Effects of CO-releasing molecules on planktonic and biofilm cells of *Pseudomonas aeruginosa*

A thesis submitted by

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Abstract

The overuse of antibiotics to treat cystic fibrosis (CF) lung infections has led to high levels of antibiotic resistance. Novel antimicrobial agents that could replace or complement current therapies are needed to fight chronic infections in CF patients. The advent of carbon monoxide-releasing molecules (CORMs) has been useful in investigating the physiological effects of CO by allowing the controlled release of CO to specific targets in mammalian systems. Recent evidence shows that the bactericidal activity of CORMs against a number of species is more effective compared to CO gas. Here, we applied ruthenium- based CORMs (CORM-2, CORM-3) and manganese-based CORM (PhotoCORM $[Mn(CO)_3(tpa\text{-}\kappa^3N)]^+$) to biofilms using a novel nebulizer technique in conjunction with a Modified Robbins Device, and compared these effects with laboratory planktonic cultures of the Gram-negative pathogen *Pseudomonas aeruginosa*. We demonstrate that both CORM-2 and CORM-3 rapidly kill planktonic cells in laboratory culture. Although CORM-3 inhibits surface-associated growth of *P. aeruginosa* by both preventing biofilm maturation and killing bacteria within the established biofilm, cells growing in a biofilm are more resistant to CORM-3 than planktonic cells. The mechanisms behind the activity of CORM-3 resistance from *P. aeruginosa* biofilm cells are examined. Poor penetration and anaerobic microenvironment may contribute to this resistance, but not starvation. Aerosolized CORM-3 treatment has a similar bactericidal effect to aerosolized colistin, a commonly used antibiotic for *P. aeruginosa* lung infections. Although CORM-2 or colistin kills bacteria within the established biofilm, the combination effect of those two antimicrobial compounds was found to be only additive against *P. aeruginosa* planktonic cells and biofilm cells. Although activated PhotoCORM exhibits slightly inhibitory effect on *P. aeruginosa* planktonic cells, the compound significantly inhibits surface-associated growth of *P. aeruginosa* by both preventing biofilm formation and killing bacteria within the established biofilm. Anaerobic cultures of *P. aeruginosa* are less sensitive to CORM-3 compared to aerobic cultures.

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Chapter 1

Introduction

1.1 Carbon monoxide (CO)

Carbon monoxide (CO) is a colourless, odourless and tasteless gas that is commonly considered as a poison and an atmospheric pollutant. The major source of CO in nature is generated by photochemical reactions in the troposphere (Weinstock and Niki, 1972). Other natural sources of CO natural combustion such as volcanoes and forest fires, and the incomplete oxidation of carbon-containing compounds during operating internal combustion engines, burning fossil fuels and iron smelting. Since Claude Bernard investigated the toxic effects of CO on dogs around 1846, its toxicity has been feared and exploited for centuries. The 2015 report from the All-Party Parliamentary Carbon Monoxide Group (APPCOG) has shown that around 40 deaths and 200 hospitalizations were caused by accidental carbon monoxide poisoning each year in England & Wales. Moreover, over 4,000 CO poisoning cases were treated in accident and emergency departments each year in England.

CO is a small molecule (molecular mass of 28) containing one carbon atom and one oxygen atom. It is very stable due to a triple bond formation which is similar to dinitrogen (N_2) and has a high bond dissociation energy of 1072 kJ mol^{-1}. At room temperature CO exists as a gas owing to its low boiling point (-191.5 °C). However, CO is able to form complexes with some transition metals that have low oxidation states (Davidge *et al.*, 2009a).

1.1.1 Gasotransmitters

Recent advances have classified CO as a gasotransmitter molecule that has important roles in biology. The gasotransmitter family also includes at present two other important biological molecules: nitric oxide (NO) and hydrogen sulfide (H_2S) (Li *et al.*, 2009,

Tinajero-Trejo *et al.*, 2013). These three molecules have similar characteristics: they are all small, membrane-permeable, toxic molecules; they are produced endogenously in small amounts in mammalian systems; and they play vital roles in the body such as signalling and messaging. Each gasotransmitter is suggested to have potential in therapeutic applications (Bannenberg and Vieira, 2009, Szabo, 2010).

1.1.2 CO in mammalian systems and bacteria

High concentrations of CO have toxic effects in mammals due to its intrinsic high affinity for the ferrous iron of haemoglobin to form carbonmonoxy-haemoglobin (COHb), which prevents the delivery of oxygen to tissues (Goldbaum *et al.*, 1975). Interaction of CO with other haem proteins causes structural modifications and alteration of their biological function that may also contribute to CO toxicity (Piantadosi, 2002).

However, Tenhunen and workers attributed to the realization that CO is an endogenous product of haem metabolism by haem oxygenase (HO). The degradation of haem by HO requires O_2 and NADPH. NADPH activates O_2 and reduces Fe^{3+} in haem to Fe^{2+}, which is necessary for the cleavage of haem. The breakdown of haem results in CO, Fe^{2+} and biliverdin (a green pigment) (Tenhunen *et al.*, 1969), which is later broken down by biliverdin reductase into bilirubin (a yellow pigment) (Wu and Wang, 2005) (see Figure 1.1). Haem breakdown can be visualized during bruising. Such an injury will initially appear purple-red as oxygenated haem is released under the skin. Haem oxygenase uses oxygen to break down haem, generating anaerobic conditions and therefore deoxyhaem, causing the bruise to appear blue. The formation of bilirubin will then lead to the bruise becoming yellow (Mann, 2012).

Endogenously-produced CO has been shown to have various cytoprotective functions including roles as neurotransmitter (Verma *et al.*, 1993), as a vasodilator (Furchgott and Jothianandan, 1991), and having anti-inflammatory (Otterbein *et al.*, 2000) and anti-apoptotic properties (Boczkowski *et al.*, 2006).

