depicted in the figure)

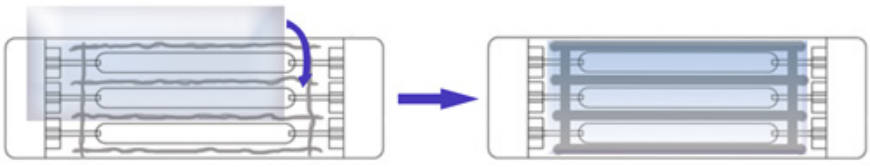

Figure 3. Illustration of the silicone glue application lines for attachment of the glass substratum. The indicated cover glass is placed over the silicone glue to attach it to the flow cell.

3. Turn the flow cell over and place it on a flat surface with the cover slip side down. Press gently on the back of the flow cell to push the cover slip onto the base of the flow cell. Turn the flow cell over and inspect for areas that are not sealed by the silicone. The handle (piston) part of a syringe can be used as a tool to gently press the glass and flow cell together.

3. Medium Bottle

1. Place a feeding silicone tube (2 mm inner diameter) in a medium bottle and insert a straight connector at the other end.
2. Add medium to the bottle (for medium content see "media" paragraph)

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Cover connector and bottle with metal foil and autoclave the medium. Make sure that the end of the supply tube is fixed above the liquid level in the medium bottle or a siphon effect may empty the medium bottle when autoclaving.

4. Connecting the Bubble Trap, Flow Cell and Pump

Assemble all tubing according to the outline in Figure 1. Use silicone tubing except for the part that goes through the peristaltic pump where Marprene tubing is applied.

1. In order to connect the tube from the medium bottle to all individual flow chambers, split a tube into the required number of inlets applied in the experiment. Use T-connectors to make the desirable number of connection tubes from the feed tube to the Marprene tubes in the pump (see Figure 1, T-connector). It is generally a good idea to keep the same sequence order of tubes and flow cells throughout the system, to facilitate identification of components in case of faults in the system.
2. Connect each individual feeding tube (2 mm in diameter) to Marprene tubes in the pump using straight connectors. Connect the Marprene tubing to a bubble trap (Figure 2b) via intermediate silicone tubing (1 mm in diameter). Make sure that the pump is connected to the inlet at the tallest part of the bubble trap.
3. Connect the resulting outlet tube of the bubble traps to the flow cell inlet (1 mm in diameter), make sure that the length of these tubings allows the flow cell to be moved to the stage of the confocal microscope (typically 1 m).
4. Place 5 mL syringes on top of the bubble traps. Close the tops with suitable caps.
5. On the flow cell outlet connect a short, approximately 40 mm piece of (1 mm in diameter), tubing and use a "reducing" straight connector to attach a waste tube (2 mm in diameter) of needed length. Place waste tubes in the waste container.
6. Importantly, the waste container must always be placed at the same level as the flow cells, never below flow-cell level. Also, make sure that the end of the waste tubing is fixed above the expected level of waste liquid to avoid flush-back due to a siphon effect when handling the flow cells.

5. STERILIZING AND WASHING THE FLOW SYSTEM

1. Remove bubble trap caps and place them in 70% ethanol to keep them sterile.
2. Run at highest pump speed to fill the system with 0.5% (v/v) sodium hypochlorite in water.
3. Place the bubble trap caps back on when the bubble traps are completely filled.
4. Tap the flow cells to remove bubbles in the flow chamber. Take care not to damage the fragile cover glass.
5. Allow the system to sterilize for 3-4 h at a flow rate of 3 mL/h/channel (0.2 mm/s linear flow rate).
6. Wash the system 2-3 times to wash out all the hypochlorite. Fill and empty the system with sterile water. Bubble traps must be emptied completely between each wash. This can be done by pumping in air until bubble traps have been emptied. After emptying, remove caps from the bubble traps before refilling the system. Replace caps after the bubble traps have been completely filled with liquid. Repeat as required.
7. Run sterile water through the system at a low flow rate (1-3 mL/h/channel) over night or proceed to the next step.
8. Connect the medium bottle to the inlet and flush the system with medium over night at low flow rate (3 mL/h/channel) at the temperature where the experiment will be performed. Note: bubble traps must be emptied for water before the system is filled with medium.

6. INOCULATION OF THE FLOW CELL

1. From an overnight culture make a dilution to a desired optical density (for *P. aeruginosa* e.g. 0.001 OD_{600nm} and 0.1 OD_{600nm} for *S. cerevisiae*).
2. Use a 0.5 mL syringe with a 27G needle to load enough inoculum to fill the chamber. 250 µL is sufficient for the flow chambers having the dimensions specified in this work (Figure 2., 40 mm x 4 mm x 1 mm).
3. Stop the peristaltic pump.
4. Clamp off the silicone tubing leading to the flow cell to prevent back flow into the system.
5. Sterilize the inoculation site on the silicone tubing by wiping it with 70% ethanol.
6. Insert the needle into the silicone tube and introduce the tip into the inlet of the flow cell. Slowly inject the inoculum into the chamber (be careful not to inject air bubbles).
7. Remove the needle and wipe the injection site with 70% ethanol followed by immediate sealing of the hole using silicone glue over the injection site.
8. Turn over the flow cell and let the micro-organisms adhere to the substratum for 1 hour without flow through the flow cell.
9. Turn the flow cell, start the medium pump (3 mL/h/channel) and take the clamp off the silicone tube.
10. The system is placed for incubation, at 37°C in the case of *P. aeruginosa* and 30°C in the case of *S. cerevisiae*.
11. Biofilm in the flow chambers can now be visualized by CLSM.

7. STAINING OF BIOFILM FOR MICROSCOPY

1. Make a dilution of the appropriate staining (e.g. 1:1000 Syto 9 live stain for *S. cerevisiae*)
2. Stop the peristaltic pump.
3. Clamp of the silicone tubing leading to the flow cell.
4. Sterilize inoculation site on the silicone tubing by wiping it with 70% ethanol.
5. Use a 0.5 mL syringe with a 27G needle to load enough staining solution to fill the chamber. 250 µL is sufficient for the flow chambers used here.
6. Insert the needle into the silicone tube and introduce the tip into the inlet of the flow cell. Slowly inject the staining solution into the chamber (be careful not to inject bubbles).
7. Remove the needle and wipe the injection site with 70% ethanol followed by immediate sealing of the injection site.
8. Leave the flow cell without flow for 15 minutes.
9. Take off the clamp and start the flow (3 mL/h/channel)
10. Acquire data with the CLSM

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Discussion

We have demonstrated a flow cell system that represents a powerful tool in biofilm investigations. Combined with 3D imaging by confocal microscopy, the system has a range of advantages in comparison to other methods of analyzing microbial biofilms by means of more traditional microscopic techniques. This system allows 3D visualization of living microbial biofilm communities without disturbance of the community. Light microscopy will not provide detailed information about niches of the biofilm and while electron microscopy provides nanoscale resolution of the biofilm, it does not allow live cell imaging.

Using the described flow channel system we have previously elucidated the spatial distribution of bacterial cells sensitive to several antibiotics[5-8] (Figure 4a), distribution of extracellular compounds, e.g. DNA[9-11] and, the distribution of motile and non-motile cells of the same species within a bacterial community[4,6,9] (Figure 4c). We envision that the flow cell system can be used to study aspects of yeast biofilms. This may be the spatio temporal distribution of yeast biofilm in response to environmental factors such as fungicides as well as identification of genes involved in yeast biofilm development. Though yeast is not known to differentiate into motile and non-motile cells, other aspects of biofilm diversification may be studies such as the morphological shift from yeast to pseudohyphal cells and the shift from haploid to diploid cells.

We have shown a system that comply with several microbial species and will work with several staining techniques. A variety of different staining probes and fluorescent proteins, such as GFP, enable specific niche investigations in the developing biofilm and is an efficient tool in analyzing the effect of antimicrobial agents or other environmental factors. The information that can be gained is very detailed (Figure 4) and features in the biofilm can be quantified with computer programs such as COMSTAT[12,13].

Overall, the most critical aspect of the protocol is the fact that it is a time-consuming process. It is also a limitation that the cells need to be able to grow on a non-fluorescent, transparent surface. Since the biofilm formed is analyzed using a confocal microscope, the depth that can be investigated is limited to a few hundred micrometres[14].There are further technical limitations inherent in the design: the system is not suited for high throughput screening, as an experienced researcher can handle at most about 15 channels per experiment, which in turn can take several days to prepare. However, antibiotics or mutants that are considered relevant for biofilm studies can initially be mass screened with other methods such as crystal violet staining before the most interesting candidates are transferred to the flow cell system. The cover glass sheets are very thin and break easily, and care should be taken when handling the systems. In addition the tubing should be examined daily during the run of an experiment; as considerable "back-growth" in the inlet tubes just upstream of the flow cells can occur. Such contamination can be solved by removing several centimeters of silicone tube from the inlet side of the flow cells, using sterile technique.

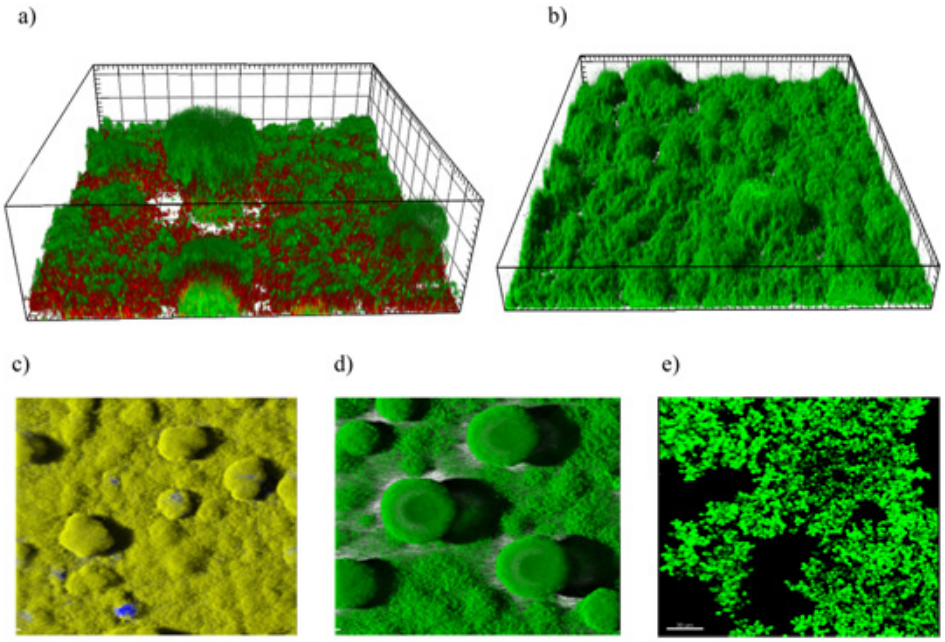

Figure 4. a) 4 day old PAO1 - GFP biofilm treated for 24h with Colistin and Propidium iodide for dead staining (red stain) b) 3D presentation of a three day old *P. aeruginosa* PAO1 (*P. aeruginosa* wild type) - GFP biofilm[6] c) 3D picture presentation of a PAO1 - CFP pilA mutant (blue) with an PAO1 wild type YFP (yellow) d) 5 day old PAO1 - GFP biofilm presented as a 3D picture e) 26 h *S. cerevisiae* (PTR3 mutant in CEN.PK background) biofilm stained with Syto-9[15].

Disclosures

No conflicts of interest declared.

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13.2 Paper II

Skolimowski, M., Nielsen, M.W., Emnéus, J., Molin, S., Dufva, M., Taboryski, R., Sternberg, C., and Geschke, O., *Microfluidic biochip as a model of the airways of cystic fibrosis patients.* Proceedings of the Thirteenth International Conference on Miniaturized Systems for Chemistry and Life Sciences, p. 1091-1093, 2009.

MICROFLUIDIC BIOCHIP AS A MODEL OF THE AIRWAYS OF CYSTIC FIBROSIS PATIENTS

Maciej Skolimowski[1,2], Martin Weiss Nielsen[1,2], Jenny Emnéus[1], Søren Molin[2], Rafael Taboryski[1], Martin Dufva[1], Claus Sternberg[2] and Oliver Geschke[1]

[1]Technical University of Denmark, Department of Micro- and Nanotechnology, Ørsted Plads, Building 345east, DK-2800 Kgs. Lyngby, DENMARK;
[2]Technical University of Denmark, Department of Systems Biology, Matematiktorvet, Building 301, DK-2800 Kgs. Lyngby, DENMARK

ABSTRACT

Here we report work towards development of a microfluidic system that can simulate the aerobic and anaerobic conditions in the human airways. The main purpose of this device is to mimic habitats of bacteria that usually attack airways of cystic fibrosis (CF) patients. The presented system enables the study of formation, growth, and dissolution of the bacterial biofilms, which is the main cause of the massive pulmonary deficiency and eventually death of CF patients. The ultimate goal for the microdevice is to replace the existing animal models (mice) in the development of new drugs and new treatment strategies in CF.

KEYWORDS: biochip, cystic fibrosis, biofilm, airways, microsystem

INTRODUCTION

The airways consist of at least three independent compartments, the conductive airways (the trachea, bronchii and bronchioles), the oxygen exchange compartment (alveoles) and a third, less recognized compartment, the paranasal sinuses (maxillary sinuses, frontal sinuses and ethmoid sinuses). In the first and the last compartment the environment is essentially anaerobic while in the alveoles the environment is highly aerated.

THEORY

To simulate the environmental conditions, especially the oxygen level which is major parameters affecting biofilm growth in CF patients, we propose the microfluidic airways model (*Figure 1*). The model consists of microchambers for bacterial biofilm growth and microfluidic channels for providing oxygen-scavenging liquid or oxygen-rich liquid. To avoid any impact on the

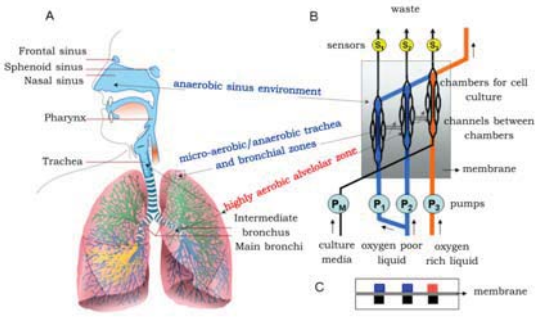

Figure 1. Human airways system, (B) top view sketch of Microfluidic Airways Model (MAM) and (C) cross-section view of MAM

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growth of microorganisms from these liquids, the compartments are separated by an oxygen permeable membrane. All media and liquid flow is controlled by external pumps. The oxygen level in the chambers can be controlled by varying the flow rates (*Figure 3*). A sensing layer for oxygen concentration measurements was incorporated at the bottom of the chambers. To provide the possibility of tracking bacterial re-infections, the bacteria growth chambers exposed to different oxygen levels were interconnected with channels (Figure 1 B).

EXPERIMENTAL

The biochip (*Figure 2*) was fabricated by micromilling of inlets and outlets and laser ablation of the channels in poly(methyl methacrylate). Poly(dimethylsiloxane) (PDMS) was used as the oxygen permeable membrane. The oxygen permeability of PDMS is a well known property, and its use for supplying oxygen in microdevices has been reported [1,2]. However, to our knowledge, no applications comprising active removal of oxygen by means of utilizing concentration gradients below oxygen saturation has been reported. This mechanism mimics the physiological oxygen exchange in the airways compartments. The lid comprising the oxygen sensing layer was fabricated by doping uncured PDMS with platinum(II) octaethylporphyrin (PtOEP), diluted with THF and spincoated to a thickness of approx. 15 µm on a 100 µm thick glass lid followed by thermal curing. Substitution of the usually used sensor matrix (ORMOSIL) [3] with PDMS allows to perform biological experiments in a 100 µm deep microchamber that is constructed from uniform material. All layers were sealed by silicone adhesive tape.

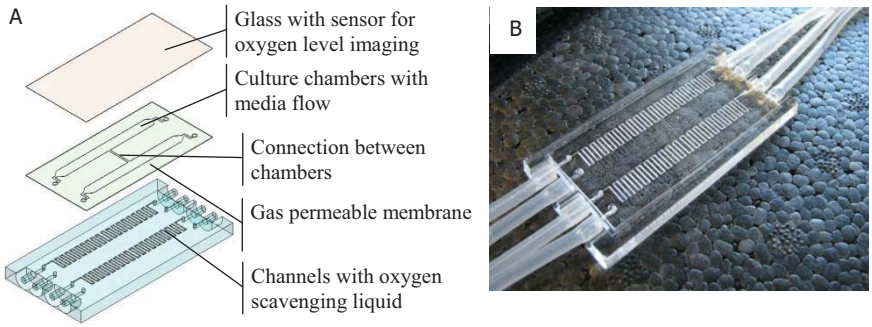

Figure 2: Designed (A) and fabricated (B) microdevice

To form the dissolved oxygen gradient in one of the culture chambers, oxygen scavenger (5% sodium sulphite water solution) was pumped through the corresponding microchannel situated beneath the membrane. The second channel was constantly flushed with oxygen saturated water.

RESULTS AND DISCUSSION

Formation of the oxygen concentration gradient inside the culture chamber was modelled, using numerical simulation with COMSOL 3.4 software (*Figure 3*), and experimentally confirmed by photoluminescence lifetime recordings on a multilabel reader Victor^2 (Perkin-Elmer Life Sciences) with the PtOEP sensing layer.

The microsystem was used for bacterial biofilm culture. A *Pseudomonas aeruginosa* strain, isolated from a CF patient (B54-1 WT-blue), was tagged with four different fluorescent proteins and inoculated. The experiment was carried out for 7 days. The biofilm was formed in 2 days and was not dissolving during the entire experiment. Confocal scanning laser microscopy was used for monitoring the biofilm formation (*Figure 4*).

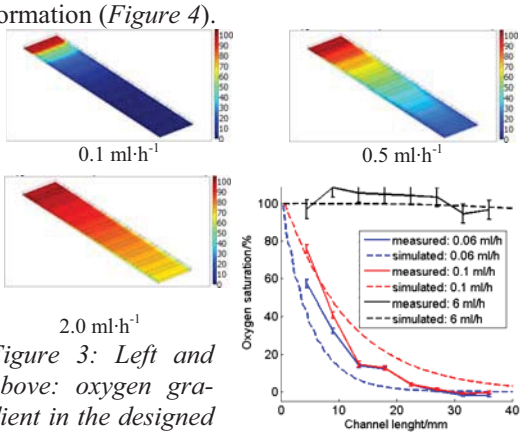

Figure 3: Left and above: oxygen gradient in the designed microdevice at various flow of the fluid in the chamber. Right: comparison between simulated and measured oxygen concentration

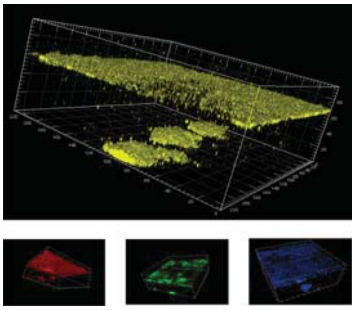

Figure 4: Confocal images of the bacterial biofilm formation in the microdevice. Cells express different fluorescent proteins (FP) (top: YFP, bottom from left to right: RFP, GFP, CFP).

CONCLUSIONS

The presented microfluidic chip was successfully used for generation of the oxygen gradient, which was easily regulated by altering the media flow rate and monitored by time-resolved photoluminescence measurements with integrated microsensor. Separation of the oxygen scavenger allows utilising the chip for cell culture.

The system was used for culturing of *Pseudomonas aeruginosa* biofilm. Future research will be focused on studies of the different antibiotic treatment strategies of bacterial biofilms.

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13.3 Paper III

Maciej Skolimowski[1], Martin Weiss Nielsen[1], Jenny Emnéus, Søren Molin, Rafael Taboryski, Martin Dufva, Claus Sternberg, Oliver Geschke: *Microfluidic dissolved oxygen gradient generator biochip as a useful tool in bacterial biofilm studies.* Lab Chip, June 2010. (DOI: 10.1039/c003558k)

Microfluidic dissolved oxygen gradient generator biochip as a useful tool in bacterial biofilm studies

Maciej Skolimowski,†*[ab] Martin Weiss Nielsen,†[ab] Jenny Emnéus,[a] Søren Molin,[b] Rafael Taboryski,[a] Claus Sternberg,[b] Martin Dufva[a] and Oliver Geschke[a]

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A microfluidic chip for generation of gradients of dissolved oxygen was designed, fabricated and tested. The novel way of active oxygen depletion through a gas permeable membrane was applied. Numerical simulations for generation of O_2 gradients were correlated with measured oxygen concentrations. The developed microsystem was used to study growth patterns of the bacterium *Pseudomonas aeruginosa* in medium with different oxygen concentrations. The results showed that attachment of *Pseudomonas aeruginosa* to the substrate changed with oxygen concentration. This demonstrates that the device can be used for studies requiring controlled oxygen levels and for future studies of microaerobic and anaerobic conditions.