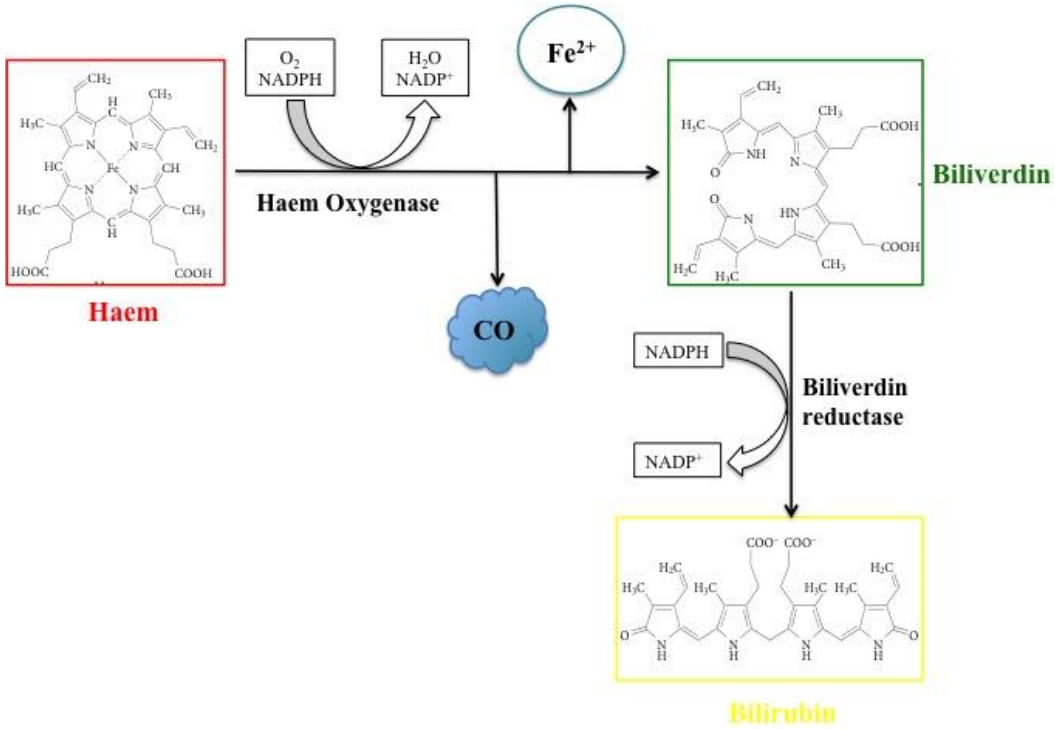


Figure 1.1 Degradation of haem by haem oxygenase. Haem is broken down to biliverdin by HO while liberating a molecule of CO and Fe^{2+}. Then biliverdin is rapidly converted to bilirubin in the presence of biliverdin reductase. Modified from Motterlini and Otterbein (2010).

In certain bacteria, CO is used as their sole carbon and energy source (Ragsdale, 2004). The metabolism of CO by some bacterial species has been comprehensively reviewed (King and Weber, 2007, Ragsdale, 2004, Oelgeschlager and Rother, 2008). This process requires enzymes such as CO dehydrogenase (CODH), so CO can be oxidized to CO_2 and generates two protons and two electrons in metabolic pathways (as shown in equation (1)).

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \qquad (1)$$

The bacteria also have the ability to detect CO through protein sensors. One example is CooA from *R. rubrum*, which is a typical example of a haem-based CO sensor (Aono *et al.*, 1996). It regulates the expression of CO-utilising genes (Shelver *et al.*, 1995), including the *coo* operon (Bonam *et al.*, 1989). Other examples include FixL in *Rhizobium meliloti* (Rodgers and Lukat-Rodgers, 2005, Gilles-Gonzalez *et al.*, 1991), RcoM from anaerobic *Burkholderia xenovorans* (Kerby *et al.*, 2008) and HemAT in *B. subtilis* (Hou *et al.*, 2000).

1.1.3 The therapeutic potential of CO

As discussed in section 1.1.2, CO shows beneficial effects within mammalian systems, which has led to research into using CO for therapeutic purposes. This can be achieved by the induction of HO-1 expression or the inhalation of gaseous CO. Especially, CO inhalation therapy shows promising benefits as a natural compound that is not metabolized by the body to produce harmful breakdown products (Motterlini and Otterbein, 2010). Recently, several clinical trials were carried out for CO inhalation therapy. For example, one phase II trial investigated the use of air containing CO gas (100 – 250 ppm) to prevent the development of postoperative obstructions of the bowel following colon surgery, while another investigated the use of CO to improve the success rate in kidney transplantation (reviewed by Mann, 2010). However, many questions must be answered before CO inhalation can be considered as a credible therapy. These include questions about the dose of CO required, and whether this is best delivered in multiple, low dose sessions, or in less frequent, larger doses (Motterlini and Otterbein, 2010).

1.2 Carbon Monoxide Releasing Molecules (CORMs)

1.2.1 CORMs discovery and development

With the increasing studies on the beneficial biological consequences of CO in the body, exploration of safe mechanisms to deliver CO into biological systems has begun. The advent of CO-releasing molecules allowed the possibility of storing CO in a solid form and then releasing CO in a controlled, targeted manner in the body (Motterlini *et al.*, 2002). These molecules provide a number of advantages over CO inhalation, such as overcoming the limitations associated with gaseous CO application and allowing controlled amounts of CO to be applied directly to tissues.

Initially, Brian Mann and Roberto Motterlini considered using transition metal carbonyls to delivery CO and commercially available carbonyl compounds were shown to have some encouraging vasoactive properties in *vivo*. However, the problems caused by these commercial compounds included insolubility in water while some compounds required photolysis to release CO. Therefore, the development of CORMs to make them versatile for using in biology was required (Mann, 2012). In order to successfully use CORMs in biological systems, CORMs should be non-toxic, water-soluble, pure and stable, and release CO at adequate rates (Davidge *et al.*, 2009a). The structure and properties of several commonly used CORMs are listed in Table 1.1, and the CORMs used in this thesis are described in more detail below. The potential for therapeutically using CORMs has been confirmed and the details will also be reviewed in this section.

1.2.1.1 CORM-2

CORM-2 (tricarbonyldichlororuthenium (II) dimer) is a ruthenium-based compound, which is insoluble in water but can be dissolved in DMSO. Motterlini and co-workers found that CORM-2 releases CO spontaneously by substitution of CO coordinated to ruthenium (Ru) with DMSO from the solvent. When dissolved in DMSO, the CORM-2 dimer immediately dissociates to form tri-carbonyl and di-carbonyl monomers

(Motterlini *et al.*, 2002). When CORM-2 solubilized in DMSO, CO is released to myoglobin at around 0.7 mol of CO per mol of the compound with a half-life of approximately 1 min (Motterlini *et al.*, 2002). Further evidence suggests that sulfite species such as dithionite dramatically increase the release of CO from CORM-2 (McLean *et al.*, 2012).

Since CORM-2 became commercially available, this compound has been widely studied. Several studies have confirmed that CORM-2 has beneficial effects *in vivo*. In human airway smooth muscle, CORM-2 inhibits proliferation of human smooth muscle cells by increased mitochondrial ROS production and decreased phosphorylation of ERK1/2 and cyclin D1 expression (Taille *et al.*, 2005). In mice kidney, CORM-2 protects against acute renal failure caused by ischemia (Vera *et al.*, 2005). CO from CORM-2 also promotes generation of reactive oxygen species (ROS) from complex III of the electron transport chain. This in turn leads to the redox state of key cysteine residues in human cardiac L-type Ca^{2+} channels, inhibiting ion transport, which has cardioprotective effects (Scragg *et al.*, 2008). In the liver of septic mice, CORM-2 reduces inflammation by attenuating LPS-induced production of ROS and NO by preventing expression of intracellular adhesion molecule-1 and activation of NF-κB (Cepinskas *et al.*, 2008). In a transplant model, pre-treatment of the kidney donor rats with CORM-2 protects renal transplants from ischemia reperfusion injury by modulating inflammation (Caumartin *et al.*, 2011). More recently, the protective effect of CORM-2 in a mouse model of acute pancreatitis has been reported. CORM-2 inhibits systemic inflammatory cytokines and decreases TNF-α generation which resulted in reducing mortality, pancreatic damage and lung injury (Xue and Habtezion, 2014).

1.2.1.2 CORM-3

A major disadvantage of CORM-2 is its poor solubility in water. In order to solve this problem, Motterlini and colleagues successfully synthesised another ruthenium-based water-soluble compound tricarbonylchloro(glycinato)ruthenium(II), known as CORM-3 ($Ru[CO]_3Cl$(glycinate)). This compound releases 1 mole of CO per mole compound to myoglobin *in vitro* with a half-life of less than 2 min. (Clark *et al.*, 2003). CORM-3 is

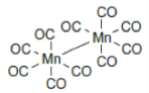
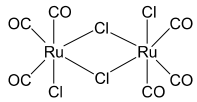
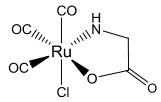
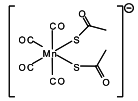
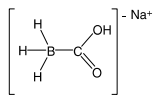
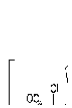
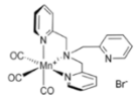
CORMs	Chemical formula	Structure	Solubility	CO release characteristics	Reference
CORM-1	$[Mn_2(CO)_{10}]$		Soluble in DMSO	Photolysis Stoichiometry of CO release has not been reported	(Motterlini *et al.*, 2002)
CORM-2	$[Ru(CO)_3Cl_2]_2$		Soluble in DMSO	Promoted by dithionite Releases 0.7 mole CO / mole CORM	(Motterlini *et al.*, 2002) (McLean *et al.*, 2012)
CORM-3	$Ru[CO]_3Cl$(glycinate)		Soluble in water	Promoted by dithionite Releases 1 mole CO / mole CORM	(Clark *et al.*, 2003) (Foresti *et al.*, 2004) (Vadori *et al.*, 2009)
CORM-371	$[Me_4N][Mn(CO)_4$(thioacetate)$_2]$		Soluble in water	Releases 1 mole CO / mole CORM Half - life 20.2 min	(Desmard *et al.*, 2012)
CORM-A1	$Na_2[H_3BCO_2]$		Soluble in water	Releases CO spontaneously The rate is pH and temperature dependent	(Motterlini *et al.*, 2005)
CORM-401	$[Mn(CO)_4(S_2CNMeCH_2CO_2H)]$		Soluble in phosphate buffered saline	Promoted by dithionite, pyridine and phosphorus ligands Release 1 mole CO spontaneously and ≤ 3.2 mole / mole CORM by ligand substitution	(Crook *et al.*, 2011) (McLean *et al.*, 2012)
PhotoCORM-CN028-31	$[Mn(CO)_3$(tpa-κ^3N)$]^+$		Soluble in water	Promoted by irradiation at 365 nm Releases ≤ 2.2 mole CO / mole CORM	(Nagel *et al.*, 2014)

Table 1.1 The structure and properties of several commonly used CORMs.

synthesised from CORM-2, with the addition of a glycine residue and a chloride coordinated to the ruthenium. Glycine was chosen as a preferable ligand as it does not contain a chiral centre, which avoids the formation of diastereomers (Mann, 2012). McLean and colleagues have explored in greater detail the mechanism of CO release in CORM-3 (McLean *et al.*, 2012). In their view, reduced myoglobin alone was not sufficient to cause CO release from CORM-3, but that dithionite, or other sulfite species, greatly facilitated CO release. Therefore, endogenous sulfite species, or other similar intracellular components, trigger CO release *in vivo*.