Introduction

The generation of concentration gradients of different chemicals in microfluidic biochips is a problem often addressed in the literature. Typically, for the water soluble components, these are generated by diffusive mixing in microchannels by splitting and merging streams with different concentrations of the molecules of interest.[1–6]

In this work we address the creation of controlled oxygen gradients in perfusion based cell culture chips. A few reports exist that involve *e.g.*, the direct oxygen generation by electrolysis of water molecules,[7] a technique that requires microelectrode fabrication and that potentially can lead to small concentrations of hydrogen peroxide, generated in the same process. A different approach is to exploit the very high diffusivity and solubility of oxygen in poly(dimethylsiloxane) (PDMS).[8–10] PDMS is a chemically inert, biocompatible and optically transparent polymer[11] that is very commonly used for fast construction of microdevices.[12,13] Its properties make it a popular material for construction of cell culture based microdevices. Furthermore it can be utilised as a component in an active scheme of oxygen removal due to its gas permeability.

Active removal of dissolved oxygen from a liquid at the macroscale can be achieved in many ways. These include bubbling liquid with nitrogen gas, electrochemical reduction of dissolved oxygen, biological consumption or chemical reaction with an oxygen scavenger.[7,14–16] At the microscale the first method would lead to extensive drying of the liquid, while the other two complicate the fabrication of such a system.

There are a few very recently published examples of the use of PDMS based microfluidic chips in which the authors are using nitrogen or nitrogen–oxygen gas mixtures to develop dissolved oxygen gradients.[17–19] The main advantage of such a solution would be the possibility of reaching dissolved oxygen concentrations above air saturation point (9.2 ppm at 293 K and 1013.25 hPa).[10] The main disadvantages are the complexity of the setup and the possibility of slowly drying out the chamber when a low liquid media flow is used.

The use of oxygen or other gas containers is difficult in cell culture labs, due to safety reasons and usually very confined space. Therefore, the application of a liquid oxygen scavenger is highly preferable over systems that rely on such gas containers in order to control the specific levels of oxygen gradients. Direct addition of an oxygen scavenging agent such as sulfite or pyrogallol to the cell culture media will alternate its chemical composition which will have a heavy impact on the cultured organisms.[20] Therefore, in this paper we propose the use of a gas permeable PDMS membrane to separate an oxygen scavenging liquid from the cell culture media. To control the oxygen gradient across the cell culture microchamber we rely on the combination of the diffusive and advective oxygen transport within the microchip. To the authors best knowledge, there is so far no scientific report on such a system applied in biological studies.

It is crucial to be able to monitor the oxygen concentration at the microscale and here the classical amperometric method is not feasible because of the bulkiness of sensors and restriction to only point measurements. The most recent progress in miniaturization of the Clark electrode[21] allows one to build an electrode microarray[22,23] which would allow monitoring gradients within microfluidic devices in the future. However, most widely used techniques for measurements of oxygen concentrations in microfluidic devices are based on optical methods, which involve photoluminescent dyes or polymer matrices doped with these probes.[24,25] The basic phenomena responsible for oxygen sensing is dynamic quenching of luminescence by molecular oxygen.[26] Most of the probes with very high quenching constants are based on either ruthenium complexes, such as tris(bipyridyl)ruthenium(II)[27] and Ru(II)-tris(4,7-diphenyl-1,10-phenanthroline)[28–30]

[a]*Technical University of Denmark, Department of Micro- and Nanotechnology, Ørsted Plads, Building 345east, DK-2800 Kgs. Lyngby, Denmark. E-mail: maciej.skolimowski@nanotech.dtu.dk; Tel: +45 4525 6887*
[b]*Technical University of Denmark, Department of Systems Biology, Matematiktorvet, Building 301, DK-2800 Kgs. Lyngby, Denmark*
† These authors contributed equally to this study.

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or metalloporphyrins, mainly platinum(II) and palladium(II) octaethylporphyrin (accordingly PtOEP and PdOEP)[31–35] and platinum(II) *meso*-tetra (pentafluorophenyl) porphyrin (PtTFPP).[32,36,37] Other probes, like Erythrosin B[38] or pyrene,[39] are also reported as suitable for oxygen sensing purposes.

The main advantage of the metalloporphyrin based sensors over complexes of ruthenium based sensors is the much higher sensitivity. This is especially important for sensing not pure oxygen, but atmospheric air saturated cell culture media, where the air saturation scale remains biologically relevant. Metalloporphyrins are non-soluble in water which is the main reason why metalloporphyrins are usually embedded in polymers or silicone sol–gel matrices.[32,40] Consequently, ruthenium complexes as water-soluble compounds can be added directly to the culture media,[19] which definitely would simplify the task of sensing the dissolved oxygen concentration of the media. Such an addition would however lead to unspecified alterations in the metabolism of the cultured cells.[10]

Here we show the development of a microfluidic device that combines chambers for cell culturing, an oxygen gradient generator and an integrated optical sensor. This device was designed for high compatibility with hardware available in most laboratories. The system allows one to perform cell culturing without directly adding an oxygen scavenger or oxygen sensing compound to the culture media. This microfluidic system is applied as a useful tool for biological studies where oxygen niches are required and measurable.

Many bacteria experience fluctuating environmental changes in their living habitats. This is caused by changes in available nutritional factors and variation in oxygen concentrations which the bacteria will have to adapt to in order to become a successful colonizer.[41]

One such bacteria is the opportunistic pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) which is a recurrent and persistent cause of lung infections in patients with the genetic disease cystic fibrosis (CF).[42] Here we use *P. aeruginosa* as the model organism due to its history as a pathogen in highly diverting oxygenated milieus. In order for this pathogen to successfully colonize the human airways it needs to cope with varying nutritional and oxygen levels.[43] Differences in oxygen levels are generated within the viscous mucus layer of the CF airways and stress the bacteria to make a respiratory switch to cope with microaerobic or especially anaerobic conditions. Anaerobic growth of *P. aeruginosa* requires the presence of alternative electron acceptors as NO_3^-, NO_2^- or arginine in which the bacteria renders to generate profound biofilm.[44,45] Conditions of varying oxygen availability within the mucus layers of the CF respiratory system is a metabolic challenge for the bacteria and here we show a device that will be highly beneficial in future studies where controlled oxygen concentrations are needed.

Methods

Design and fabrication

The fabricated device is comprised of five layers (Fig. 1). The first two bottom layers (coloured red in Fig. 1A) are made of a very low oxygen permeable material (PMMA, Röhm GmbH & Co. KG, Germany, oxygen permeability: 1.35 barrers[46]). They contain inlets and outlets in order to connect the microchannel (300 μm width, 200 μm depth, 184 mm total length) and culture chamber. The microchannel and culture chamber were fabricated by laser ablation (48-5S Duo Lase carbon dioxide laser, SYNRAD Inc., USA). The third layer, the membrane, is made of 60 μm thick oxygen permeable material (PDMS, Dow Corning Corp., USA), obtained by spincoating (60 s, 800 rpm) uncured PDMS on an ETFE substrate (Tefzel® Fluoropolymer Film, 200LZ, DuPont de Nemours (Luxembourg) S.A., Luxembourg) followed by curing in 70 °C for 1 h. The ETFE substrate was used here as a temporary solid support for the membrane and was subsequently removed after integration of the membrane into the microchip. The fourth layer, a 150 μm thick cell culture chamber, was fabricated by laser ablation in the low oxygen permeable ETFE foil. The culture chamber was sealed with a glass slide covered with the oxygen sensing dye. The sensing layer was fabricated by doping uncured PDMS with PtOEP (Sigma-Aldrich Denmark A S^{-1}) diluted in THF. The solution was spincoated (60 s, 3000 rpm) to a thin film (thickness approx. 8 μm) on the 120 μm thick glass slide and cured at 70 °C for 20 min. All layers were sealed by silicone adhesive tape (ARcare® 91005, Adhesive Research, Inc., Ireland) and integrated with the base module which contains the milled (Mini-Mill/3PRO, Minitech Machinery Corp., USA) fluidic connectors (Fig. 1B). The microchip was designed to fit with outer dimensions to the microscope slide standard (76 × 26 mm).

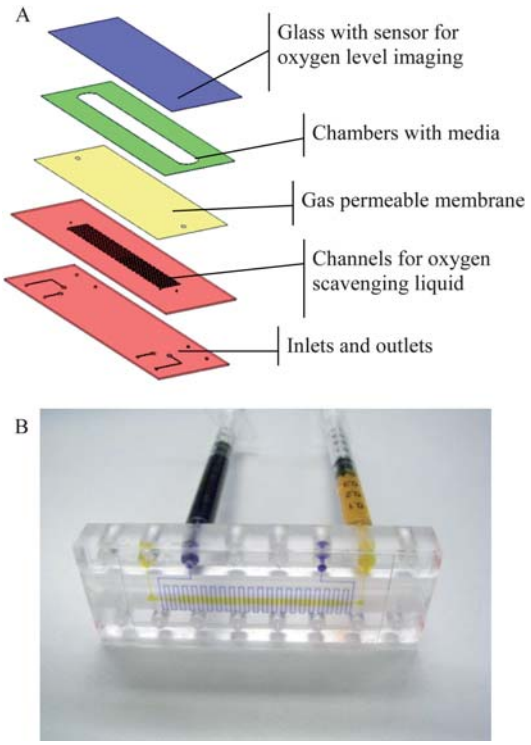

Fig. 1 Schematic presentation of the different layers of the device (A) and the fabricated microdevice (B). Here dyes are injected to visualize the microstructures of the assembled system (blue for channel and yellow for chamber).

Numerical simulations

The oxygen concentration gradients were numerically simulated in COMSOL Multiphysics 3.5a in accordance to the chamber geometry of the designed device. For the numerical simulation, the following conditions were assumed: the density and viscosity of the liquid are similar to water (1000 kg m^{-3} and 0.001 Pa s, respectively), a maximum concentration of oxygen in water (saturation point at standard conditions) of 0.281 mol m^{-3}, a diffusion coefficient of oxygen in water of 1.9×10^{-9} m^2 s^{-1}, a diffusion coefficient of oxygen in PDMS of 4.1×10^{-9} m^2 s^{-1}, oxygen solubility in PDMS 0.18 cm^3(STP) cm^{-3} atm.[8] The equilibrium between oxygen in water and in PDMS can be described by eqn (1). The rate constant of oxygen transport from water to PDMS (k_1 in eqn (1).) was set as 1.0×10^{-2} m s^{-1} [47] and the rate constant (k_2) for the reverse flux was set as 1.7×10^{-3} m s^{-1}.

$$O_2^{water} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O_2^{PDMS} \quad (1)$$

The ratio of these two rate constants, under steady state condition, is equal to the ratio of oxygen concentrations in water and in PDMS (2). This can otherwise be expressed as a partition coefficient (K_p) between the oxygen in PDMS and in the water phase. This implicates the linear correlation between oxygen concentrations in these two phases (3).

$$\frac{k_1}{k_2} = \frac{O_2^{PDMS}}{O_2^{water}} = K_p \quad (2)$$

$$O_2^{PDMS} = \frac{k_1}{k_2} O_2^{water} \quad (3)$$

The oxygen flux at the PDMS–water boundaries was modelled according to eqn (4). The signs at rate constants depend on the direction of oxygen diffusion.

$$flux_{O_2} = \pm k_2 \cdot C^{PDMS}{}_{O_2} \pm k_1 \cdot C^{water}{}_{O_2} \quad (4)$$

To simplify the simulation we additionally assumed a rapid reaction of the oxygen at the interface between the membrane and the oxygen scavenging liquid (0% oxygen concentration at this interface as a boundary condition) (Fig. 2). This condition can be achieved with an adequate flow of the scavenging liquid and the addition of a catalyst.[15] The liquid flow in the chamber varied from 1 μl min^{-1} to 500 μl min^{-1}, which corresponds to a Péclet number for dissolved oxygen from 0.6 (diffusive transport) to 290.4 (advective transport).

Photoluminescence lifetime measurements of the oxygen gradient

To form the gradient of dissolved oxygen in the growth chamber, an oxygen scavenger (10% sodium sulfite solution with 0.1 mM $CoSO_4$ as catalyst, both from Sigma-Aldrich Denmark A S^{-1}) was pumped by a syringe pump (model 540060, TSE Systems GmbH, Germany) through the microchannel situated beneath the membrane with a constant flow rate of 20 μl min^{-1}, while the growth chamber above the membrane was flushed with atmospheric air saturated water with varying flow rates according to the values used in the numerical simulations.

Photoluminescence lifetime measurements of the PtOEP based sensor were carried out on a multi-titre plate reader (Wallac Victor2, Perkin-Elmer Life Sciences, USA). The entire microchip was mapped to the sectors of a 384 well plate (Fig. 3). The excitation wavelength was 340 nm and the emission wavelength was 640 nm.

In order to correlate the photoluminescence lifetime measurements with the oxygen concentration, a two point calibration was made. The phosphorescence decay curves for the

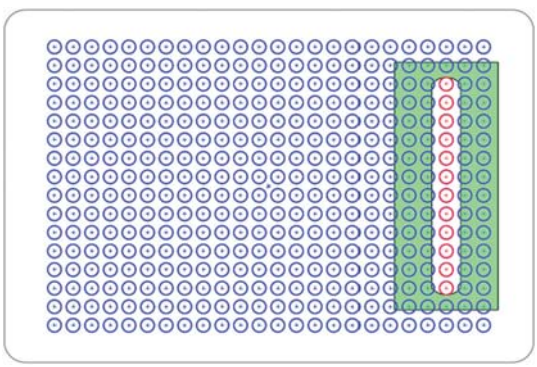

Fig. 3 The chamber mapped to the sector of a 384-wells microplate (the green area is the biochip, blue circles are the 384 microplate wells while the red circles are the oxygen gradient measurement points).

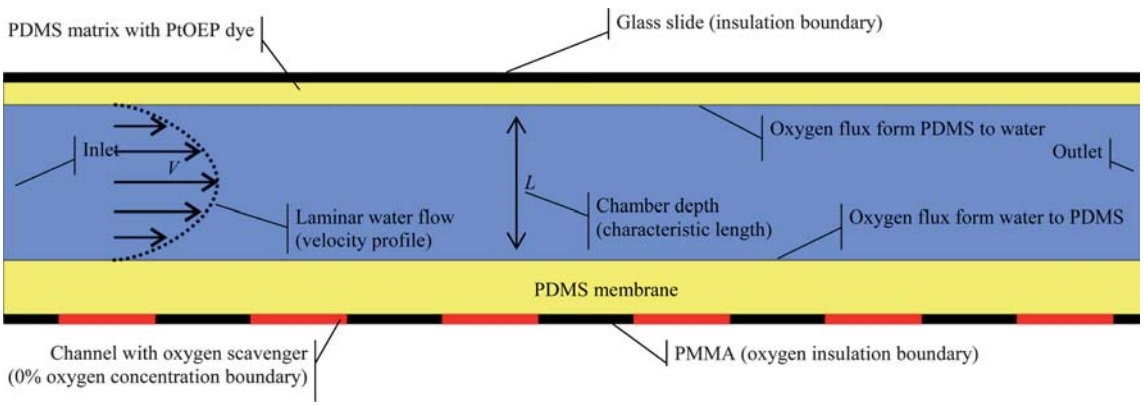

Fig. 2 Schematic model of the oxygen gradient generation in the microdevice.

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sensing layer flushed with oxygen free and air saturated water were recorded.

For calculation of the oxygen gradient, in total 12 location points were used. For each measurement location, the decay of the phosphorescence curve was determined by reading the intensities after excitation pulses at 5, 10 15, and 20 μs and then with 10 μs intervals to 170 μs. In total, 20 points were used for the determination of each phosphorescence decay curve. The monoexponential model of the phosphorescence decay was assumed according to eqn (5), where $I(t)$ is the phosphorescence intensity at time t, I_0 is a pre-exponential factor and τ is the phosphorescence lifetime.

$$I(t) = I_0 \cdot e^{-\frac{t}{T}} \quad (5)$$

The data were fit to the model using MATLAB 2008a software and phosphorescence lifetime was determined for every location point. In order to convert the phosphorescence lifetime to the dissolved oxygen concentration, a two-point calibration was done by measuring phosphorescence lifetimes of oxygen free and atmospheric air saturated water.

Cultivation of *Pseudomonas aeruginosa*, PAO1 within the microfluidic chip

Pseudomonas aeruginosa strain PAO1[48] tagged with a green fluorescent protein (GFP)[49] was grown in the flow chambers of the device. The individual chambers were either set up with water in the channels as a control or with oxygen scavenging liquid. PAO1 biofilms were cultivated in chambers irrigated with FAB medium. For the initial attachment experiment the media was supplemented with 0.3 mM glucose and for the long term cultivation experiments with 10.0 mM sodium citrate. Several studies have demonstrated that FAB media supplemented with sodium citrate as the sole carbon source promote a carpet-like biofilm instead of microcolonies and mushroom-like structures. This generates a better possibility of quantitative comparison of the bacterial biomass developed under different oxygen environments.[50]

The flow chambers were inoculated by injecting 500 μl overnight culture diluted to an OD_{600} of 0.005 into each flow chamber. This inoculum ensured that the entire culture chamber was filled. To drive the growth of bacterial biofilm on the glass side the chip was turned upside down without flow for 1 h after inoculation. The media flow was subsequently started at the flowrate of 10 μl min^{-1} using a peristaltic pump (Watson Marlow 205S, Watson-Marlow Inc, USA), directly followed by detection of attached bacteria by confocal laser scanning microscopy (CLSM).

In order to illustrate biofilm development in different oxygen environments the long term cultivation of PAO1 was performed for 4 days using sodium citrate supplemented FAB media. All steps were carried out at 37 °C.

Microscopy and image analysis

Microscopic observations and image acquisitions were performed on a Zeiss LSM 510 CLSM (Carl Zeiss, Jena, Germany). Detectors and filter settings were set for monitoring GFP. Confocal images of the 1 h initial attachment was taken using the 63×/1.4. For each indicated time point 7 pictures were taken in random locations of the specific oxygen saturation point. For the long term experiments, pictures were taken at day 4 using the 40×/1.4 Plan-Neofluar oil objective. The first location for the long term biomass measurements was chosen at an oxygen concentration of 97%. Each consecutive measurement was performed in intervals of 1 mm down the oxygen gradient (see Fig. 8 and Fig. 9 later). All images were processed using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

For quantification of biomass the COMSTAT software was used. COMSTAT defines biomass as a biomass volume divided by the substratum area (μm^3 μm^{-2}).[50]

Results

Description of the system

We have fabricated a multilayer system where we incorporated: a PDMS immobilised oxygen sensing probe, a culture chamber, a gas permeable membrane and a microfluidic channel for oxygen scavenging.

The purpose of the microfluidic channel is to constantly flush the PDMS membrane with an oxygen scavenging liquid. This allows oxygen transport across the membrane by diffusion from the growth chamber to the microchannel. Here the oxygen is irreversibly consumed in a rapid chemical reaction. The oxygen gradients generated within the chamber can be monitored by the oxygen sensing probe.

The culture chamber was used for *P. aeruginosa* cultivation in different dissolved oxygen environments.

Numerical simulations of the oxygen gradients

In order to verify the generation of dissolved oxygen gradients within the microdevice, numerical simulations were performed. The gradient can be observed in a horizontal as well as a vertical plane. Since PAO1 strain forms biofilm on the glass side of the chamber only the oxygen gradient from the top horizontal plane was taken into consideration (Fig. 4).