In order to study the potential of using CORM-3 as a therapeutic agent, several studies have reported using CORM-3 *in vivo*. In mice, CORM-3 prevents cardiac allograft rejection and protects myocardial cells and isolated rat hearts in ischemia reperfusion injury (Clark *et al.*, 2003). This involves the activation of HO-1, sGC and ATP-dependent potassium channels. Mice treated with CORM-3 also have limited myocardial ischaemia-reperfusion injury *in vivo* (Guo *et al.*, 2004). CORM-3 also has significant vasodilatory properties, which are induced at concentrations of CORM-3 as low as 25 – 50 μM (Foresti *et al.*, 2004). CORM-3 inhibits human platelet aggregation (Chlopicki *et al.*, 2006). CORM-3 causes reduction of microglia activity in strokes and other neuroinflammatory diseases (Bani-Hani *et al.*, 2006) and prevents cardiac allograft rejection in mice (Vadori *et al.*, 2009). In a more recent study, Abid and co-workers have observed the mice that treated with CORM-3 (50 mg kg^{-1}) once daily for three weeks mimicked the action of endogenous CO by inhibiting pulmonary vascular remodelling, so CORM-3 is a potential therapeutic treatment in pulmonary hypertension. (Abid *et al.*, 2014).

1.2.1.3 PhotoCORM-CN028-31

PhotoCORM-CN028-31 ($[Mn(CO)_3(tpa\text{-}\kappa^3N)]^+$) is a novel water-soluble photo-activated CORM that releases CO on exposure to UV irradiation at 365 nm and is stable in solution in the dark (Nagel *et al.*, 2014). This compound releases CO in a precisely controlled manner. In addition, this PhotoCORM has manganese as the CO carrier and so is more biologically favourable compared to ruthenium-based CORMs. However, there are

several disadvantages associated with this compound, such as the potential damage to tissues cause by irradiation.

Limited studies of this compound exist so far in the literature, but Nagel and colleagues have shown that the growth of *Escherichia coli* K-12 was inhibited by this PhotoCORM when cultures were exposed to UV light. However, no toxicity was observed in cultures kept in the dark. Pronounced inhibition of cultures growing in the presence of a non-fermentable carbon source upon activation of the PhotoCORM in situ, together with the demonstration of CO binding to *E. coli* terminal oxidases suggested that the mechanism of action of PhotoCORM is related to the inhibition of respiration more than the toxicity exerted by the metal-coordinated backbone (Nagel *et al.*, 2014).

Additionally, other relevant PhotoCORMs were also investigated for their potential as novel therapeutic agents. One group of PhotoCORMs was based on monocationic manganese(I) tricarbonyls $[Mn(CO)_3(tmp)]^+$. These are stable in the dark and do not affect the viability of HT29 human colon cancer cells, but typically release 2 CO groups per molecule upon irradiation at around 360 nm, and in this activated form, significantly reduce the viability of such cells (Niesel *et al.*, 2008). Researchers have shown that this PhotoCORM with a targeting peptide has the potential to target specific cancer cells (Pfeiffer *et al.*, 2009). The linkage of $[Mn(CO)_3(tmp)]^+$ to silicium dioxide nanoparticles have the potential to deliver CORMs into solid tumours without adversely affecting CO release (Dordelmann *et al.*, 2011). Another group of PhotoCORMs is based on the sodium salts of the water soluble tungsten carbonyl complex $Na_3[W(CO)_5(TPPTS)]$ (Rimmer *et al.*, 2010). These are stable in aerated media, but release approximately 1 CO upon irradiation; further CO is then released due to aerobic oxidation. These PhotoCORMs have been suggested as suitable for topical applications using UVA as an excitation source. More recently, a manganese-based PhotoCORM has been described that has therapeutic potential (Ward *et al.*, 2012). Particular advantages of this CORM are that it releases 3 CO molecules from each molecule, is activated by light from an LED at 400 nm, and is not toxic to RAW 264.7 murine macrophages before and after irradiation.

1.2.1.4 The future of CORM development

The future design of CORMs and work to characterize them should pay special attention to understanding the mechanism of CO release from CORMs. It is desirable for CORMs to have high CO release ratios, so that less drug needs to be administered in order to achieve a therapeutic effect. In addition, future CORM design needs to focus on making CORMs more suitable for use as medical drugs. Currently we have water-soluble, bioactive CORMs such as CORM-3 and CORM-A1, but in order for CORMs to be a realistic therapy they must have highly attuned absorption into tissues and excretion from them, have higher biocompatibility and contain no potentially harmful components (Motterlini and Otterbein, 2010).

It is also important that we gain a better understanding of how CO from CORMs or the CORM itself enters cells (Mann, 2012). For example, it is not known whether CORMs enter via specific transporters, or by passive diffusion. Furthermore, little is known about the fate of the CORM backbone or the released CO after it has entered cells (Mann, 2012). However, Raman microscopy has shown that when HT29 human colon cancer cells are exposed to CORM, most of the CORM localizes around the nucleolus (Meister *et al.*, 2010).

It is also vital that future CORMs are designed with the availability of reliable, control (inactive) molecules in mind. Currently, despite the reported use of three different control molecules for CORM-3: inactive CORM-3 (iCORM-3), myoglobin inactivated CORM-3 (miCORM-3) and $RuCl_2(DMSO)_4$, there is no control molecule for this compound for which the chemistry is completely understood, or that mimics the *in vivo* breakdown products of this CORM precisely.

1.2.2 The therapeutic effects of CORMs

As mentioned above, much research focuses on using CO as a therapeutic agent due to its beneficial effects in mammalian systems. CORMs were designed to delivery CO to the site where it is required in a safe and efficient way (Johnson *et al.*, 2003, Motterlini

and Otterbein, 2010, Romao *et al.*, 2012). Here I will give only a brief overview of this area of research.

1.2.2.1 Vasoactive properties

CORMs are considered to have great potential in regulating vasoactive processes in a variety of diseases (Motterlini and Otterbein, 2010). CORM-2 causes vasodilation and reduces hypertension in rat aorta, whereas the control molecule $RuCl_2(DMSO)_4$, which is structurally similar to $[Ru(CO)_3Cl_2]_2$, but with the carbonyl groups replaced with DMSO, is unable to cause these effects, suggesting that CO, rather than the CORM backbone is the causative agent (Motterlini *et al.*, 2002). Importantly, no cytotoxicity was experienced in vascular smooth muscle cells treated with up to 420 µM CORM-2 over 3 h. CORM-3 also has significant vasodilatory properties, which are induced at concentrations of CORM-3 as low as 25 – 50 µM (Foresti *et al.*, 2004). A major advantage of CORM-3 is that it has relatively low toxicity: concentrations less than 500 µM are not toxic to porcine aortic endothelial cells or to primate peripheral blood mononuclear cells *in vitro* (Vadori *et al.*, 2009). Furthermore, the non-metal CORM, CORM-A1 causes significant but slow-acting and sustained vasodilation of precontracted aortic rings, thereby mimicking endogenous CO, while the inactive compound (iCORM-A1) does not.

1.2.2.2 Anti-inflammatory properties

CORMs are also considered to have great potential as anti-inflammatory agents (Motterlini and Otterbein, 2010). Several CORMs have been shown to decrease levels of anti-inflammatory intermediaries and to reduce levels of TNF-α and NO in LPS stimulated macrophages (Sawle *et al.*, 2005). CO delivered by CORMs may also be beneficial in the treatment of inflammation associated with arthritis. CORM-3 reduced levels of inflammatory cytokines in joint tissues and reduced the recruitment of inflammatory cells, joint inflammation and degradation of cartilage in animal models of arthritis (Ferrandiz *et al.*, 2008). In addition, CORM-2 has been shown to substantially reduce the inflammatory response induced by cytokines in human colonic epithelial cells. This includes reduced expression of nitric oxide synthase-2 (NOS-2) and subsequent

production of nitrite as well as differential-regulation of inflammatory genes involved in intestinal inflammation and cancer progression (Megias *et al.*, 2007).

1.2.2.3 The effects of CORMs on organ transplantation

Much research has been undertaken into the protective potential of CO in organ transplant systems, and it has been found to have significantly beneficial effects (Akamatsu *et al.*, 2004). An early study into the biological activity of CORM-3 investigated cardiac allograft rejection in mice. This study showed in the presence of CORM-3, but not an inactive control compound, greatly increased the survival rate of transplanted hearts (Clark *et al.*, 2003). CORMs have also been shown to prevent ischemia reperfusion injury in intestinal grafts via a sGC-dependent mechanism (Nakao *et al.*, 2006b). The reduction of pro-inflammatory cytokines by CORMs is also thought to be important in increasing the survival rate of transplant patients (Nakao *et al.*, 2006a). In addition, CORMs and saturated solutions of CO added to the organ preservation solution have been shown to greatly benefit the health of the transplanted organ (Sandouka *et al.*, 2006, Musameh *et al.*, 2007, Pizarro *et al.*, 2009). The mechanisms by which CO and CORMs induce these effects is not fully understood, but there is evidence that sGC (Nakao *et al.*, 2003), cytochrome p450 (Nakao *et al.*, 2008) and the reduction of inflammatory cytokine levels (Nakao *et al.*, 2006a) are involved.

1.2.2.4 Limitations to the therapeutic use of CORMs

Despite the many potential advantages of CORMs compared to CO gas as a therapeutic agent, they also have some disadvantages. For example, consideration must be given to the fate of the CORM backbone following the release of CO. It is important that the resulting compound, and any residual breakdown products are not toxic, and that they are not metabolised by the body to subsequently produce toxic products. CORM-2 and CORM-3, which have potent therapeutic effects, both contain ruthenium, which is not found naturally in the body and therefore they are not biological favourable.

1.2.3 Antimicrobial activity of CORMs

In contrast to the numerous studies into the therapeutic effects of CO and CORMs, more limited research has done into antimicrobial activity of CORMs. A variety of CORMs has been used against bacteria, including CORM-2, CORM-3, CORM-A1, CORM-371, CORM-401 and several PhotoCORMs (Davidge *et al.*, 2009b, Desmard *et al.*, 2009, Desmard *et al.*, 2012, McLean *et al.*, 2013, Murray *et al.*, 2012, Nagel *et al.*, 2014, Nobre *et al.*, 2009, Nobre *et al.*, 2007, Rana *et al.*, 2014, Tavares *et al.*, 2013, Tavares *et al.*, 2011, Wilson *et al.*, 2013). Here I only address the antimicrobial activity of two commonly studied CORMs: CORM-2 and CORM-3. Note that recent data on CORM-3 casts doubt on the previous assumptions that CO release is a/the major cause of antimicrobial activity (Southam *et al.*, 2018).

1.2.3.1 The antimicrobial effects of CORM-2

Work by Nobre and colleagues provided the first evidence about the antibacterial action of CORM-2. They tested the effect of CORM-2 on the growth and viability of both *E. coli* and *S. aureus* cells under aerobic and anaerobic conditions (Nobre *et al.*, 2007). Their results showed a significant killing after exposure cell cultures to 250 μM CORM-2. CO release was claimed to be the major cause of the bactericidal effect as CORM-2 had no effect in the presence of the CO scavenger haemoglobin. However, CO release was not detected in the media containing CORM-2. Moreover, CORM-2 was more toxic to aerobic *E. coli* cultures than anaerobic *E. coli* cultures in an initial 30 min exposure, but this trend reversed after the addition of CORM-2 (Nobre *et al.*, 2007).

Nobre and co-workers later carried out another investigation on the effect of CORM-2 on transcriptional regulation in *E. coli* cells (Nobre *et al.*, 2009). In agreement with the previous finding that CORM-2 was more toxic to *E. coli* under anaerobic conditions, CORM-2 had greater effect on the transcriptome of *E. coli* anaerobic cultures. They also showed CORM-2 had effects on genes involved in methionine metabolism and biofilm formation of *E. coli* (Nobre *et al.*, 2009).

Based on the confirmation that CORM-2 has bactericidal effect on *E. coli* cells, Tavares and colleagues studied the stimulation of formation of reactive oxygen species (ROS) as the mechanism behind its (Tavares *et al.*, 2011). Their research appeared to show that ROS content was increased in *E. coli* cells treated with CORM-2. The generation of ROS in the presence of CORM-2 also resulted in damage to DNA. Importantly, CORM-2 generated hydroxyl radicals *in vitro* (Tavares *et al.*, 2011).