The oxygen gradients vary with the media flow rate (Fig. 4) due to changes in the ratio of advective and diffusive transport. This ratio can be described by the Péclet number (*Pe*) (see eqn (6) and Fig. 2 where L is the characteristic length, V is the velocity the media and D is the diffusion coefficient of oxygen in water). In the first case where the flow rate of the media is low

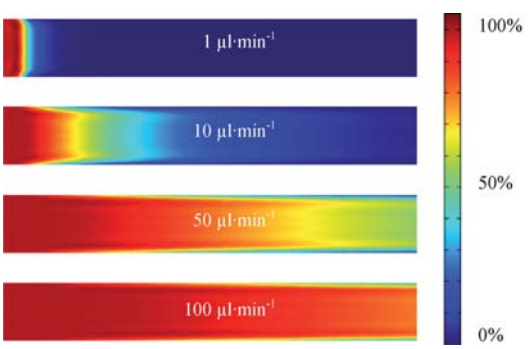

Fig. 4 Simulations of the oxygen saturation gradients in the growth chamber according to the various flow rates of culture media.

(1 μl min^{-1}, $Pe = 0.6$), only a very short horizontal distance within the chamber is needed to remove almost all the oxygen. When the flow rate within the chamber is increased, the oxygen saturation at the end of the chamber increases accordingly (2% at 10 μl min^{-1} ($Pe = 6$), 43% at 50 μl min^{-1} ($Pe = 29$), 63% at 100 μl min^{-1} ($Pe = 58$) and 88% at 500 μl min^{-1} ($Pe = 290$) (last not shown in Fig. 4)).

$$Pe = \frac{L \cdot V}{D} \quad (6)$$

Photoluminescence lifetime measurements of the oxygen gradients

A two point calibration of the PDMS immobilised oxygen probe was made to be able to calculate oxygen gradients generated within the microfluidic device. The determined phosphorescence lifetimes for oxygen free and atmospheric air saturated water were: 69.7 ± 0.4 μs and 8.2 ± 0.1 μs, respectively. These measurements allowed us to make a correlation between the photoluminescence lifetime and oxygen concentration (Fig. 5).

The comparison between the numerically simulated and measured oxygen concentration gradients were made in order to validate both the theoretical model and the experimental data (Fig. 6). The simulation curves were plotted as oxygen saturation in water from the middle chamber cross-section *versus* the length of the channel. For each curve, twelve measurement points were used according to the spots marked in Fig. 3.

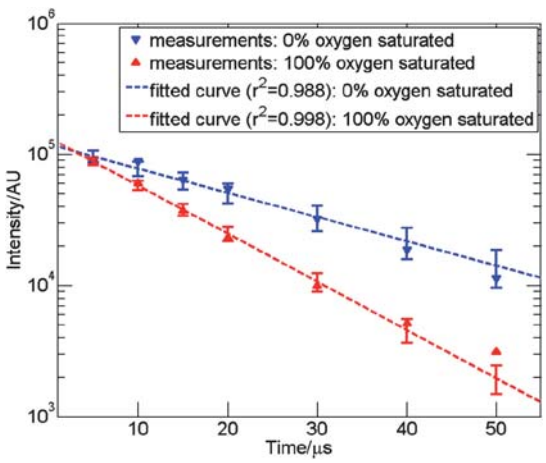

Fig. 5 Phosphorescence decay curve for oxygen free and atmospheric air saturated water (see equation 5).

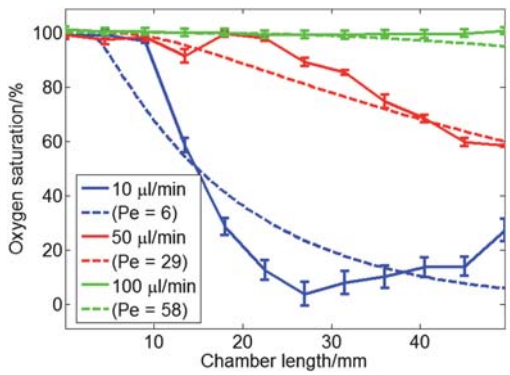

Fig. 6 Comparison between simulated (dashed lines) and measured (solid lines) oxygen concentration gradients in the culture chamber at different media flow rates.

The measured oxygen gradients follow the data obtained by the numerical simulations (Fig. 6). The variance coefficients between simulated and measured oxygen concentrations were 2.2%, 5.9% and 13.5% for, accordingly, 100, 50 and 10 μl min^{-1} media flow rates. The measured oxygen concentrations were slightly higher than the simulated ones at high flow rates and slightly lower at low flow rates. These differences can be explained by the flexibility of the PDMS membrane, which was not taken into consideration in the numerical simulations, for the sake of model simplicity, The membrane is slightly deflecting due to hydraulic pressure differences between the chamber and the channel with oxygen scavenger. This affects the chamber depth (L, Fig. 2), oxygen diffusion length and consequently the oxygen gradient.

According to both the numerical simulations and experimental measurements of the oxygen concentrations, we have shown that it is possible to control the oxygen gradient by merely alternating the media flow.

Reduced initial attachment and biofilm growth in the microdevice

To investigate the effect of the oxygen concentration on the growth of the *P. aeruginosa* PAO1, we cultivated this strain in the chip with a defined media known to support aerobic growth. We hypothesised that the lack of an alternative electron acceptor would affect the growth pattern of PAO1 under low oxygen concentrations. As a reference point for comparison purposes, we arbitrarily chose the culture chamber section with 20% of atmospheric air saturation (microaerobic conditions).

Following one hour of attachment under this low oxygen concentration, the attachment capacity of PAO1 to the PDMS surface was highly reduced. As shown in Fig. 7, PAO1 prefers to attach to the PDMS surface (shown 146.6μm × 146.6μm area) during conditions of higher air saturation. The attachment characteristics of PAO1 was furthermore investigated by time-lapse confocal microscopy. In a setup where the cells were allowed 1 h of initial attachment under atmospheric air saturated media, it was shown that the cells readily leave their attachment site after the media is reduced in oxygen concentration (data not shown).

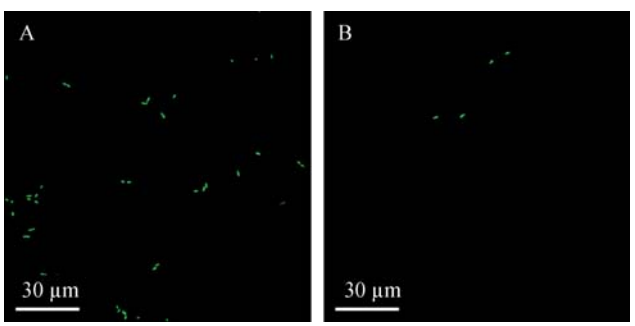

Fig. 7 Initial attachment of *P. aeruginosa* strain PAO1 at the end of microchambers recorded after 1 h of attachment: (A) media saturated with atmospheric air, (B) 20% of dissolved oxygen saturation.

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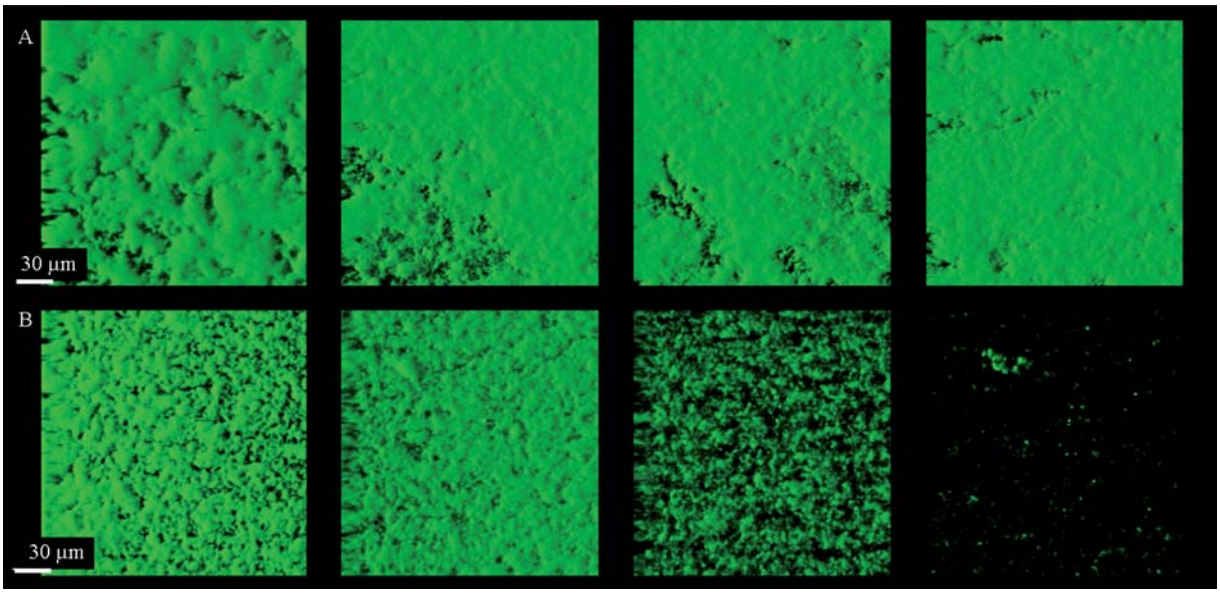

Fig. 8 Four day old biofilm of *P. aeruginosa* PAO1 cultivated in FAB media supplemented with 10 mM sodium citrate under: (A) atmospheric air saturation, (B) oxygen saturation (from left to right): 97%, 79%, 60% and 41%.

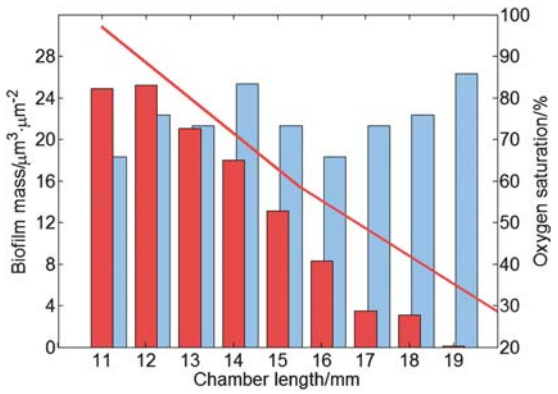

Fig. 9 Comparison of bacterial biomass developed in different oxygen environments. The red bars represent the PAO1 biomass within the culture microchamber under the oxygen gradient (red line). The blue bars represent PAO1 biomass produced in the atmospheric air saturated media.

In order to quantitatively compare the effect of different oxygen environments on PAO1 growth we used a FAB media supplemented with sodium citrate as the sole carbon source. We measured bacterial biomass formed after 4 days of cultivation in the microchambers. Under aerobic conditions (Fig. 8A) an even distribution of carpet-like biofilm is spread over the substratum. The drop in oxygen availability has a high impact on the cells ability to form a stable and evenly distributed biofilm (Fig. 8B). Fig. 9 shows clear correlation between PAO1 biomass and the oxygen gradient generated within the culture chamber.

Discussion

One of the main goals of our work was to design a device that was fairly easy to fabricate and handle. In order for a device to be a successful asset to a wide range of functional purposes in a laboratory, the need for complicated setups and supplies should be reduced as much as possible. In this paper we presented a developed system which easily produces controllable oxygen gradients within a culture chamber.

We exploit the advantages of an oxygen scavenging liquid over methods which involve flushing the channel with oxygen–nitrogen gaseous mixtures.[19] This significantly reduces the complexity of the setup and need of additional equipment.

Since PDMS is highly permeable, not only to O_2 and N_2 but also to water vapour, the use of pressurised gases would cause extensive drying of the culture media during long term experiments. In the case of low media flow rates, this could lead to severe changes in media osmolarity[51] and introduce bubbles within the culture chamber.[52]

The use of an oxygen tank in the laboratory is furthermore a serious safety threat to the personnel, therefore any solution which would eliminate it from the setup is highly desirable.

Furthermore we have produced a system which is cheap in expenses for oxygen scavenging due to very small amounts of chemicals needed.

The designed microfluidic system was integrated with a thin film sensor which was used for the detection of dissolved oxygen concentration gradients within the cultivation chamber. The oxygen concentrations were monitored by photoluminescence lifetime measurements. This specific way of monitoring oxygen concentration is compatible with several different detection methods as micro plate readers and fluorescence lifetime imaging microscopes.

Since bleaching of the oxygen probe by short excitation pulses, as used in time-resolved spectroscopy, is much lower than in the case of excitation with continuous light, the proposed thin film sensor can be used for long term experiments.

Prolonged exposition of the culture chamber to a strong light, used for oxygen probe excitation, would be harmful for the cultivated cells.[53] Therefore, the short excitation pulses used in

time-resolved spectroscopy techniques are preferable over continuous light excitation.

Furthermore, the choice of not using dissolved oxygen probes generate a system in which the influence of the probes on the cells[10] is completely eradicated. A significant advance over previous approaches,[19] where the authors created a system in which oxygen probes were present within their setup, is that we generated a highly biocompatible chip where the toxicity of the probes is eliminated.

For the making of the thin film sensor, we chose a PDMS matrix over the organically modified silica (ORMOSIL), which was widely used as a sensor matrix in previous works.[32] This substitution allows one to perform biological experiments without exposing the cultivated organism to two different environments (a hydrophilic glass-like surface in the case of ORMOSIL and a hydrophobic silicone rubber surface in the case of PDMS). Having these two surfaces with radically different properties could bias biological experimental data. This is especially important in studies of bacterial biofilm formation, where adherence will vary according to hydrophilicity of a specific surface.[54] However in other applications, especially for mammalian cell cultures, the use of ORMOSIL could be advantageous due to its chemical inertness[55] and the possibility of relatively easy surface modifications.[56]

Moreover, the cracking problem, reported for low molecular mass ORMOSIL precursors[40] is not present in PDMS films. Another advantage of the PDMS based matrix is the rapid fabrication time. The curing time for thin PDMS films is only 20 min while gelation and drying time of ORMOSIL based matrices, reported in the literature, can be as long as 2 weeks.[34]

The results from photoluminescence lifetime measurements of the dissolved oxygen gradients, which was generated within the culture chamber, correlated well with the results obtained from the numerical simulation. This does not only confirm the correctness of the mathematical model of the oxygen transport in the microfluidic system but also the reliability of time-resolved luminescence measurements done using the proposed PtOEP film sensor.

We consider that the discrepancies between simulated and measured oxygen concentrations observed at some media flow rates are due to deflection of the PDMS membrane. In order to include this effect in the numerical simulations one would need to incorporate one of the stress–strain models for a hyperelastic material. In the literature one can encounter a limited number of examples with numerical simulations of PDMS deflection.[57,58] However, to our best knowledge, there is no literature data describing how the gas permeability of PDMS is changing according to the strain.

When the only changes in dissolved oxygen levels are due to the chemical reaction with scavenger and diffusion–advection transport, the numerical simulations can predict the approximate shape of oxygen gradients within the chamber of the microchip. However, when this is not the case, *e.g.* when the chamber is used for cell culturing or when the material properties are not well characterised, a simple mathematical model cannot precisely predict the oxygen consumption. In such a case it will be necessary to use an integrated sensor.

The designed system was successfully applied to support growth of *P. aeruginosa* PAO1 biofilm. PAO1 responded to the low oxygen concentration environment by a reduced number of attached cells to the surface of the microchamber after the one hour adherence period. This indicates that the oxygen concentration within the microenvironment of the attachment site is highly important for PAO1 to effectively bind to this surface. The specific response to the low oxygen availability shows that PAO1 under flow conditions seems to lack the ability to effectively form an irreversible attachment.

In the CF lungs environment, the bacteria are living in a mucus matrix with the presence of DNA, proteins and other factors[59] which are highly diverting over the course of infection. This is currently not possible to fully grasp in the experimental conditions. However the most important factor in the chronically infected CF patients is the oxygen tension.[45] The presented device makes it possible to generate such differences in oxygen levels and is here proven to make an excellent platform for this kind of experimental setup.

Concluding remarks

In this paper we presented a novel approach for oxygen gradient generation within a microfluidic biochip. The system was successfully fabricated by micromilling, laser ablation and spin-coating techniques.

The simple layer-by-layer design allows the incorporation of several desirable elements as the integrated gas permeable membrane which we exploited by an oxygen scavenging liquid to produce the desired oxygen environments. The experimental data correlated as expected to the numerical simulations performed.

Stability and biocompatibility experiments were successfully performed over a long term biofilm culture, which proves the system as an excellent foundation for oxygen dependent studies.

We successfully made a system that does not rely on toxic dyes or difficult setups, but was built to be easy and safe to operate as well as highly mobile and compatible with common used biological laboratory equipment such as microscopes and multi-titre well plate readers.

In future CF biofilm studies, desired to be investigated in different oxygen environments, this platform could furthermore be used with the incorporation of other biologically important factors, like DNA and proteins. This would be a great advance towards mimicking the CF airways.

Acknowledgements

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13.4 Paper IV

Skolimowski, M[1]., Nielsen, M.W[1]., Abeille, F., Skafte-Pedersen, P., Sabourin, D., Fercher, A., Papkovsky, D., Molin, S., Taboryski, R., Sternberg, C., Dufva, M., Geschke, O. and Emnéus, J, *Modular microfluidic system as a model of cystic fibrosis airways*. Submitted to Biomicrofluidics, May 2012

Modular microfluidic system as a model of cystic fibrosis airways

M. Skolimowski,[1,2,a,b)] M. Weiss Nielsen,[1,2,b)] F. Abeille, [1,2] P. Skafte-Pedersen,[1] D. Sabourin,[1] A. Fercher,[3] D. Papkovsky,[3,4] S. Molin,[2] R. Taboryski,[1] C. Sternberg,[2] M. Dufva,[1] O. Geschke[1] and J. Emnéus[1]

[1]Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsted Plads, Building 345B, Kgs. Lyngby, DK-2800, Denmark

[2]Department of Systems Biology, Technical University of Denmark, Matematiktorvet, Building 301, Kgs. Lyngby, DK-2800, Denmark

[3]Biochemistry Department, University College Cork, Cavanagh Pharmacy Building, Cork, Ireland

[4]Luxcel Biosciences Ltd., BioInnovation Centre, UCC, College Road, Cork, Ireland

A modular microfluidic airways model system that can simulate the changes in oxygen tension in different compartments of the cystic fibrosis airways was designed, developed and tested. The fully reconfigurable system composed of modules with different functionalities: multichannel peristaltic pumps, bubble traps, gas exchange chip and cell culture chambers. We have successfully applied this

[a] Author to whom correspondence should be addressed. Electronic mail: maciej.skolimowski@nanotech.dtu.dk.

[b)] M. Skolimowski and M. Weiss Nielsen contributed equally to this work.

system for studying the antibiotic therapy of *P. aeruginosa*, the bacteria mainly responsible for morbidity and mortality in cystic fibrosis, in different oxygen environments. Furthermore, we have mimic the bacterial reinoculation of the aerobic compartments (lower respiratory tract) from the anaerobic compartments (CF sinuses) following an antibiotic treatment. This effect is hypothesised as the one on the main reasons for recurrent lung infections in CF patients.

I. INTRODUCTION

The human airways are complex multi-compartmental habitats for infectious bacteria. In healthy humans, the majority of the airways are essentially kept sterile as a result of highly efficient clearing mechanisms[1]. In cystic fibrosis (CF) patients, this clearing mechanism is severely impaired and bacterial infections inflict deteriorating health and becomes the major cause of mortality in these patients[2, 3].

The human airways consist of at least three independent compartments, the conductive airways (the trachea, bronchi and bronchioles), the oxygen exchange compartment (alveoli), and a third, less investigated compartment, the paranasal sinuses (maxillary sinuses, frontal sinuses and ethmoid sinuses). In the first and the last compartment, the environment is essentially anaerobic[4], while the alveoli are highly aerated.

In healthy individuals, the conductive airways are constantly cleared by mucociliary transport of entrapped microorganisms[5]. As a result, very few bacteria will ever reach the alveoli. Bacteria that do evade this clearing mechanism will rapidly be cleared by the actions of the immune system. The sinuses also have a mucociliary clearance mechanism although not as effective as the one found in the conductive airways. Large concentrations of bacteria

can be found widely spread throughout the sinuses, in particular in cases of common colds, etc[6].