In addition, researchers have reported on the antimicrobial action of CORM-2 on commensal *E. coli* strain, Bang and co-workers evaluated the effect of CORM-2 on several clinical isolates of *E. coli* (Bang *et al.*, 2014). The strains used were multidrug resistant strains producing extended-spectrum β-lactamases (ESBLs). CORM-2 inhibited the growth of ESBL-producing *E. coli* and killed the cells within 4 h (at a concentration of 500 μM). The bactericidal effect of CORM-2 was much greater than with nitrofurantoin, an antibiotic used against ESBL-producing *E. coli* (Bang *et al.*, 2014). This study revealed the potential application of using CORMs in the clinic for treatments of bacterial resistant strains.

More recently, Qiu and colleagues examined the effect of CORM-2 on an *E. coli* sepsis model in mice (Qiu *et al.*, 2015). CORM-2 suppressed bacterial vitality and toxicity by damaging bacterial structure. The survival rate of mice with *E. coli* sepsis in a dose-dependent fashion increased after CORM-2 intervention. Meanwhile, no effect was observed with the CO-depleted control iCORM-2. CORM-2 also enhanced bacterial clearance in mouse livers, lungs, spleens and kidneys. These beneficial effects of CORM-2 may also associated with the modulation of key genes by CORM-2 in *E. coli* cells.

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen of animals, plants and humans (Bodey *et al.*, 1983). The first examination of the bactericidal activity of CORM-2 against *P. aeruginosa* planktonic cells was by Desmard and co-workers (Desmard *et al.*, 2012). A very low concentration of CORM-2 (10 μM) inhibited bacterial growth. 100 μM CORM-2 killed *P. aeruginosa* planktonic cells and reduced oxygen

consumption. The bactericidal effect of CORM-2 was prevented by the presence of N-acetyl cysteine (NAC) (Desmard *et al.*, 2012).

P. aeruginosa infections are particularly difficult to treat due to this organism's ability to form biofilms, which offers protection against antimicrobials (for details see section 1.3.1). The first investigation of the antimicrobial effect of CORM-2 against *P. aeruginosa* biofilms was by Murray and colleagues (Murray *et al.*, 2012). They showed that addition of CORM-2 prevented biofilm maturation and destroyed established biofilms. The combination of CORM-2 with tobramycin, an established antibiotic used in *P. aeruginosa* infections, was more effective at preventing biofilm maturation. Additionally, CORM-2 (50 - 200 μM) disrupted *P. aeruginosa* biofilm formation on human bronchial epithelial cells, and these concentrations are not cytotoxic to the cells. Importantly, Murray and colleagues concluded that the inhibitory activity of CORM-2 observed in this study was not attributed to ROS formation, due to the detection of ROS even though addition of cysteine inhibited CORM activity in cells (Murray *et al.*, 2012).

The therapeutic properties of CORM-2 led Tavares and co-workers to investigate the effect of CORM-2 on *Helicobacter. pylori*, a major pathogen causing gastric ulcer diseases (Tavares *et al.*, 2013). This study showed that CORM-2 effectively killed all *H. pylori* strains even those resistant to antibiotics. Control experiments with the addition of the control compound iCORM-2 showed no bactericidal effect, revealing that the bactericidal effect was attributed to CO release. In addition, the combination of antibiotic and sub-lethal doses of CORM-2 enhanced the ability of killing *H. pylori* cells. Moreover, *in vivo* studies done in human macrophages showed the ability of *H. pylori* to infect murine macrophages was inhibited by CORM-2 (Tavares *et al.*, 2013).

1.2.3.2 The antimicrobial effects of CORM-3

The first demonstration of the antibacterial action of CORM-3 against both *E. coli* and *S. aureus* cells was also by Nobre and co-workers (Nobre *et al.*, 2007). Their work revealed a significant decrease on the viability of *E. coli* and *S. aureus* cells after exposure cell

cultures to a higher concentration of CORM-3 (400 μM) compared to CORM-2 (250μM). However, aerobic *S. aureus* cell cultures were more resistant to CORM-3. Similarly to CORM-2, the antibacterial actions of CORM-3 also depended on CO release due to CORM-3 lost bactericidal effect in the presence of CO scavenger haemoglobin; CORM-3 also not released CO when dissolved in the media (Nobre *et al.*, 2007).

Davidge *et al.* found that, even though CORM-3 was bactericidal against *E. coli* cells under both aerobic and anaerobic conditions, CORM-3 completely inhibited the aerobic cell cultures at lower concentrations (100 μM) than required under anaerobic conditions (200 μM). Meanwhile, after exposure of cell cultures to CORM-3 for 2h, 30 μM CORM-3 was needed to reduce the number of viable cells 10-fold under aerobic conditions, whereas 100 μM CORM-3 had no effect on the cell viability under anaerobic conditions. Interestingly, CORM-3 but not the equivalent concentration of CO-saturated solution prevented bacterial growth. Ruthenium from CORM-3 was found to accumulate greater inside *E. coli* aerobic cells than in anaerobic cells, which may partly explain the higher toxicity of CORM-3 against aerobic cell cultures. Furthermore, CORM-3 was shown to inhibit bacterial respiration accompanied by the formation of CO adducts of terminal oxidases. Importantly, transcription in aerobic cultures was shown to be affected more by CORM-3 than in anaerobic conditions. The most affected gene in the presence of CORM-3 was *spy*, an envelope-stress-induced periplasmic protein. The *spy* gene was up-regulated more than 100-fold anaerobically, and 26-fold aerobically (Davidge *et al.*, 2009a).

A subsequent time-resolved transcriptional analysis compared the effects of a sub-lethal concentration of CORM-3 (40 μM) with the control compound (inactivated CORM-3 (iCORM-3)) on gene expression in aerobically grown *E. coli* (McLean *et al.*, 2013). In this study, they found the control compound evoked a much smaller transcriptomic response than CORM-3, with only 2% of the genome significantly affected, compared to 23% at 40 min in the presence of CORM-3. Furthermore, both of the compounds can rapidly enter the cells, but iCORM-3 accumulated at much lower levels than CORM-3, indicating that the smaller effect of iCORM-3 may be caused by poor cellular penetration.