Cystic fibrosis patients suffer from a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The affected gene codes for the CFTR protein, which is a chloride channel that is present in the epithelial cell membrane. Reduced or absent function of the CFTR will lead to highly reduced secretion of chloride and accordingly water over the cell membrane[7]. A direct consequence of this defect is that the mucus layer in the conductive airways becomes very viscous and the mucociliary clearance mechanisms are highly impaired[3, 8]. This results in frequent and recurrent infections of the CF airways, with the risk of pneumonia. As the bacteria infect the lungs in large numbers, the immune system tries to eradicate the infection but with a reduced effect since the bacteria are embedded in mucus and more or less recalcitrant to the cellular defence[9]. Instead the lung tissue is gradually damaged by the on-going immunological exposure, eventually leading to massive pulmonary deficiency and death[10]. In the clinic, the infections can be treated with cocktails of antibiotics, which can reduce or sometimes eradicate the infectious agents[11]

Bacterial airway infections in patients with a normal mucosa are relatively easy to treat with antibiotics. This is unfortunately not the case for CF patients and the myriad of infections they acquire during their lifetime leave each patient with a high need for recurrent antibiotic treatments. This is a multifactorial phenomenon and there are a lot of theories that try to explain this[12-14]. The most obvious reason for a treatment failure is the hindered diffusion of the antimicrobial agent through the thick and viscous mucus layer[15, 16]. However, according to recent findings, the main reason may reside in limited oxygen availability in some parts of the airways[17, 18]. These highly different oxygen environments are due to the

human physiology of the airways and furthermore upheld by the consumption from epithelial and immune cells in the local surroundings.

As *Pseudomonas aeruginosa (P. aeruginosa)* infections are almost inescapable in CF patients, especially in older patients, this makes *P. aeruginosa* an important organism for studies of "oxygen" phenomena[19]. *P. aeruginosa* is a facultative anaerobic bacteria[20] with reduced growth[21] and metabolic activity[18] at low oxygen levels. Antibiotics such as tobramycin, ciprofloxacin, and tetracycline preferentially kill the physiologically active bacteria living at high oxygen levels (aerobic environment), while colistin is more effective on the physiologically inactive bacteria growing in an anaerobic environment[10, 22].

An antibiotic treatment has the potential to clear a lung infection, yet after a few months the same bacteria are very likely to reappear, possibly as a result of reinoculation from the anaerobic environment of the sinuses[23]. In this context the sinuses could very well serve as a reservoir for "sinus" bacteria, which are difficult to treat with antibiotics and can cause the reinfection of otherwise cleared patients.

The now classical ways of studying CF related bacterial infections, primarily *P. aeruginosa*, are either to use animal models or to grow the bacteria in flow-cell systems.

A number of different animals have been tried as models of chronic infections in CF patients[24]. This includes rats[25-27], guinea pigs[28], cats[29] and rhesus monkeys[30, 31]. However the most important animal model is a mouse[32-35]. The use of an animal model is expensive and rise ethical concerns[36, 37]. Furthermore, animal models for CF related infections are still not ideal, mainly due to immunological differences between man and e.g. mouse. Mice do not acquire spontaneous and chronic infections as seen with human CF patients[38]. CFTR knock-

out mice do not show the same mucus accumulation as seen in the lower airways of CF patients[39, 40].

In flow-cell based systems, the bacteria are allowed to form a biofilm on a surface, as in the airways, and can then be monitored using a confocal microscope[11, 41]. However, this is not a suitable model for the human airways as they are subdivided into aerobic and anaerobic compartments.

The advancement in micro- and nanofabrication and assembly, as well as better understanding of microfluidics, has made possible the development of devices for modelling different tissue organs. Thanks to these devices, the control over the environment, relations and interactions between the cells and tissues at microscale with high spatial and temporal resolution can be achieved[42, 43]. The yet small but fast growing number of microdevices that can mimick different and even entire organs have been reported. These include blood vessels[44], bones[45], muscles[46], liver[47-50], brain[51], guts[52], kidneys[53, 54], endothelia[55] and blood-brain barrier[56].

In the field of mimicking the human airways, Huh *at al.*[57, 58] proposed a microfluidic device that can simulate injuries to the airways epithelia done by liquid plug flow. Recently, Huh *at al.* proposed a model of the vacuoles in the lung[59]. In this work the phagocytosis of planktonic Escherichia coli cells by neutrophils on the epithelial surface was shown.

In our previous work[21] we have shown the possibility of using a poly(dimethylsiloxane) (PDMS) membrane and an oxygen scavenging liquid to control the oxygen gradient within cell culture microchambers. However, according to the authors' best knowledge, the microfluidic model of different compartments of the human airways that

would allow to observe the influence of the microenvironment of these compartments on the recurrence of CF related infections has not been reported previously.

Therefore, the aim of this work was to make a model system, which simulates the three compartments of the airways to better understand the interplay between them. Using this artificial airways model, we can look into the bacterial details in the three compartments, their transmitting interaction, and the states of the bacterial inhabitants before, during and after antibiotic treatment.

II. MICROFLUIDIC AIRWAYS MODEL

The three sections of human airways: the conductive airways (trachea, bronchi and bronchioles), which are considered micro-aerobic, the highly aerobic gaseous exchange compartments (the alveoli) and the compartments of the paranasal sinuses, which are basically anaerobic (FIG. 1 a), are reproduced in this Microfluidic Airways Model (MAM). This is realized by constructing cell culture microchambers with different oxygen levels (FIG. 1 b). A microchamber with atmospheric air oxygenated media (aerobic environment) is connected by a channel to a microchamber with culture media saturated below the atmospheric air saturation level (micro-aerobic environment). This chamber is consecutively connected to a chamber with deoxygenated media (anaerobic environment). The connections between the chambers as well as outlets can be closed and opened and the actual oxygen level in the compartments is determined by an oxygen probe. The entire system is actuated by peristaltic micropumps.

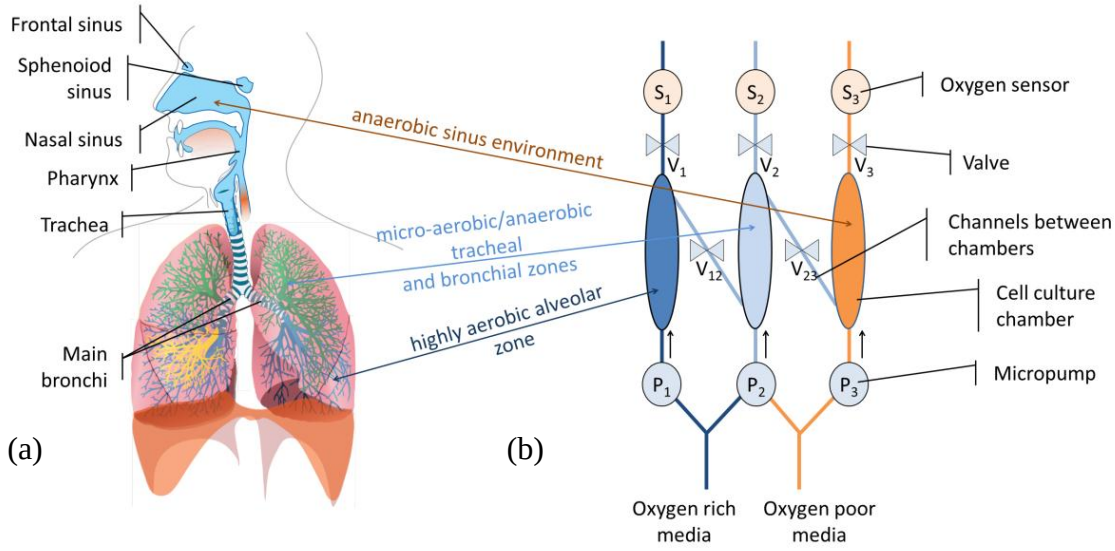

FIG. 1. (a) The human airways system[60] and (b) the Microfluidic Airways Model (MAM)

III. DESIGN

The above model was implemented, as a modular system comprised of the following distinct modules: a modified previously described multichannel microfluidic peristaltic pump[61, 62], a bubble trap, gas exchange and cell culture chambers (FIG. 2). The system allows to simultaneously cultivate cells in 8 chambers (3 of them facilitate aerobic environment, 2 micro-aerobic environment and 3 anaerobic environment).

These modules can be attached to form a microfluidic platform. The detailed design of each of these modules and the platform is available in the supplementary materials at [URL will be inserted by AIP].

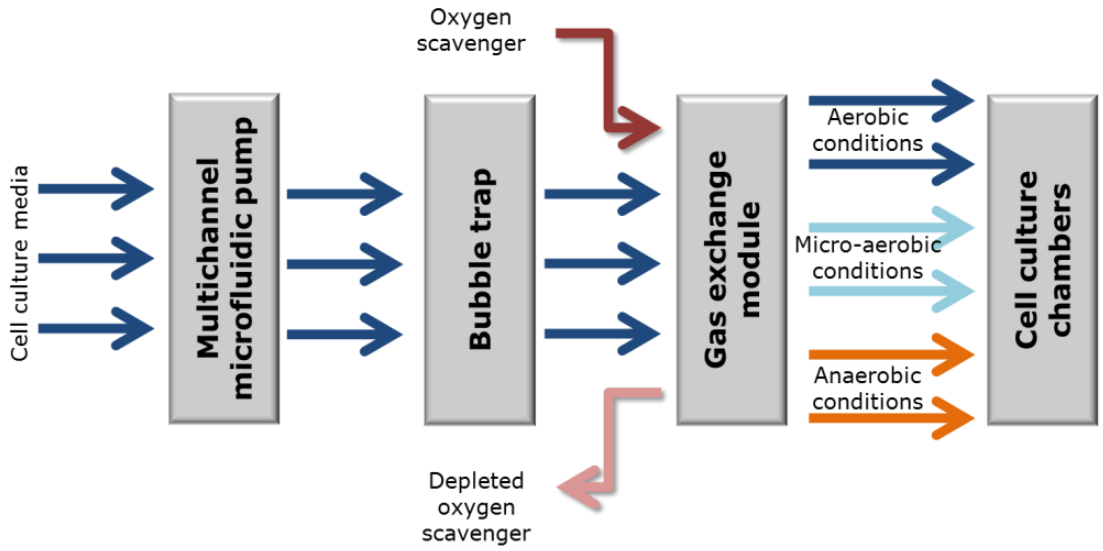

FIG. 2. Modular implementation of MAM

IV. MATERIALS AND METHODS

A. Microfabrication

The material of choice for most of the parts was polycarbonate (PC) (Nordisk Plast A/S, Denmark). This material exhibits low oxygen permeability (1.8 barrer[63]), very good mechanical and optical properties[64] as well as being resistant to alcohols and oils, which is important for sterilization and microscopy. The structures in PC were obtained by micromilling (Mini-Mill/3PRO, Minitech Machinery Corp., USA) followed by tetrahydrofuran vapour assisted bonding (50°C, 1.5 MPa).

PDMS inlays were fabricated by casting the silicone mixture (Sylgard 184, Dow Corning Corp., USA) against the milled mould. The PDMS parts were bonded to PC using silicone adhesive tape (ARcare® 91005, Adhesive Research, Inc., Ireland). The tape was cut into the desired shape using laser ablation (48-5S Duo Lase carbon dioxide laser, SYNRAD Inc., USA).

The gaskets used for sealing the modules to the platform, as well as check valves, were fabricated by milling in fluoroelastomer VITON A (J-Flex Rubber Products, UK).

B. Cultivation of *P. aeruginosa* strains

The *P. aeruginosa* laboratory strain PAO1 was used for all biofilm experiments. The PAO1 strain was originally isolated from a burn wound[65]. PAO1 was fluorescently tagged at a neutral chromosomal locus with GFP or mRFP1 with miniTn7 constructs as previously described[66]. A *P. aeruginosa* medium (FAB)[41, 67] supplemented with 0.3 mM glucose and 65 mM KNO_3 (FAB-GN) was used for biofilm cultivation. All biofilms and batch cultures were grown at 37°C. PAO1 was pre-cultured overnight in Luria Bertani (LB) media and prepared for inoculation in FAB-GN media. The overnight culture was diluted to an OD_{600} of 0.01 and subsequently 100 µl was inoculated with a Gilson P200 pipette through the designed inlets. In order to visualise the bacteria migration, the anaerobic culture chambers were inoculated with the GFP tagged strain and microaerobic and aerobic chambers were inoculated with the mRFP1 tagged strain. The device was left upside down for an hour without media flow. Following the one hour incubation period, the media flow was started at 500 $\mu l \cdot h^{-1}$ per channel. After 48 hours of cultivation in the growth chambers the media was exchanged to media supplemented with 50 $\mu g \cdot ml^{-1}$ of the antibiotic, Ciprofloxacin (Sigma-Aldrich, Denmark A/S). 24 hours antibiotic treatment was followed by staining with 1 µM SYTOX® Blue dead cell stain (Molecular Probes, Invitrogen, Denmark). The chambers were left for 48 hours running on media without Ciprofloxacin. This was done in order to subsequently evaluate the cells ability to migrate between oxygen gradients. Interconnection was made

between the chambers and cells were allowed to migrate for 24 hours before analysed with confocal laser scanning microscopy.

The interconnections between chambers were made with PVC tubing attached to PEEK connector plugs that were inserted into the specific chambers with different oxygen levels.

C. Dissolved oxygen level control

In order to control the dissolved oxygen levels in the cell culture media, one of the PDMS gas exchange module inlays was supplied with an oxygen scavenger (10% sodium sulphite solution with 0.1 mM $CoSO_4$ as catalyst, both from Sigma-Aldrich Denmark A/S). The second inlay was left open to atmospheric air.

Determination of the oxygen levels in the cell culture chambers was achieved using the phosphorescent oxygen-sensitive nanoprobe based on Platinum(II)- tetrakis-(pentafluorophenyl) porphine (PtTFPP) dye[68] (Luxcel Biosciences, Ireland). The oxygen levels were determined by: (i) phosphorescence intensity and lifetime measurements on an Axiovert 200 wide-field microscope (Carl Zeiss, Germany) upgraded for phosphorescence lifetime imaging (LaVision Biotec, Germany), and (ii) phosphorescence lifetime measurements on a multi-label plate reader (Victor2, Perkin-Elmer Life Sciences, USA). The imaging experiments were performed as described in[69] using pulsed excitation with a 390 nm LED and emission collection at 655±50 nm. Plate reader measurements were performed as described in[68], using excitation at 340 nm and emission at 642 nm. For such measurements the device was inoculated with non-fluorescent *P. aeruginosa* laboratory strain PAO1, then maintained under a flow of medium, containing 0.01 $mg.ml^{-1}$ of probe, for 12h and then washed with medium. Thus, biofilms stained with the phosphorescent probe were produced

in the device, which can be used to monitor oxygenation and conduct biological experiments for several days.

The correlation between the phosphorescence intensity or photoluminescence lifetime and dissolved oxygen concentration was determined by two-point calibration: at 0% and 100% of atmospheric air oxygen saturation in the culture media. The 0% atmospheric air oxygen saturation was obtained by supplementing the glucose containing culture media with glucose oxidase (Sigma-Aldrich Denmark A/S) as previously described in[70]. Each cell culture chamber was calibrated separately.

D. Confocal microscopy and image analysis

Confocal fluorescence images were taken with a Leica TCS SP5 microscope using a 50x/0.75W objective. 4 random pictures were taken from each chamber. Settings for visualization of the probes were: 514 nm excitation and 613-688 nm emission for mRFP1; 488 nm excitation and 517-535 nm emission for GFP; 458 nm excitation and 475-490 nm emission for SYTOX® Blue dead stain. All images were processed by the Imaris 7 software package (BITPLANE AG, Zürich, Switzerland).

V. RESULTS

A. Integration of the modular microfluidic system

The microfluidic modules described in supplementary material at [URL will be inserted by AIP] were successfully fabricated and assembled on a microfluidic platform (FIG. 3 a). The system was integrated with 16-channel peristaltic micropump. The pump was

actuated by two motors obtained from commercially available LEGO® Mindstorms® NXT 2.0 robotic kit[71] (The LEGO Group, Denmark) (FIG. 3 b)[62].

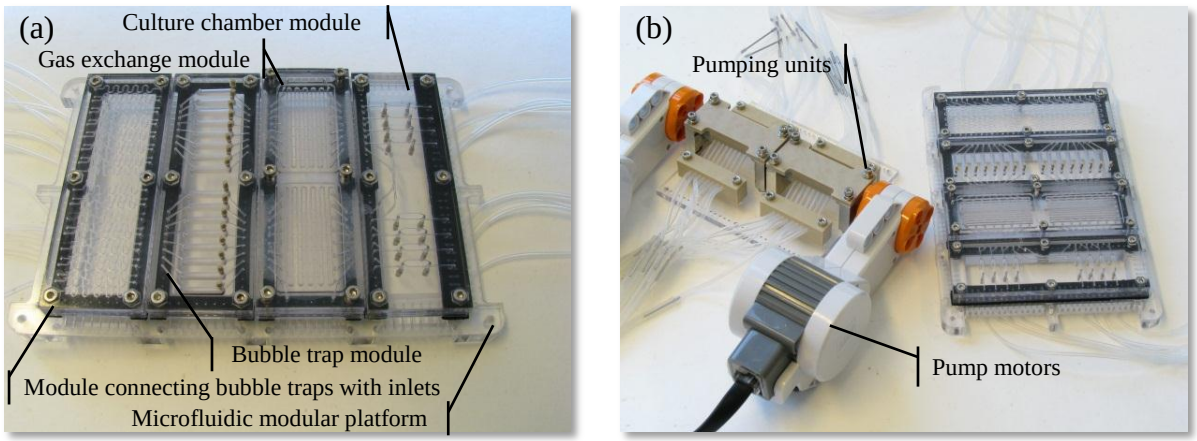

FIG. 3. (a) Microfluidic platform with modules and the peristaltic micropump (b).

B. Determination of the dissolved oxygen level

The differences between the phosphorescence intensity of the *P. aerugionsa* biofilm, stained with the PtTFPP nanoprobe, in the aerobic and anaerobic environments was investigated in order to determine the oxygen removal efficiency from the culture media using the gas exchange module (FIG. 4). The phosphorescence intensity increased approximately two-fold in anaerobic conditions as compared with aerobic conditions.

A two-step calibration curve was established by measuring The photoluminescence lifetime of the nanoprobe in oxygen free and atmospheric air-saturated media. The photoluminescence lifetime was determined to be 54.5±1.3 μs (oxygen free media) and 29.7±0.6 μs (and atmospheric air-saturated media). Oxygen concentration in atmospheric air-saturated media was determined to be 0.281 mM [72]. The measurements were performed at

room temperature. Assuming a reversible collisional quenching model[73], the Stern-Volmer constant was determined to be 2.97 mM^{-1}.

The photoluminescence lifetimes of the nanoprobe in the chambers, resembling aerobic, micro-aerobic and anaerobic environments, were: 30.5 μs, 35.8 μs and 51.1 μs, respectively, which corresponds to 94.2%, 62.7% and 7.9% of atmospheric air-saturation of the culture media according to the two-point calibration curve.

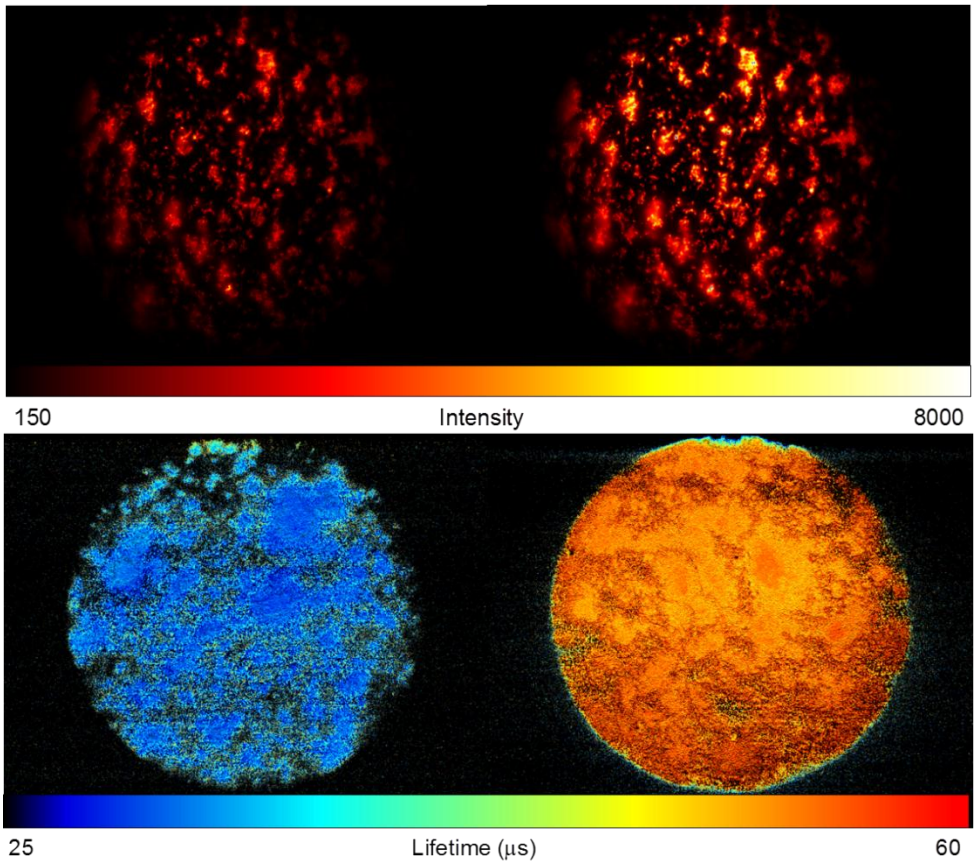

FIG. 4. Phosphorescence intensity (top panel) and lifetime (bottom panel) images of the *P. aeruginosa* biofilm stained with the PtTFPP nanoprobe in the culture chambers with high (left) and low (right) oxygen concentration.