Importantly, this study also identified that one target of CORM-3 and iCORM-3 activity is sulphur metabolism (McLean *et al.*, 2013).

Wilson and colleagues provided another study of the effects of CORM-3 on microbial respiration (Wilson *et al.*, 2013). In this study, addition of CORM-3 to cultures of *E. coli* resulted in a period of respiratory stimulation followed by inhibition, while higher concentrations of CORM-3 resulted in increased stimulation for a shorter time period, followed by more marked inhibition. It was also found that incubation of CORM-3 with the bacterial suspension under anoxic conditions for increased time periods resulted in greater inhibition. They suggested that this observation was due to CORM-3 enabling the movement of potassium or sodium ions into the cell, which resulted in less back pressure from positive charges outside of the cell, and therefore favouring proton extrusion coupled to respiration (Wilson *et al.*, 2013).

The potential of CORM-3 as an antimicrobial agent for treatment of *P. aeruginosa* related infections was first confirmed by significantly inhibiting the growth of an antibiotic-resistant *P. aeruginosa* clinical isolates in the presence of very low concentrations (0.5 – 10 μM). CORM-3 (10 μM) was as effective deceasing the bacterial count to the same extent as the antibiotic amikacine (50 mg l^{-1}, or 0.8 mM), and more effective than another antibiotic ticarcilline (50 mg kg^{-1}). Moreover, CO is known to bind to cytochrome *c* oxidase in *P. aeruginosa* (Parr *et al.*, 1975); spectroscopic analysis of *P. aeruginosa* cells after exposure to CORM-3 revealed the formation of CO-adducts of terminal oxidases. The antimicrobial effect of CORM-3 was mediated by the inhibition of respiration, and probably not linked with ROS overproduction. The addition of thiol compounds cysteine, glutathione and the thiol donor *N*-acetylcysteine (NAC) prevented the effects of CORM-3 on *P. aeruginosa* cell growth. Furthermore, this group investigated CORM-3 activity *in vivo*, and found that CORM-3 had a direct bactericidal effect on a model of PAO1 bacteraemia in mice (Desmard *et al.*, 2009). In later work by the same group, they found that CORM-3 exerted a significant bactericidal effect on *P. aeruginosa* PAO1 together with decreased oxygen consumption (Desmard *et al.*, 2012).

1.2.4 The mechanisms behind CORM bactericidal activity

Despite CORMs being promising antimicrobial agents, many questions about how CORMs kill bacteria still remain in this field. Initially, inhibition of respiration was considered to be a likely cause of the bactericidal properties of CORMs, and several pieces of evidence support this hypothesis. CO from CORM-3 has been shown to bind rapidly to the terminal oxidases of the aerobic respiratory chains of *E. coli* and *P. aeruginosa*, and a reduction in oxygen consumption in the later has been observed following treatment with this compound, prior to a decrease in viability. In contrast, there is evidence that inhibition of respiration is not the main cause of killing by CORMs. In *C. jejuni*, CORMs have been shown to inhibit respiration, without adversely effecting growth or viability, and transcriptomic analysis of *E. coli* treated with CORM-3 suggests that while respiratory genes are greatly affected by this compound, the expression levels of a wide variety of non-respiratory genes are also significantly affected. Furthermore, the growth and viability of anaerobic cultures is also adversely affected by treatment with CORM-3 and CORM-2. An alternative hypothesis is that CORMs are bactericidal because they generate ROS. Again there are several pieces of evidence both for and against this suggestion. Research has shown that CORM-2 generates hydroxyl radicals *in vitro*, and that *E. coli* mutants unable to resist oxidative stress are hypersensitive to killing by this compound. However, other work has found no evidence of ROS formation by CORM-3 or CORM-2.

1.3 The bacterial biofilm

A biofilm could be simply defined as a community of microorganisms bound to a surface, or to each other. When a cell switches from a planktonic mode of growth to a biofilm mode of growth, it undergoes a phenotypic change that leads to self-aggregation and transition to a lifestyle different from its planktonic counterparts (Costerton *et al.*, 1995).

Biofilms are ubiquitous. In nature, biofilms can be found in freshwater systems, including rivers, abattoir effluents, marine environments, desert rocks and leaves (Costerton *et al.*, 2007). In human, a lot of human infections are involved with biofilms, such as otitis media,

chronic bacterial prostatitis, cystic fibrosis, and periodontitis *etc.* (Donlan and Costerton, 2002). Moreover, biofilm formation can result in several medical problems, and one of the most common consequences is the growth of biofilms on medical devices. For example, biofilm growth on an implanted device can result in chronic infections such as conjunctivitis (van Bijsterveld and Jager, 1996), urinary tract infection (Trautner and Darouiche, 2004) or pelvic inflammatory disease (Pal *et al.*, 2005). Another common example is biofilm growth on contact lenses which can lead to microbial keratitis (McLaughlin-Borlace *et al.*, 1998). McLaughlin-Borlace *et al.* studied patients with microbial keratitis, and observed biofilms on both the surface of contact lens and contact lens storage cases, which caused contaminations on both lenses and lens solutions (McLaughlin-Borlace *et al.*, 1998). Conversely, certain body tissues in healthy individuals are actually protected from extraneous organisms by biofilms. Thick biofilms in the intestine are viscous moving populations that occupy binding sites on endothelial cells that would otherwise be targets for pathogenic adhesions. In the intestine, they also play important roles in digestion, gut physiology and metabolism. Through a series of intestinal biopsies, Hartley et al were the first to discover that commensal bacteria adhere to the large intestine (Hartley *et al.*, 1979). They found that *E. coli* was present at densities of 10^3 - 10^7 cells per gram of tissue. Furthermore, the effect of biofilms on industry is also large and wide-ranging. Examples include biofilm-related animal and plant diseases have detrimental effects on the farming industry, spoiling crops and lowering yields, an example being mastitis, which affects milk production (Melchior *et al.*, 2006). Biofilm growth on water pipes is a potential problem, especially in drinking water systems (Juhna *et al.*, 2007).