C. Cultivation of *P. aeruginosa* strains in different oxygen environments

PAO1 biofilm formation was analysed 24 hours after inoculation. In order to follow the trail of each specific population following each inoculation, we used different fluorescent tagged versions of PAO1. The initial 24 hour cultures, under different oxygen environments in presence of nitrate as an alternative electron acceptor, gives rise to highly equivalent biofilm formations within the oxygen compartments (FIG. 5 a-c).

The green confocal image (FIG. 5 a) originates from GFP tagged bacteria and was cultivated under the lowest oxygen saturation. The red biofilm derives from mRFP1 tagged bacteria and was cultivated in microaerobic (FIG. 5 b) and aerobic conditions (FIG. 5 c). However, under the tested conditions in a minimal media, the biomass within first 24 hours of growth reached highly equivalent magnitudes of biomass regardless of oxygen tension. Under anaerobic conditions, supplemented with nitrate as electron acceptor, PAO1 has in LB media shown to develop 3 fold more biomass[74].

In order to evaluate the efficiency of the antibiotic ciprofloxacin on PAO1 biofilms under conditions on differentially lowered oxygen availability in a FAB-GN media, each chamber was challenged with the same ciprofloxacin concentration. After 24 hours of incubation each chamber received media supplemented with 50 $\mu g \cdot ml^{-1}$ of the antibiotic ciprofloxacin for a period of 24 hours. The treatment would present the differences in the effectiveness of the antibiotic in a developing PAO1 biofilm. Dead cells in the biofilms were visualized by staining each chamber with Sytox® blue dead cell stain (FIG. 5 d-f). The effect of the ciprofloxacin on the PAO1 biofilm was highly dependent on the oxygen environment

PAO1-GFP biofilm was much more susceptible to the antibiotic treatment than biofilm formed under higher oxygen concentrations. The antibiotic concentration was chosen to eradicate the majority of cells in the establishing biofilms, though low enough to allow surviving cells. PAO1 had in that sense been established enough to produce a healthy biofilm and represent a community associated environment.

Following the 24 h treatment with ciprofloxacin, the chambers were taken off the antibiotic containing media for 48 hours and connections between the different oxygen environments were made (FIG. 5 g-i) (see connection details in the supplementary materials at [URL will be inserted by AIP]). This enabled tracking of the bacteria in a novel way that has previously not been possible. We setup the system in a way to follow in which direction, if any, the surviving bacteria would move. As the ciprofloxacin treatment had been stopped the only difference between the chambers were the differences in oxygen concentrations. The small green clusters on FIG. 5 a) and i) come from the GFP tagged bacteria. This proves that PAO1 moves from chambers with low oxygen tension (FIG. 5 g) to microaerobic (FIG. 5 h) and aerobic chambers (FIG. 5 i).

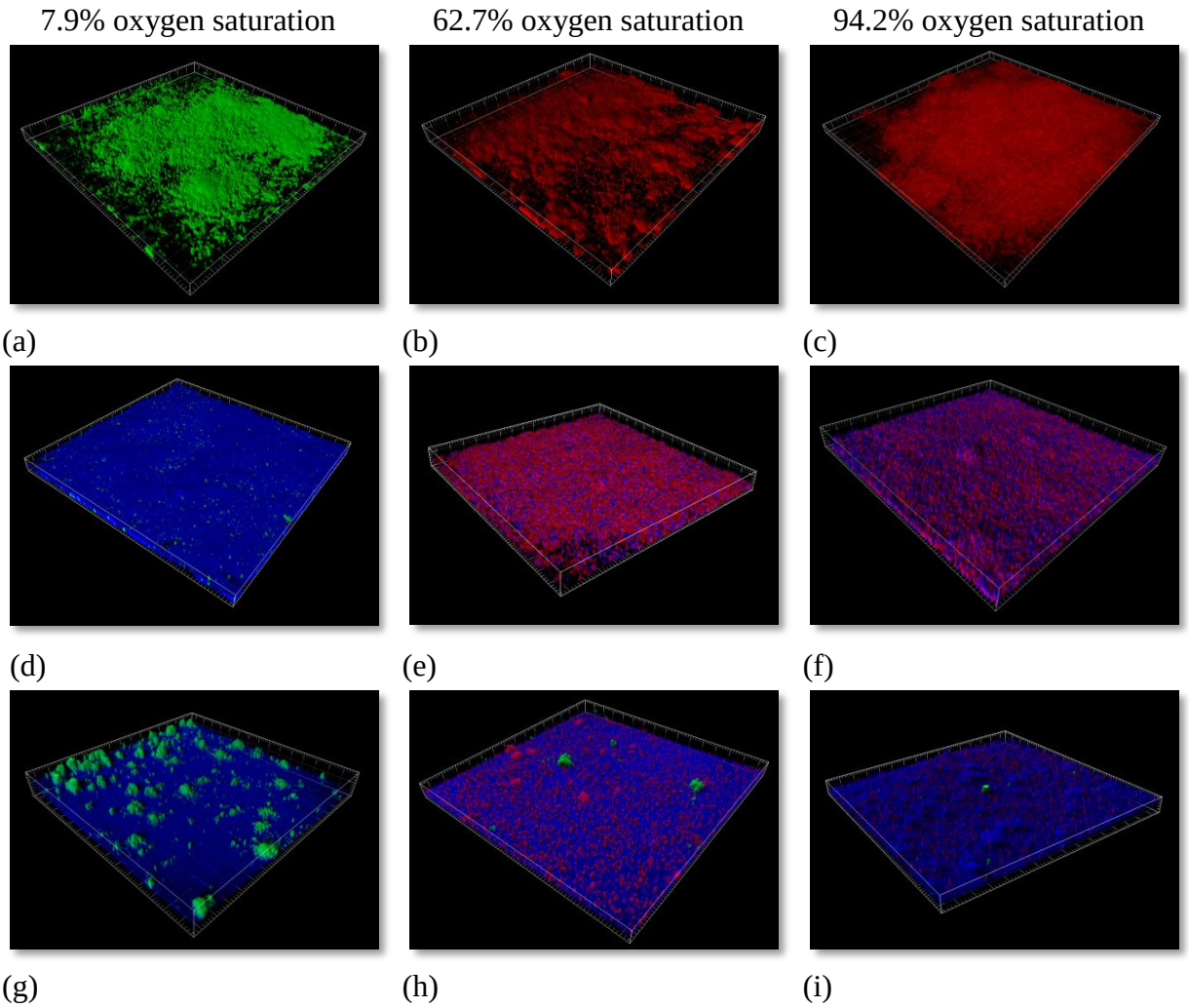

FIG. 5. 3D representation of the PAO1 biofilms at different oxygen saturation in FAB-GN media (minimal media supplemented with nitrate). PAO1 expresses either the fluorescent protein GFP or mRFP1. a-c): 24 hours old biofilms in FAB-CN media. d-f): 48 h after inoculation the cells were challenged with 50 $\mu g \cdot ml^{-1}$ Ciprofloxacin for 24 hours and then stained with dead stain SYTOX® Blue. g-i): Interconnected chambers of the different oxygen saturation environments.

VI. DISCUSSION AND CONCLUSIONS

In this paper we describe design, fabrication, working principle and application of a highly complex modular microfluidic system. Integration of different modules, bringing in such important functionalities as multichannel fluid control, bubble trapping, gas control -

exchange and bacterial culturing on a microfluidic lab-on-a-chip system, has been shown to be successfully achieved. The modularity allows addition and removal of the different functionalities. The design permits easy reconfiguration and tailoring of the system to match particular needs. In case of malfunctions in a single module, the system benefits from its modular construction and allows uncomplicated exchange of the broken module without the need for fabrication of other essential parts of the system. This is particularly important in the field of life science microfluidic systems, in which not yet all of these components are suitable for mass production. Furthermore it allows quick prototype testing of different system configurations.

The microfluidic system in its present configuration enables comparison of changes between anatomically driven oxygen tensions in different compartments of the CF airways model, as well as full control and sensing of dissolved oxygen levels. By making the system compatible with common substrates such as microscope slides and multitier plates, it enables research staff to use standard laboratory equipment such as standard microscopes and multitier plate readers.

Furthermore, the microfluidic CF airways model permits to freely reconfigure connections between oxygen rich and oxygen depleted regions without bringing restrictions to the researcher in the design of experiments. It enables to mimic some different conditions and diseases in patients suffering from CF, such as clogging of the ostia in recurrent sinusitis[4, 75] or the development of mucus plugs[76] in the bronchioles. These experiments were not previously possible to perform in standard *in vitro* flow-cells models for biofilm studies. *In vivo* models will usually not allow precise control of such important conditions.

Moreover, the use of this microfluidic system, instead of a CF airways animal model, is cheaper, safer and easier to handle for researchers. Importantly, it furthermore does not raise any ethical concerns, which is the case for the use of animal models in medical research.

We demonstrated the application of our microfluidic airway model for studying *P. aeruginosa* PAO1 under different oxygen levels in response to treatment with ciprofloxacin. We have in this way explicitly shown that the system is an asset in reliable and controllable biofilm evaluations for treatment with antibiotics at reduced oxygen concentrations. Importantly, such a system allows testing of very small volumes thereby minimizing the use of large amounts of expensive antimicrobials. PAO1 survival was shown to be highly dependent on the amount of oxygen available during the antimicrobial treatment. This corresponds very well with previous studies where it was shown that higher metabolic rate, in nitrate supplemented media under anaerobic conditions, lead to a lower survival rate of the bacteria[77].

We have shown that PAO1, under lowered oxygen concentrations, migrates towards higher oxygen concentrations even in nitrate supplemented media. Nitrate, which serves as final electron acceptor for anaerobic nitrate respiration (denitrification), does not seem to be favoured in the presence of oxygen. This scenario can mimic the reinoculation of the lower respiratory tract, previously cleared by the antibiotic treatments, with bacteria from the sinuses. This effect is hypothesised as the main reason for recurrent infections in CF patients[17].

Next generation microfluidic systems should focus on *in vivo*-like microenvironmental cues (mucus accumulation, mucus constituents, air-liquid interface,

stress factors, etc.) that would lead to more profound understanding of the bacterial role in CF pathology.

VII. ACKNOWLEDGEMENTS

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Supplementary materials

I. DETAILED DESIGN OF THE MICROFLUIDIC MODULES

A. Multichannel peristaltic micropump

The design of the multichannel peristaltic micropump was based on a previously reported system[1, 2] with two major changes. In the new design (FIG. 1), the previously used poly(dimethylsiloxane) (PDMS) inlays were exchanged with thin-walled poly(vinyl chloride) (PVC) tubing with 0.63 mm internal diameter (medical grade, Thoma Fluid, Reichelt Chemietechnik GmbH + Co, Germany). The module (FIG. 2 a) was equipped with two separate micropumping units (FIG. 2 b). Each unit is capable of actuating up to 8 channels. In this way the capacity of each module is a total of 16 individual channels. The replacement of the PDMS inlays with PVC tubing allows us to skip the laborious process of the inlay moulding. Besides, PVC is a much more durable material than PDMS which makes the entire system more reliable. PVC is also known to be far more resistant to organic solvents and does not absorb hydrophobic agents from aqueous media as PDMS does.

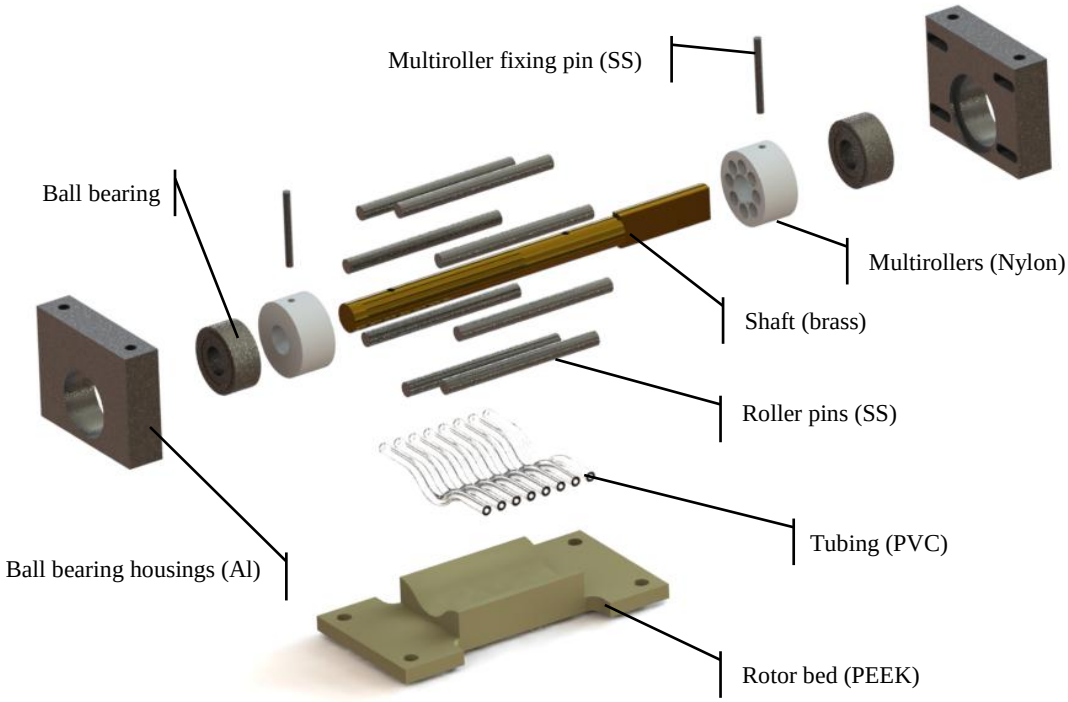

FIG. 1 Exploded view of the multichannel peristaltic micropump. The type of material for each fabricated part is indicated in parenthesis, the acronyms stands for: SS – stainless steel, Al –aluminium, PEEK – poly(ether ether ketone), PVC - poly(vinyl chloride), PC – polycarbonate.

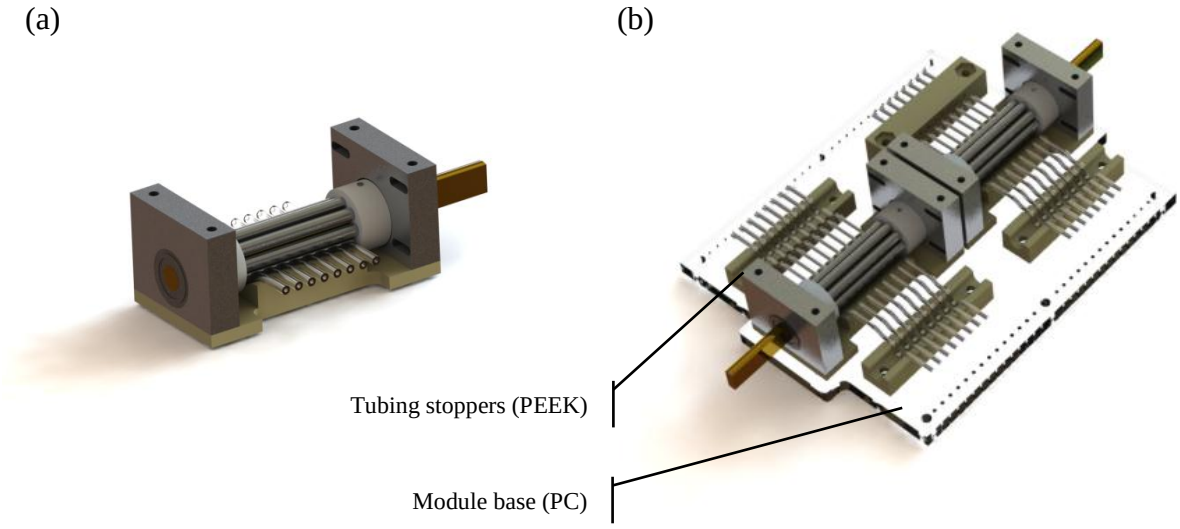

FIG. 2 Multichannel peristaltic micropump: (a) assembled micropumping unit, (b) module with two micropumping units facilitating 16 channels.

B. Bubble trap module

The array of bubble traps was designed according to previously reported work[3]. The entire module was made in polycarbonate (PC). Each bubble trap has a volume of 56 mm^3

and is equipped with a tapered opening on the top which serves as a vent. It can be closed or opened with the plug in order to release entrapped air bubbles (FIG. 3).

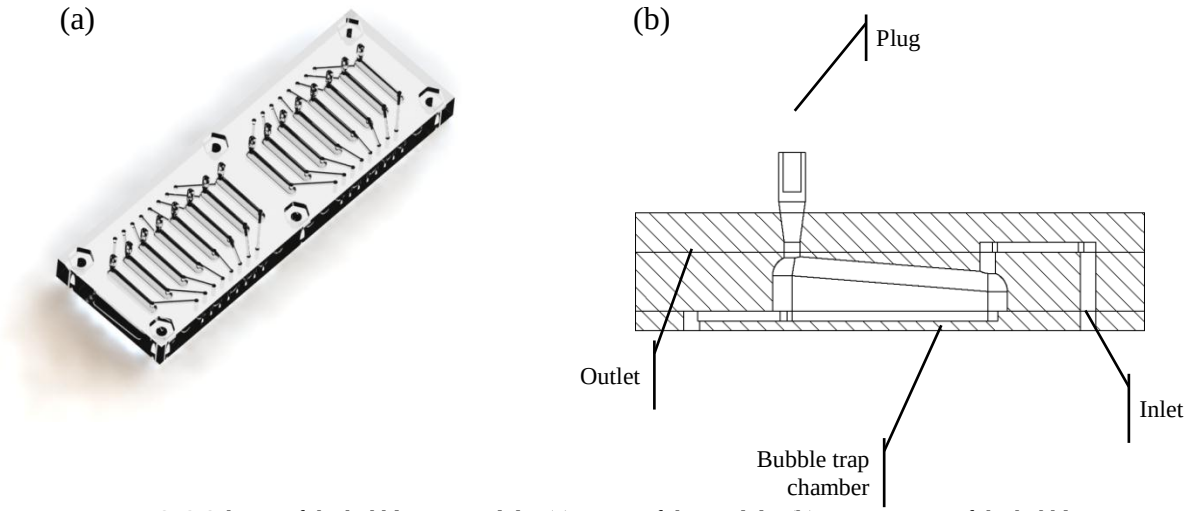

FIG. 3 Scheme of the bubble trap module: (a) image of the module, (b) cross-section of the bubble trap.

C. Gas exchange module

The gas exchange module allows oxygen saturation or desaturation of a desired culture media. The working principle of the module was communicated in our previous report[4]; here described briefly. It is based on the oxygen diffusion through gas permeable membranes made from PDMS. The culture media flows through the channels fabricated in PDMS. The channels were patterned with groves, similar as reported in our previous work[5], in order to improve mixing of the media. The oxygen can diffuse from the culture media through the integrated membrane to the channel filled with oxygen scavenging liquid. The scavenger (sodium sulphite) reacts with the available oxygen. In the case, where the channels are filled with atmospheric air, the media is ensured to be saturated with oxygen. The PDMS inlays are enclosed in low gas permeability scaffold, made form PC, in order to separate the permeable material from the atmosphere (FIG. 4).

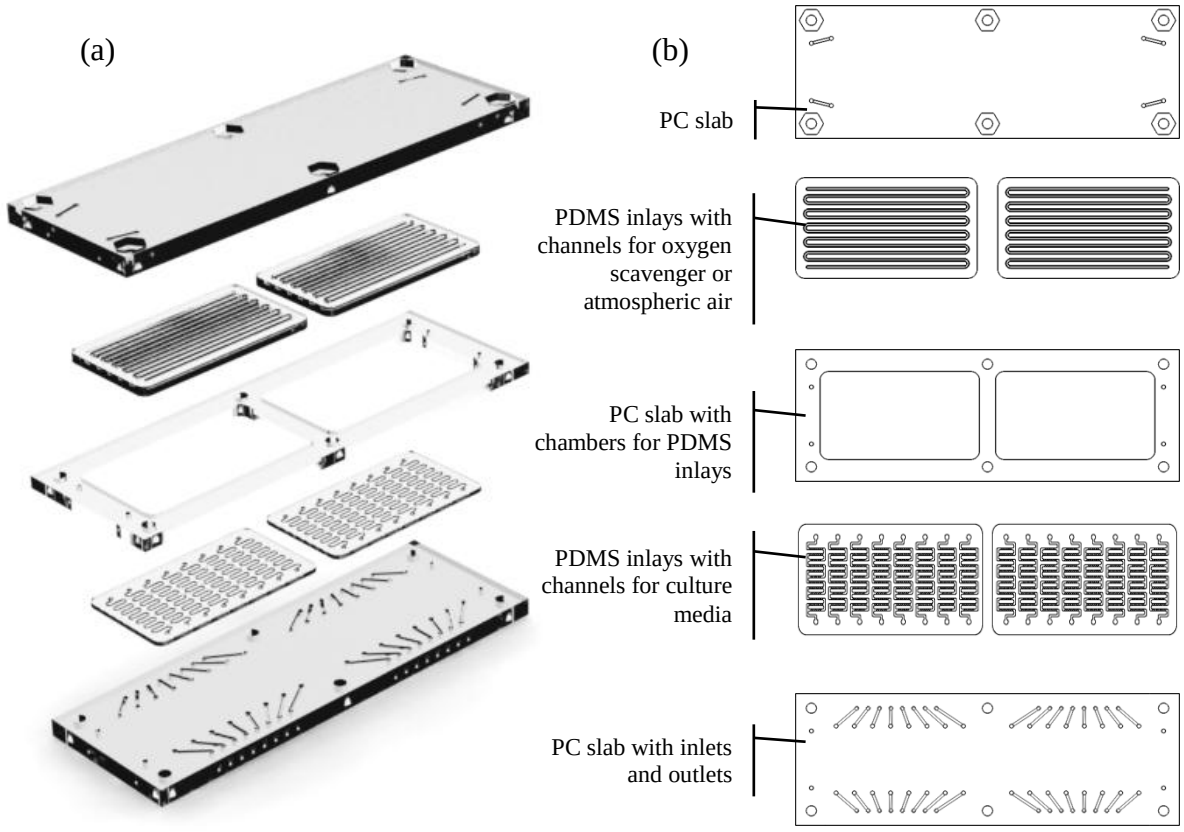

FIG. 4 Scheme of the gas exchange module: (a) exploded view of the module, (b) top view for on the module layers.

D. Culture chambers module

The culture chambers were designed to implement three different oxygen saturation environments (FIG. 5). The module consists of 8 chambers that can be interconnected by detachable PVC tubing. The volume of each chamber is 4.2 µl and the area for cells attachment equals to 12.7 mm^2. Each chamber has additional tapered openings: one near its inlet and the other near its outlet (FIG. 6). The opening near the inlet of the chamber is used as the inoculation site. It can be opened and closed with a plug in the same manner as the vents in the bubble trap module. Instead of plugs, these openings can be equipped with fittings and tubing in order to interconnect two chambers with different oxygen environments.

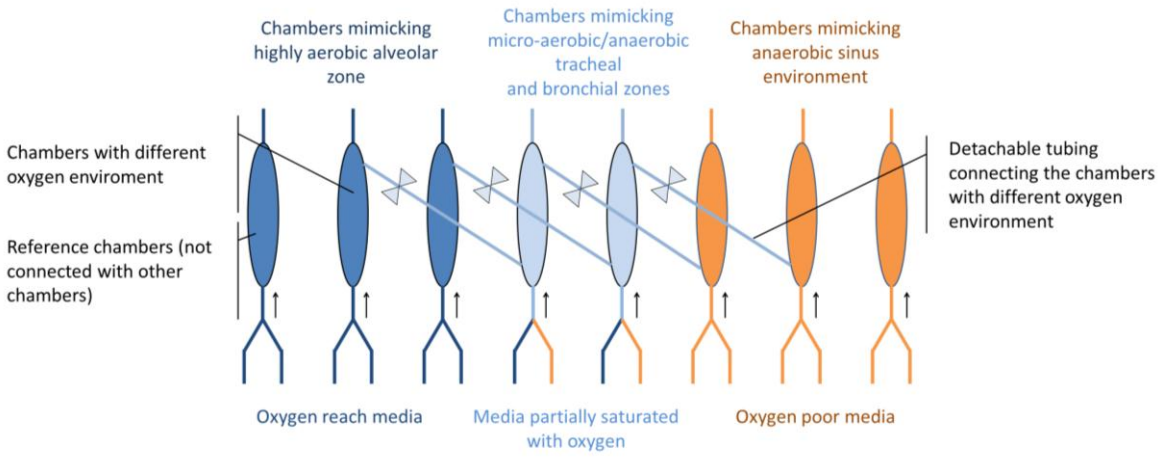

FIG. 5 The scheme of the cell culture chamber module facilitating different oxygen environments.

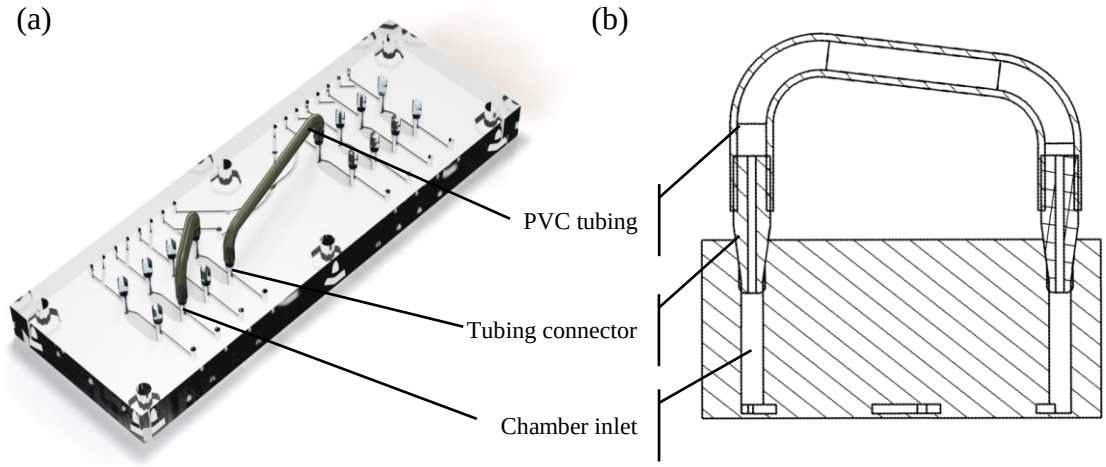

FIG. 6 Cell culture chamber module: (a) scheme of the module with plugs and tubing interconnecting different chambers (for clarity of the image, only two interconnections are shown), (b) cross-section of the fitting and tubing.

E. Check microvalve

The working principle of the designed check microvalve was based on a previous report[6]. An array of 28 check microvalves was fabricated in 1 mm thick fluoroelastomer (FIG. 7 a), the same material that was used to fabricate the seals. The microvalves are clamped in-between the microfluidic modules and the platform. Each microvalve has a chamber with a 150 μm thick membrane (FIG. 7 b). In the membrane there are two holes with Φ = 500 μm spaced 1.59 mm from each other (centre to centre). Such spacing is large enough to cover the holes of the microfluidic modules when the membrane is in a relaxed

state. Membrane deflection is only possible inward the chamber thereby only allowing media flow in one direction (FIG. 7 b).

The purpose of the microvalve is to stop the backflow from the outlet tubing while allowing the user to inoculate the system using standard laboratory micropipettes. The membrane thickness, as well as the size and spacing between the holes, was optimised in order to get the opening pressure low enough enabling the use of a micropipette (typically below 1 kPa).

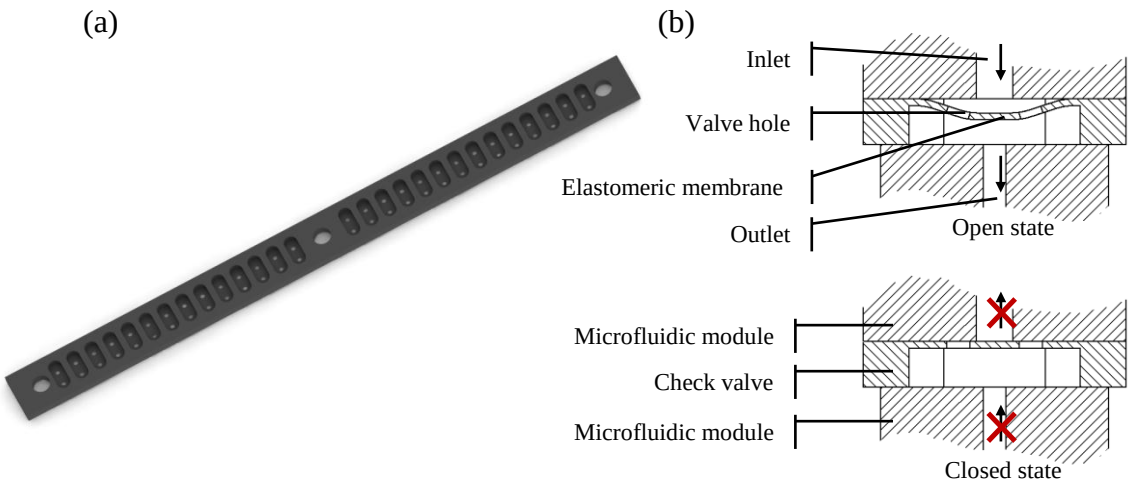

FIG. 7 Check microvalve: (a) scheme of the module with 28 independent microvalves, (b) the working principle of the valve

F. Modular platform

The platform was designed to comply with the microplate footprint standards[7, 8]. This allows the device to be used together with a broad range of commonly used laboratory equipment such as microtitre plate readers and microscopes. The platform can be equipped with up to 4 modules with a microscope slide size (76x26 mm). Modules can be serially interconnected by 28 independent channels at the long edges of a module. Additionally, each module has 8 inlets/outlets at each of the short edges. The platform has 28 independent inlets and the same amount of outlets (FIG. 8 a).

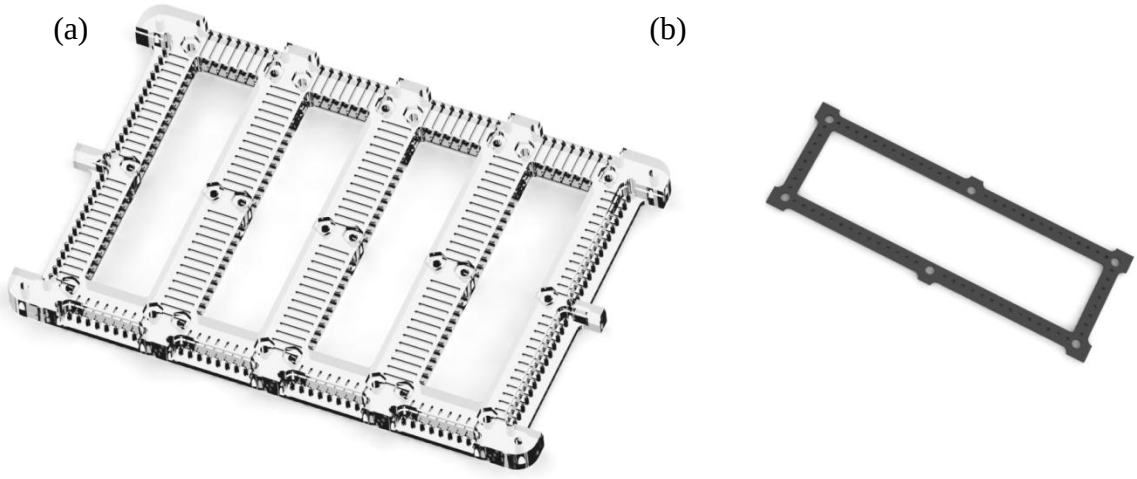

FIG. 8 (A) Microfluidic modular platform, (B) gasket used to seal the modules with the platform

Modules are attached to the platform by screws and nuts (FIG. 9) and all the microfluidic connections between the modules and platform are sealed by gaskets made from fluoroelastomer (FIG. 8 b).

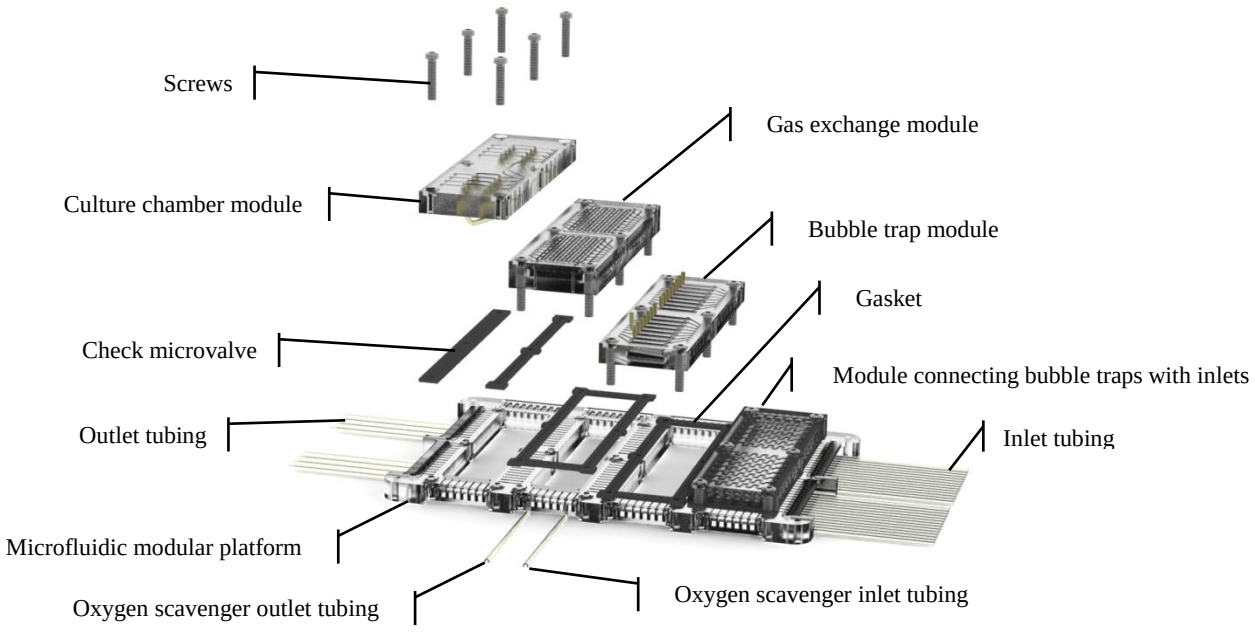

FIG. 9 Exploded view of the microfluidic platform with modules

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13.5 Paper V

Skolimowski, Nielsen, M.W., M., Abeille, F., Lopacinska, J., D., Molin, S., Taboryski, R., Sternberg, C., Dufva, M., Geschke, O. and Emnéus, J., *Microfluidic model of cystic fibrosis bronchi.* Accepted for Proceedings of the Fifteenth International Conference on Miniaturized Systems for Chemistry and Life Sciences, 2011

MICROFLUIDIC MODEL OF CYSTIC FIBROSIS BRONCHI

M. Skolimowski[1,2*], M. W. Nielsen[1,2], F. Abeille[1,2], J. Lopacinska[1], S. Molin[2], R. Taboryski[1], O. Geschke[1], C. Sternberg[2], M. Dufva[1] and J. Emnéus[1]

[1]*Technical University of Denmark, Department of Micro- and Nanotechnology, DENMARK*

[2]*Technical University of Denmark, Department of Systems Biology, DENMARK*

ABSTRACT

In this paper we report a microfluidic model to simulate the bronchi of a cystic fibrosis (CF) patient. The biochip is comprised of two cell culture chambers separated by a membrane. On top of the membrane an alginate hydrogel is formed in order to simulate the thick mucus layer spotted in a CF bronchi. In the bottom chamber a monolayer of epithelial cells are cultured to simulate the bronchi tissue. By inoculating the *pseudomonas aeruginosa* PAO1 strain to the hydrogel layer one can simulate bacterial infections commonly subjected to the CF patient, and the system can be applied for the studies on antibiotic treatment of bacterial infection related to CF.

KEYWORDS: cystic fibrosis, bronchi, model, microfluidic, microfabrication

INTRODUCTION

Here we report work towards a microfluidic system that simulates the cystic fibrosis (CF) bronchi and the impact of the mucus layer on the treatment of bacterial infections. The classical way of studying CF related bacterial infections, primarily *Pseudomonas aeruginosa*, is by growing them in flow-cell systems [1,2]. In these flow cells bacteria are capable of forming biofilm, as in the airways, and can then be monitored using confocal microscopy [3]. However, the bacteria in CF patient bronchi are not subjected to a constant flow of nutrients as in flow-cell based systems. Instead they embed in the mucus that covers the bronchi epithelia through which the nutrients and metabolites are diffusing (Figure 1A). Moreover, the content of the mucus highly affects bacterial attachment and biofilm growth. Consequently, the biological response for biofilm drug treatments can be altered by changes in the mucus [4].

THEORY

While the human primary bronchi have a relatively large lumen diameter (order of magnitude of centimetres), the respiratory bronchioles are about 1 mm in diameter. Therefore, in order to be able to mimic it as closely as possible to the *in vivo* conditions, a microfluidic system is required. The presented microfluidic model of the CF bronchi consists of two chambers separated by a microporous membrane (Figure 1B). The membrane is the underlying support for a hydrogel, which mimics the mucus layer in the CF bronchi. The chamber below the membrane simulates the artery and supplies the media with nutrients and transports the metabolites. These compounds are provided further to the top chamber by diffusion through the membrane and hydrogel. On the bottom side of the membrane epithelial cells are cultured while on the top part of the hydrogel the bacteria cells are inoculated. In order to simulate antibiotic treatment, the media in the bottom channel can be supplemented with drugs. This supplementation can be performed in cycles, which would mimic drug dosage to CF patients. The introduction of the human sub-bronchial gland cell line (Calu-3) without the layer of hydrogel can simulate the normal bronchi while the same system but with the hydrogel can simulate the bronchi of CF patients.

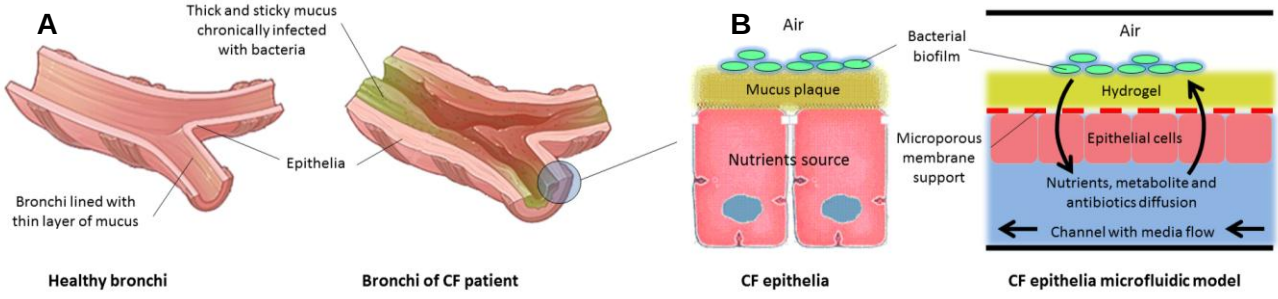

Figure 1: (A) Bronchi of the healthy individual and CF patient. (B) The microfluidic model of CF epithelia.

EXPERIMENTAL

The biochip (Figure 2) was fabricated in polycarbonate (PC) by micromilling (Mini-Mill/3PRO, Minitech Machinery Corp., USA). The PC membrane with 0.45 µm pores was inserted between the milled parts and bonded (tetrahydrofuran vapours assisted bonding, 3.5 MPa, 50°C).

The hydrogel layer was formed by introduction of 0.3% sodium alginate to the top chamber and 0.1 M $CaCl_2$ solution to the bottom chamber. The thickness of the hydrogel was controlled by focusing the sodium alginate stream with PBS (Figure 3). In order to visualise the hydrogel thickness, the sodium alginate was stained with 6-aminofluorescien according to the receipt by Strand *et al*. [5].

The biochip was thoroughly washed with PBS followed by cell culture media (DMEM, 10% FBS). Calu-3 cells with a density of $5 \cdot 10^5$ cells/ml were seeded on the membrane in the bottom chamber.

The inoculum (OD_{600} = 0.01) of the *P. aeuruginosa* PAO1 strain tagged with GFP was introduced into the upper chamber to mimic the bronchi infection (Figure 5). The culture was performed for 3 days in the incubator (5% CO_2, 37°C) (HERAcell incubator, Heraeus, Germany) with perfusion of culture media through the lower chamber with flow rate 0.3 $ml \cdot h^{-1}$.

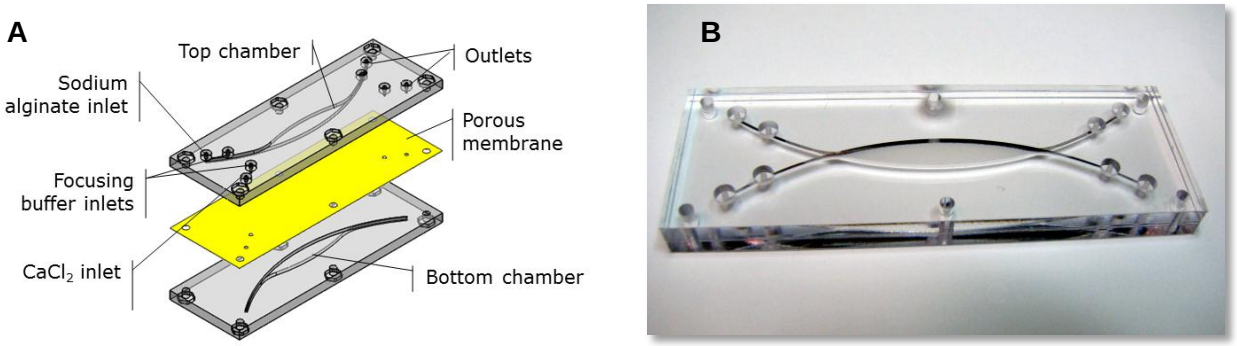

Figure 2: (A) The 3D model of the biochip consist from 3 layers. (B) Fabricated and bonded biochip.

RESULTS AND DISCUSSION

The obtained thickness of the hydrogel layer in the top chamber was 270±20 µm (Figure 4). The focusing buffer was actively pushing the sodium alginate through the membrane, therefore a thin (below 100 µm) layer of hydrogel was formed on the other side of the membrane. The vertical transport of the calcium ions through the membrane was purely diffusional.

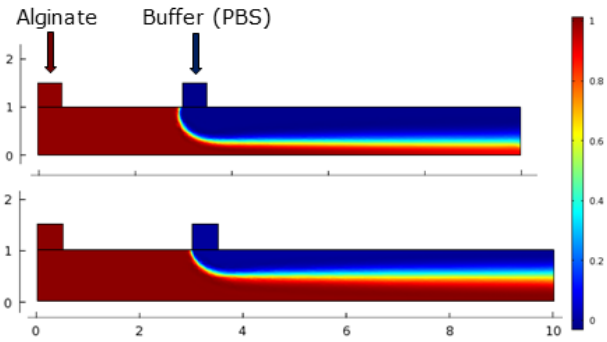

Figure 3: Numerical simulation of the formation of hydrogel layer in the top chamber. The thickness of the layer can be controlled by changing the ratio between the sodium alginate and focusing buffer flow rates.

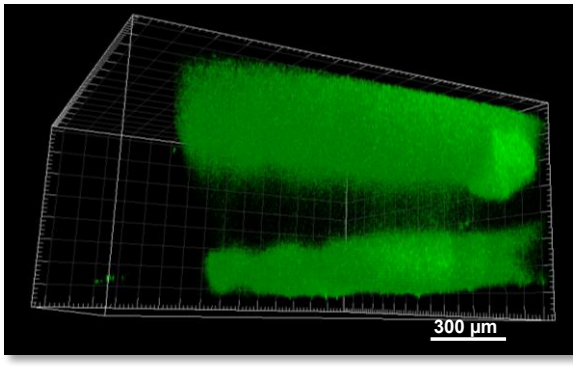

Figure 4: Confocal image of the hydrogel formed in the biochip. Green fluorescence represents calcium alginate and the gap between is the porous PC membrane. The alginate was stained according to [5].

The Calu-3 cells were cultured in the bottom chamber of the system. After reaching the 70% of confluency (3rd day of culture) the *P. aeruginosa* PAO1 strain was inoculated to the upper chamber. The bacteria were allow to form biofilm in the hydrogel for 3 days (Figure 5A). The Calu-3 cells were stained with live/dead stain (Calcein AM/PI) in order to visualise the viable and necrotic epithelia (Figure 5B).

The future application of the presented CF bronchi model lies in simulation of antibiotic treatment of CF related bacterial infection. The simulation of the treatment can be performed by supplementation of the culture media with drugs. The results expected from this should be a better understanding of the problems in the treatment of the chronically infected CF patient.

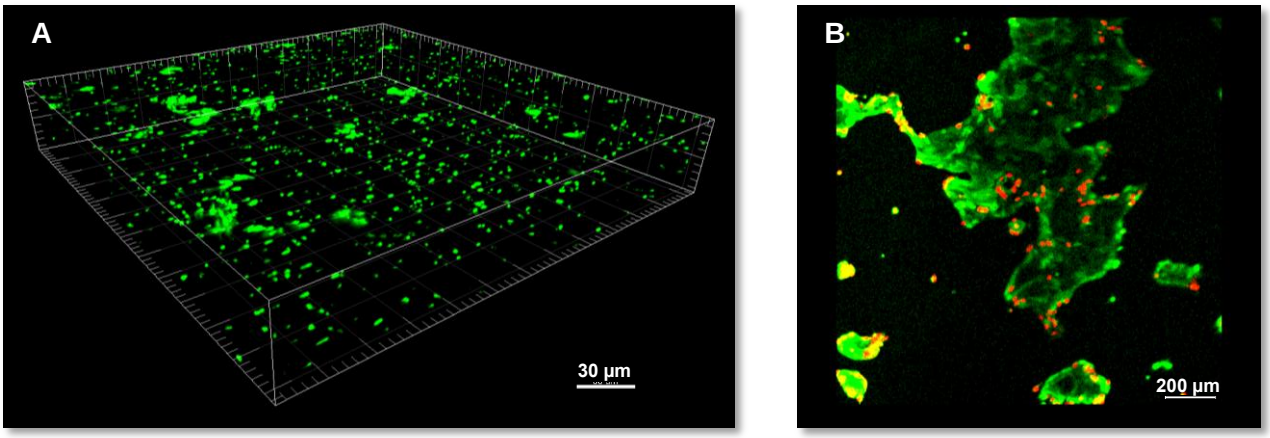

Figure 5: (A) Confocal image of the P. aeruginosa PAO1 strain tagged with the GFP growing in the hydrogel. (B) Fluorescence image of Calu-3 cells stained with live/dead stain (Calcein AM/PI) growing below the hydrogel.

CONCLUSION

We have successfully designed and fabricated a microfluidic biochip which can be used as a model of the CF bronchi. The co-culture of the Calu-3 cells and *P. aeruginosa PAO1* strain using the constructed biochip was shown. By alternating the thickness of the hydrogel layer, the presented model can be used in the future for comparative studies of the antibiotic treatment of bacterial infections in normal and CF patients. This system is a significant advancement in the mimicking of the airways function on the chip reported earlier [6].

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CONTACT

*M. Skolimowski, tel: +45 4525 6997; maciej.skolimowski@nanotech.dtu.dk

13.6 Paper VI

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Microfluidic system for *in vitro* study of bacterial infections in cystic fibrosis bronchi

Abeille, F. *, Skolimowski, M. *,**, Nielsen, M.W. **, Lopacinska, J. *, Molin, S. **, Taboryski, R. *, Sternberg, C. **, Dufva, M. *, Geschke, O. * and Emnéus, J. *

* Technical University of Denmark, Department of Micro- and Nanotechnology, Ørsted Plads, Building 345B, DK-2800 Kgs. Lyngby, Denmark;
Tel: +45 4525 6887; E-mail: maciej.skolimowski@nanotech.dtu.dk

** Technical University of Denmark, Department of Systems Biology, Matematiktorvet, Building 301, DK-2800 Kgs. Lyngby, Denmark

Keywords

microfluidic, biochip, cystic fibrosis, Pseudomonas aeruginosa, bronchi, model, bacterial infection, hydrogel

Abstract

Here we present our work on a microfluidic device that mimics the bronchi in people suffering from cystic fibrosis. To imitate the mucus of the diseased bronchi, a 200 µm thick hydrogel is formed in a controlled manner on the top of a microporous membrane. This membrane is also the support for the attachment of the epithelial cells, to mimic the bronchial tissue. In the hydrogel, *P. aeruginosa* are inoculated to recreate the scenario of bacterial infections. Compared to flow- or static cell culture systems our microdevice allows better imitation of the nutrient supply through the bronchial bio-layers.

Eventually, nutrient driven development of bacterial growth and the viability of the epithelial cells are characterized by confocal laser scanning microscopy.

Introduction

Cystic fibrosis

Many severe infections in humans affect the airways. Different types of pneumonia are major causes of morbidity and mortality in patients with various immuno compromising conditions. Ineffective anti-microbial therapies often fail to remove the infecting microbes. One of the most severe genetic diseases affecting the human airways, is cystic fibrosis (CF) [1].

CF patients suffer from a genetic defect in the CFTR (Cystic Fibrosis Transmembrane Regulator) gene that hinders the salt transport over the cell membranes [2]. This leads to high salt concentration in the epithelia and low concentration in the mucus, which stops water from rehydrating it [3]. The mucociliary clearance mechanism is impaired and results in frequent infections in the CF airways with increased risk of pneumonia[4]. Since the bacteria can infect the airways unhindered and thrive in the CF mucus, the immune system tries to eradicate the increasing infecting populations. The success of the immunological battery is highly reduced since the bacteria are embedded in mucus and more or less recalcitrant to the attacks [5]. Instead the lung tissue is gradually damaged by the ongoing immunological exposure [6], eventually leading to massive pulmonary deficiency and death.

Several works exist on the mimicking of the human airways using microsystems: some focused on the reproduction of the mechanical function of the alveoli [7-9] while others were more oriented on the regulation of mucin production by NCI-h292 epithelial cells [10]. *Huh et al.* [7] proposed recently a model of the vacuoles in the lung. In this work the phagocytosis of planktonic *Escherichia coli* cells by neutrophils on the epithelial surface was shown.However, according to the author's best knowledge there is no system that mimics the bacterial habitats in the CF bronchi.

Microfluidic model of the cystic fibrosis bronchi

The classical way of studying CF related bacterial infections, primarily *Pseudomonas aeruginosa*, is by growing them in static systems [11] or in flow systems [12-14]. In the static culture systems, the amount of nutrients provided to the cells is decreasing in time as the consequence of bacterial consumption. Consequently, in such a system the media have to be renewed regularly but render a system with higher throughput than the flow cell culture. In the flow cells, nutrients and waste are constantly being exchanged to the biofilm forming bacteria. However, the bacteria in CF bronchi are not subjected to a constant flow of nutrients. Instead they are embedded in the mucus that covers the bronchi epithelia through which the nutrients and metabolites are diffusing (Fig. 1).

The human primary bronchi have relatively large lumen diameter (in the order of centimeters), whereas the respiratory bronchioles can be smaller than 1 mm in diameter [15]. Therefore, in order to respect this order of dimension a microfluidic system is required, which is the strategy pursued in this paper.

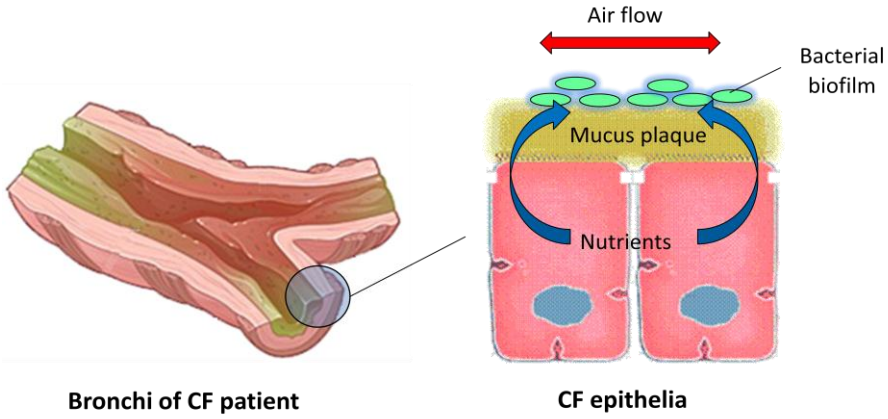

Fig. 1. The bronchus of a CF patient: Cross section and microfluidic dynamics of the CF bronchi

Materials and methods

Design and procedure

The *in vitro* CF bronchus consists of a chamber divided into two parts (bottom chamber - top chamber) by a porous membrane (Fig. 2). The membrane is used as a solid support for a hydrogel on top and for attachment of epithelial cells beneath. The hydrogel mimics the mucus residing inside the bronchi and epithelial cells the bronchial epithelial tissue. In the top chamber, bacteria can be inoculated to simulate a bacterial infection taking place in chronically infected CF patients [16]. The bottom chamber mimics an artery, provides media with nutrients and transportation of the metabolites. These compounds are supplied further to the top chamber by diffusion through the epithelia layer, the membrane and the hydrogel.

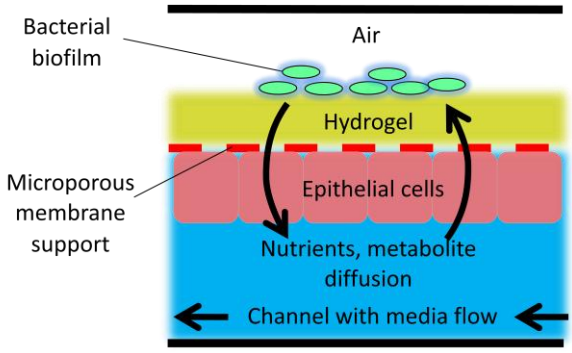

Fig. 2. Model for mimicking of the CF bronchus.

The immitation of the *in vitro* bronchus model, as described above, followed a specific procedure:

To ensure the sterility inside the device, the entire system was flushed for 30 minutes with 70% ethanol and then flushed for another 30 minutes with 5% hydrogen peroxide (Sigma Aldrich A/S, Denmark).

The hydrogel (calcium alginate) was formed by a chemical reaction on top of the membrane. Following the formation of the desired thickness of alginate gel, epithelial cells were injected in the bottom chamber as described below in the "Epithelial cell culturing" section. Here the cells were allowed to adhere to the membrane as the device was orientated in an upside down position (top chamber down and bottom chamber up) for 24 hours. Once the bronchial cells reached a confluence of 90% (the 2nd day after seeding), bacteria were inoculated in the top chamber to recreate the scenario of a bacterial infection.

Fabrication of the microfluidic chip

The main feature of the chip is the chamber in the center where the CF bronchus is mimicked. It has an elliptic shape with a perigee of 2 mm, an apogee of 21 mm and a thickness of 2 mm. This shape enables to easily remove the bubbles that may get inside the device. The membrane dividing the chamber is a hydrophilic poly(tetrafluoroethylene) (PTFE) membrane (BCGM 000 10, Millipore A/S, Denmark). It is 50 µm thick with pores of 0.4 µm diameter. Two inlets and two outlets of 0.5 mm in diameter are connected to the upper chamber by a 1 mm thick and 0.5 mm wide channels, the same for the bottom chamber (Fig. 3). The different inlets are used to inject different solutions (see the "Hydrogel formation" section). In practice only one of the outlets from each channel is useful but for a symmetry matter more of them are created.

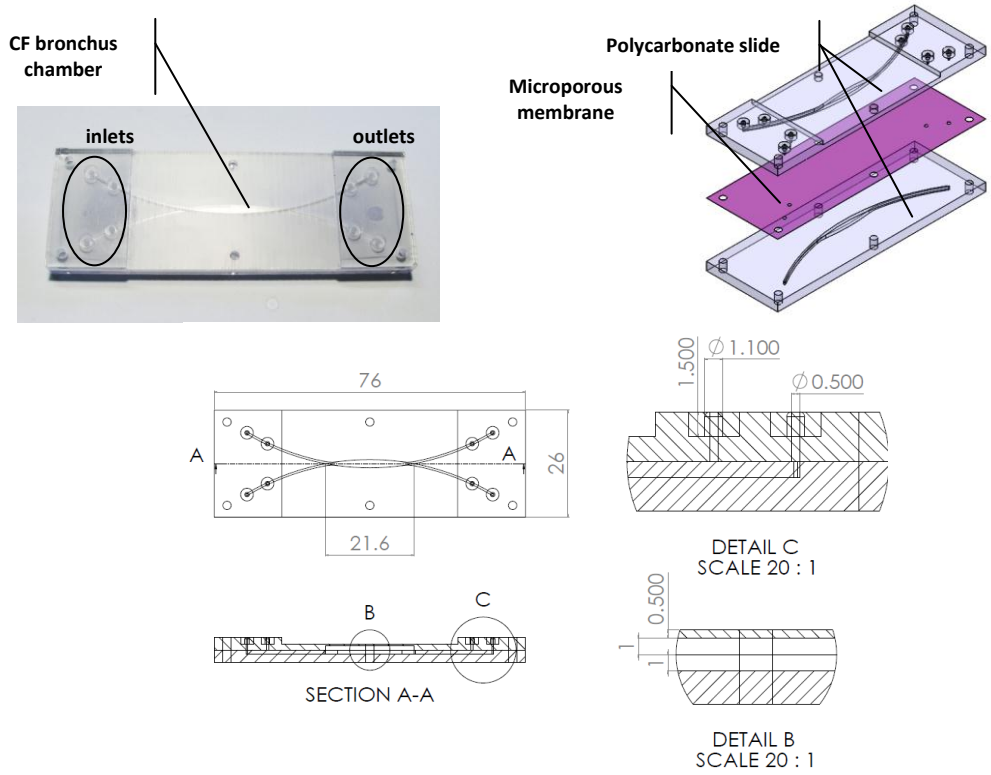

Fig. 3 Top left: Picture of the microfabricated device. Top right: Exploded view of the microfluidic chip. Bottom: Sketches of the top and the side and view of the two assembled parts of the microfluidic chip.

Each chamber with inlets, outlets and channels (except for the porous membrane) were fabricated by micromilling (Mini-Mill/3PRO, Minitech Machinery Corp., USA) in two polycarbonate (PC) slides (Nordisk Plast A/S, Denmark). Each of the slides has the dimensions of a microscope slide (76x26 mm) with a thickness of 3 mm.

The polycarbonate constituting the cover of the chamber was milled down to 0.5mm in thickness to allow the use of high magnification microscope objectives for bacteria observation within the culture chamber (top chamber). Both sides of the PTFE membrane were exposed to air plasma (70 W, 6 mbar) for 30 s to later facilitate the attachment of the epithelial cells. The PC slides were exposed to tetrahydrofuran (THF) vapors for 5 min. Eventually, the PTFE membrane was inserted between the two polycarbonate slides followed by thermal bonding (using bonding press PW 10 H, P/O/Weber, Germany) at 50°C with 4 MPa for 30 min.

Hydrogel formation

A 100 mM $CaCl_2$ solution (Sigma-Aldrich Denmark A/S) was used to cross-link a 0.5% (w/v) solution of sodium alginate (Sigma-Aldrich Denmark A/S) in order to form the calcium alginate hydrogel. A phosphate buffer saline (PBS) solution (Sigma-Aldrich Denmark A/S) was used as buffer.

The hydrogel formation within the chamber was performed in the following order (Fig. 4):

1. Perfusion of both chambers with the PBS solution for 6 min at a flow rate of 10 ml·h^{-1}. This ensures that all chemicals previously used for sterilization are washed out.
2. Sodium alginate is perfused through the top chamber at a flow rate of 5 ml·h^{-1}. In the bottom chamber PBS is kept at the same flow rate of 5 ml·h^{-1}. This procedure is carried out for 6 min.
3. The flow of PBS in the bottom chamber is then exchanged with a solution of $CaCl_2$ at a flow rate of 5 ml·h^{-1} for 10 min. In this way, calcium ions can diffuse through the membrane to cross-link with the sodium alginate to form the gel.
4. After the 10 min, the flow of sodium alginate in the top chamber is exchanged with a flow of PBS at 5 ml·h^{-1} for 10 min to remove weakly cross-linked alginate. The diffusion of calcium ions going on strengthens the hydrogel.
5. The chambers are subsequently flushed with PBS at a flow rate of 1 ml·h^{-1} for 30 min.

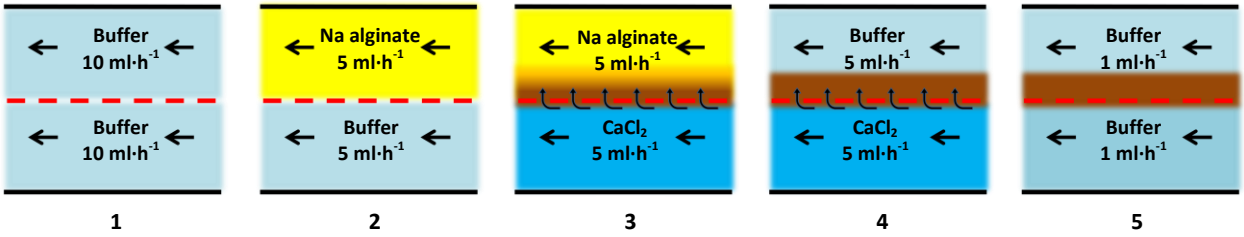

Fig. 4. Process for the hydrogel formation.

In order to determine the thickness of the hydrogel, experiments with stained sodium alginate were conducted. Aminofluorescein was covalently bound to the α-L-guluronate residues constituting the alginate by following a particular staining technique [17]. However, no stained alginate was used in the system while cell culturing was performed inside the device.

Epithelial cell culturing

Calu-3 cells (Faculty of Pharmaceutical Sciences, University of Copenhagen, Denmark) were cultured in 25 cm² tissue culture flasks (Sarstedt Inc., USA) with DMEM (Dubelcco's Modified Eagle Medium) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) , 50 µg·ml^{-1} of Gentamicin and 100 U·ml^{-1}/100 µg·ml^{-1} of Penicillin/Streptomycin. The media was changed every second day. Once the cells reached a stage of 90% confluence they were passaged by using Trypsin-EDTA solution for 10 min at 37°C.

From the harvested cells a concentration of $8 \cdot 10^5$ cells·ml^{-1} was prepared for seeding inside the chip using a syringe. Cells were inoculated in the bottom chamber where the cells were allowed to adhere to the membrane. This was done by placing the device in an upside down position (top chamber down and bottom chamber up) for 24 hours in a cell culture incubator (5% CO_2, 37°C) (HERAcell incubator, Heraeus, Germany). Eventually, the microfluidic chip remained in the incubator

with a continuous media flow of 0.3 ml·h^{-1} for both chambers until a cell confluence of about 90% was obtained.

Calu-3 viability was checked by using a 40 nM concentration of Calcein AM to stain living cells and a 1.5 µM concentration of Propidium Iodide (PI) to stain dead cells. Calcein and PI respectively stain the cells with a green and red fluorescence as shown in Fig. 6.

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich Denmark A/S.

Bacteria cell culture

The *P. aeruginosa* laboratory strain PAO1 was used in all experiments involving bacterial cultivation or co-cultivation [18]. PAO1 was grown at 37°C in shaken conditions overnight in Luria Bertani (LB) media. Preparation for inoculation was done by diluting to an OD_{600} of 0.01 in 0.9% NaCl. 500 µL was inoculated in the top chamber and the cells were allowed to attach without any media flow delivered through the system for an incubation period of 1 hour. Following the incubation, the flow was resumed at 0.3 ml·h^{-1} in DMEM media with 10% of FBS without antibiotics.

PAO1 was expressing the green fluorescent protein (GFP) tagged by the miniTn7 insertion method [19].

Microscopy and data analysis

A confocal laser scanning microscope (CLSM) (Leica TCS SP5 from Leica Microsystems A/S, Denmark) was used to acquire images of cells and hydrogel. Detectors and filters were set for monitoring Calcein-, GFP- and PI fluorescence. Images of Calu-3 cells and hydrogels were performed with a 10x/0.3 air objective. Bacteria were imaged with a 63x/0.70 air objective.

In order to evaluate the thickness of the hydrogel, the pixels with an intensity higher than 25% of the maximum intensity value were taken into consideration.

All the pictures were treated and analyzed by using the Imaris 7.1.1 software package (BITPLANE AG, Zürich, Switzerland).

Results

Hydrogel layer

The thickness of the hydrogel layer depends on the time in which the Ca^{2+} ions are able to diffuse into the top chamber. The longer they can diffuse, the further up they are able to cross-link the alginate in the top chamber. Thus, controlling the time of diffusion allows better control of the gel thickness that one wants to achieve. A thickness of about 200 µm was chosen for imitation of the mucus layer. To obtain this specific thickness, 10 min of diffusion were required during the 3rd step of the protocol of "Hydrogel formation" section (Fig. 4). Confocal imaging revealed that the formed hydrogel was 230 µm (± 20 µm) thick (Fig. 5A).

During the optimization of the hydrogel formation, several experiments allowed the determination of a diffusion time range: 5 min of Ca^{2+} diffusion lead to an approximate hydrogel thickness of 100 µm of hydrogel (Fig. 5B) whereas 15 min filled up the top chamber (Fig. 5C).

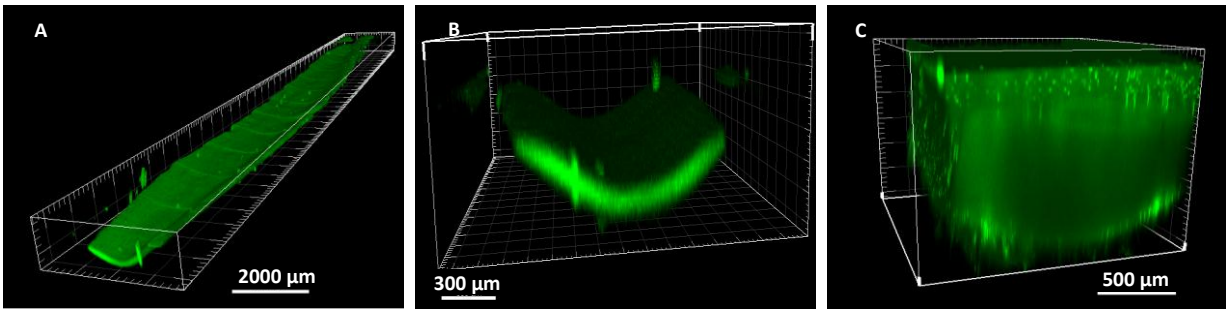

Fig. 5. A. Stained hydrogel characterized by CLSM B. 100 µm thick hydrogel obtained after 5 min of Ca^{2+} diffusion. C. Top chamber completely filled after 15 min of Ca^{2+} diffusion.

Co-culture of the Calu-3 epithelial cells and the *P. aeruginosa* PAO1 strain

The growth of the Calu-3 cells was successfully performed inside the microfluidic device. The cells reached a confluence of about 90% in 2 days (out of the 24 hours needed for attachment). To check the viability the cells were stained with Calcein AM and PI. Confocal microscopy revealed that less than 1% of the cells were necrotic after 3 days of culturing (Fig. 6). This resembles normal Calu-3 culturing viability in a regular tissue culture flask (data not shown).

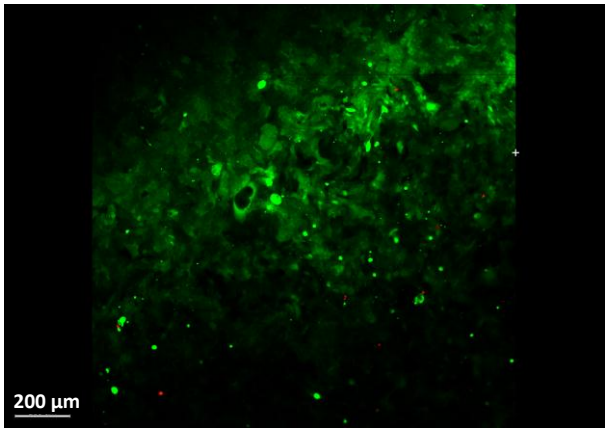

Fig. 6. Viability of the epithelial layer after 3 days of culture.

PA01 formed biofilm on top and inside the hydrogel layer (Fig. 7). Because the pores in the calcium alginate are much bigger (raging from tens to hundreds of microns) [20] than the size of bacteria, single bacteria could easily get inside and start forming microcolonies.

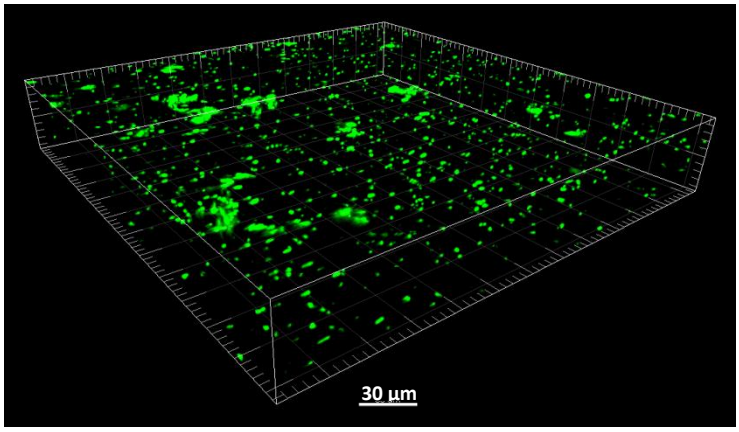

Fig. 7. 3D visualization of PA01-GFP embedded in the calcium alginate matrix at day 5.

Discussion

We demonstrated a fast and easy way to integrate the PTFE porous membrane. No specific setup needs to be used or optimized if we compare to *in situ* membrane integration [21]. No alignment or specific treatment was required for this membrane in regards to the bonding step. The surface treatment of polycarbonate parts using THF vapors and the thinness of the membrane (50 µm) enabled strong and durable bonding, across the membrane, between the PC slides.

The term "hydrogel thickness" used here includes the membrane size, the thickness of the gel below and above the membrane. The thickness below the membrane is though negligible (< 2 µm). Indeed because of the low diffusion coefficient of the alginate [22] a very low amount of it diffuses beneath the membrane and transforms into calcium alginate. However, the alginate beneath did not affect the attachment and development of the Calu-3 cells.

As mentioned in the introduction, there are two main ways to culture cells: the flow cell culture [12-14] and the static cell culture [11].

However, these types of culture do not reflect the conditions in which bacteria can develop in niches of the human body as in the severe case of the CF airways. As explained in the microfluidic model of the CF bronchi, the nutrients are provided to the bacteria by diffusion across different bio-layers from a cyclic flow (i.e. the blood stream). With this in mind, the proposed microfluidic system represents a significant step forward in the study of lung infections since such mimicry has not been reported in the literature yet.

Moreover, this *in vitro* chip makes microscopy observation possible which is not the case when using *in vivo* models. In addition, there is a very strong pressure from public societies, states, international organizations and from the industry to replace, reduce and refine the research conducted with the use of animals [23, 24]. This strategy is known as the "3 Rs" principle [25, 26]. Therefore, our developed microfluidic system can play an important role in the replacement of animal models used for medical experiments related to for instance CF.

The chip provides a new way to simulate bacterial infections in the airways. In the future, it can also provide a novel bio-mimetic approach regarding drug treatment. Antibiotics can be delivered in two different manners. One would be to use a gaseous form to be delivered to the upper chamber to reproduce treatment using spray antibiotics. On the other hand, a liquid form of the antibiotic injected in the lower chamber would simulate intravenous delivery through the blood stream.

The device offers great opportunities to improve the bio-mimicry. The biological resemblance between the mucus and the hydrogel can be enhanced by altering the composition of additives as mucins and DNA [27, 28]. Such improvement will offer a better model for studying the response of a biofilm community to drug treatments [29]. In addition, the chip could be coupled with our previous system [30] to recreate the different type of dissolved oxygen conditions inside the airways. Finally our device brings great promises in the mimicry of other human organs where the mucus is involved, e.g. the gut or the stomach.

Summary & Conclusion

In this paper, we have demonstrated the fabrication of a robust and reliable microfluidic device. This device enables the setup of an *in vitro* microfluidic chip based mimicry of an infected human CF bronchus. The recreation of the scenario leading infection of the bronchi is based on a new approach of bacteria culture to be closer to their *in vivo* growth: nutrients diffuse through different bio-layers to feed the bacteria embedded in the mucus-like matrix.

Inside the system, the imitation of the CF bronchus was performed by incorporating Calu-3 cells and a hydrogel, beneath and on top of a microporous membrane, respectively. The formation of the hydrogel was controlled in order to mimic a mucus layer as thick as 200 µm. Calu-3 cells, mimicking the epithelial tissue, were cultured for 3 days to reach 90% of confluence. A bacterial infection was simulated by inoculating *P. aeruginosa* PA01 in the hydrogel. The formation of bacterial microcolonies was observed 2 days later. Confocal microscopy allowed *in vitro* imaging of the different cells to assess of their growth and viability.

For future experiments, such device can enable to simulate and observe the biofilm response to drug treatments taken by oral or intravenous ways. Also, mucin and DNA could be added in the hydrogel to study the impact of the mucus composition on the bacterial infection.

Acknowledgment

We would like to acknowledge for the funding of PhD stipends by the Technical University of Denmark. Furthermore we would like to thank Betinna Dinitzen, Maria Læssøe Pedersen and associate professor Hanne Mørck Nielsen from the Faculty of Pharmaceutical Sciences at the University of Copenhagen, for their help and supply of the Calu-3 cell line.

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14 Concluding remarks and key findings

In vitro systems are essential for our possibility to gain insight into the expressed genetic makeup of microbes. The airways of cystic fibrosis patients, is a habitat in which inhaled micro-organisms battle for survival against other micro-organisms, defense mechanisms and treatments with antimicrobial compounds. *P. aeruginosa* has persisted in the CF community for decades and has evolved into a successful colonizer of the CF airways. The environmental shifts contribute to the diversification of the bacteria as they adapt to the specific niches of the airways. The mucus of the CF lung in combination with the biofilm mode of growth drives adaptation to life in varying oxygen tensions.

The airway environments and physiological differences between CF patients and healthy individuals have been the main inspiration for this thesis.

The versatility of the flow cell system was demonstrated to comply with several microbial organisms (paper I). I successfully produced a visual method publication in order to simplify the understanding of the highly complex flow cell biofilm system. The flow cell system was adapted to support *S. cerevisiae* biofilm formation by using Polyvinyl coverslips. *S. cerevisiae* biofilm formation is normally observed on Poly-styrene surfaces, this polymer does however have a tendency for high autofluorescence and does not conform with confocal microscopy [310]. The flow cell system was shown to be a good *in vitro* model system for studying fungal biofilms with *S. cerevisiae*. Nonpathogenic *S. cerevisiae* strains can be used to found the genetic understanding for biofilm formation by pathogenic *Candida* species. *Candida albicans* and *Aspergillus fumigatus* infections frequently arise in hospitalized patients with biofilms located on medical devices and often isolates in sputum samples from CF patients [132, 311]. The flow cell system could contribute to the understanding of underlying molecular mechanisms of biofilm formation, resistance mechanisms, cell-cell communication for *Candida* infections and interactions with CF associated bacterial pathogens [312].

In this thesis work, an immense amount of work has gone into the development and not least prototype testing that has been the groundwork for the presented systems. The initial approach in developing the new systems for investigating bacterial biofilms originated form my work with the flow cell systems (presented in paper I) and the knowledge of the generated oxygen niches in the CF airways. A microfluidic approach was employed to fabricate devices that would enable control of dissolved oxygen concentrations in the growth medium for microbial biofilm studies. The initial approach was to develop a microfluidic chip that would create a dissolved oxygen gradient through the entire growth chamber. The system was designed in a layer-by-layer fashion (paper II and III). The incorporation of a gas permeable membrane enabled the utilization of an oxygen scavenging liquid to generate the desired oxygen gradient in the growth chambers. The control of the oxygen environment was merely managed by the flow rate of the biofilm media. Oxygen sensing was enforced by incorporation of an oxygen sensitive sensor in a thin PDMS film on the cover glass. The oxygen photoluminescence lifetime measurements were done with a microtiter plate reader, as the chambers were mapped to the coordinates of a 384 well microtiter plate. The design of the microfluidic chip was intended to comply with most laboratory settings and equipment. Monitoring PAO1 biofilm formation under the set growth conditions proved the systems functionality. PAO1 responded to the low oxygen environment by a reduced number of attaching cells. As the culture media only supported growth with oxygen as electron acceptor, PAO1 responded with an immense reduction in biomass compared to atmospheric saturated culture media. Analyzing biofilms formed in the system under lowered oxygen availability does however include a variable that needs to be taken into consideration, as the local nutrient levels also influence the biofilm formation.

To create a system that could mimic the individual oxygen niches of the CF airways, a modular microfluidic system was developed (paper IV). Individually based functionalities to each module generates a united system that incorporate e.g. bubble traps, grow chambers, micro-mixer and an oxygen scavenging module. As individual growth chambers are designed to be interconnected, the system enables documentation of migration patterns

following antimicrobial treatment, as with the presented ciprofloxacin. The presented system is set for efficient evaluations of the effects of microbial responses and development in oxygen specific niches. The system operates with requirements of small volumes and thereby only consuming small amounts of the tested antimicrobials, which is highly desirable when expensive antimicrobial candidates are tested. Compared to the systems presented in paper II and III, the modular based system eradicates the variable of nutrition levels as each specific chamber only differs in oxygen concentrations. The inter-connections of the system can resemble the believed scenario of reinfection of the lower airways from the sinuses following antibiotic treatments [133].

The most significant CF defining consequences is the accumulation of viscous mucus in the airways of the patients, generating a 3D environment that *in vivo* would support antibiotic tolerance and evasion of the immune system. To mimic this phenomenon, a device to simulate the mucus covered bronchial epithelia was developed (paper V and VI). The system enables epithelial cells to be cultured on the integrated membrane separating the two chambers within the model chip. Furthermore, a mucus substitution matrix, in the shape of an alginate hydrogel, was successfully introduced and stirred to form on the "airway" side of the membrane. The system enables culturing of both epithelial and bacterial cells as they occur in the CF airways.

The presented models can be considered as a giant leap forward in developing *in vitro* models for CF research that allow biofilm investigations in more CF relevant oxygen niches. A further development that would allow a modular based "bronchi chip" to be combined with the oxygen scavenging module, would be a substantial contribution the CF related biofilm research.
The presented systems would desirably answer questions about adaptability to specific generated niche environments. An intriguing experimental setup would be more long-term experiments with boosted antibiotic treatments to evaluate the phenotypic diversification in the different oxygen compartments and migration patterns. Furthermore, it would be interesting to investigate mucoid vs. non-mucoid strains in the micro-compartmental setups with possible

introduction of white blood cells to evaluate their efficiency towards the different phenotypes.

The developed systems can bring a substantial contribution to studies of a wide range of biofilm forming microbes. The bronchi chip is an interesting system that can be used to mimic other epithelial linings as the stomach and the gut. Another interesting setup would be a microfluidic interpretation of the stomach lining for *Helicobacter pylori* investigations to evaluate the interaction and the disruption of the gastric mucosal epithelium.

The systems presented in this thesis have circumvented the problematic scenario of generation an observable and controllable oxygen environment. In future the development of microfluidic systems to mimic human tissues will probably in the future replace a large part of animal tests and lead to cheaper methods for drug screenings.

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