Biofilm development and structure vary greatly due to differences in the physical and chemical nature of the substrates and the surrounding environment, as well as the species composition of the biofilm. There are four general stages in the formation of extracellular biofilms: reversible-irreversible attachment, microcolony formation, biofilm maturation and dispersal (Figure 1.2) (Kostakioti *et al.*, 2013). The formation of an extracellular biofilm starts with bacterial adherence on a surface. This includes reversible and irreversible attachments that can be affected by some species-specific factors. Initially, reversible attachment is a weak interaction effected by surrounding hydrodynamic forces

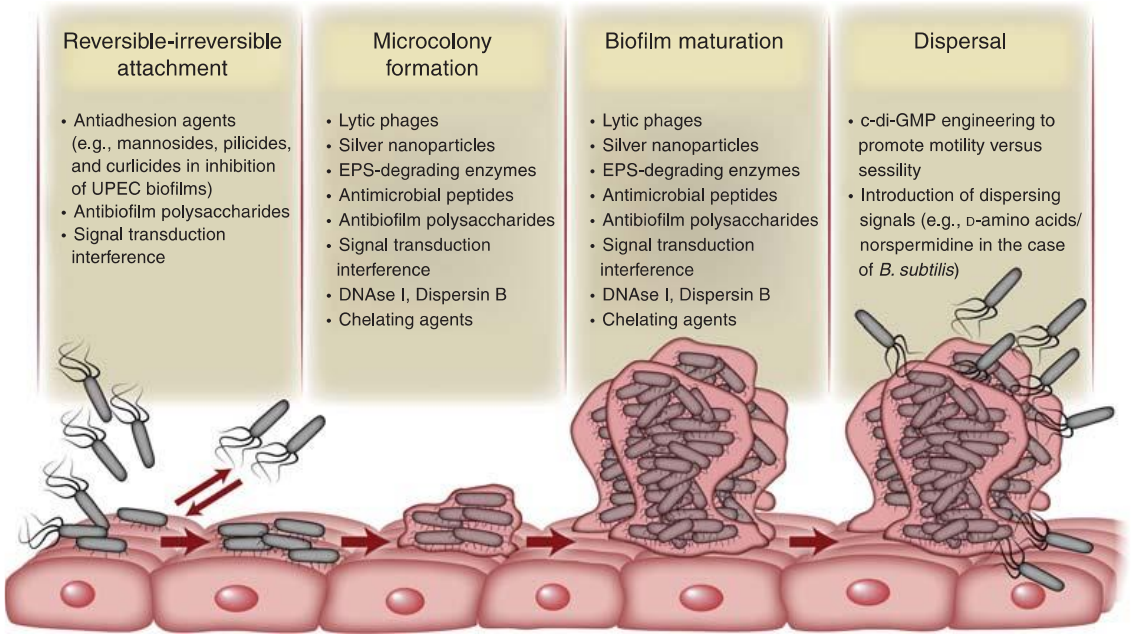


Figure 1.2 Biofilm development. The general stages in biofilm development and the inhibiting and/or disrupting biofilm formation strategies at each stage (Kostakioti *et al.*, 2013).

(Beloin *et al.*, 2008, Donlan, 2002). Cells toward or backward from the contact surface can be driven by medium properties (nutrients, pH, temperature) and bacterial cell surface components (Donlan and Costerton, 2002). If the bacteria can endure hydrodynamic forces and remain adhering on the surface, they can attach to the surface irreversibly. The next stage of biofilm development is biofilm maturation. The exopolysaccharide matrix (EPS) encloses the biofilm in this stage. The EPS, as the name suggests, consists of a highly hydrated sticky mixture of polysaccharides excreted by members of the community to 'cement' its cells to each other and to the surface. As well as providing a means to adhere to a surface, the EPS serves as a store of carbon substrates and water, a protective barrier from the effects of physical, chemical and biological challenges and a medium for communication and genetic exchange. EPS components are vary depending on the environment conditions (Harmsen *et al.*, 2010). In the final stage of biofilm development, the cells may escape from the matrix that is known as the detachment/dispersal of cells. Besides passive dispersal factor such as shear stresses, environmental changes can lead bacteria to decide whether remain biofilm mode growth or resume a planktonic lifestyle (Kostakioti *et al.*, 2013).

Biofilm development is a complex process that requires co-operation and communication. Evidence for the role of quorum sensing (QS) in biofilm formation has accumulated in recent years. QS is a communication system existed in a bacterial community, due to the production of the signalling inducer compounds such as N-acyl homoserine lactone (AHL) and a corresponding receptor. The transcription of certain genes is activated when the inducer binds to the receptor. When cells are in sufficient proximity to each other, inducer concentration is increased, so the receptor is activated. There are several QS mechanisms employed by bacteria. Most Gram-negative bacteria use the LuxI/LuxR system. The product of *luxI* is responsible for the synthesis of a homoserine lactone (HSL) which, when accumulated in sufficient concentrations, is detected by the product of the *luxR* gene, and activates transcription of a set of target genes (Madigan, 2012).

Antibiotics are the primary treatment strategic for bacterial infection. However, it was realized in the early 1980s, following failed antibiotic treatment in cystic fibrosis patients,

that growth in a biofilm increased the resistance of its residents to antibiotics (Costerton *et al.*, 1981). Since then, it has been widely accepted that biofilm bacteria are less sensitive to antibiotics compare to their planktonic counterparts. The reason that biofilms are so much more resistant than planktonic cells is still not clear, but several hypotheses have been proposed. It may be that the penetration of the antibiotic into interior of the biofilm is restricted by the biofilm itself, particularly the protection from the EPS, which causes poor antibiotic penetration (Lewis, 2001). Another possible mechanism is the formation of persister cells, they are specialised antibiotic-tolerant spore-like cells developed in the biofilm. Even if a large proportion of susceptible cells die in the biofilm during antibiotic treatment, persisters will still be protected from immune attack by the EPS (Lewis, 2007). Other hypotheses also include nutrient limitation and slow growth, adaptive stress responses (Stewart, 2002). These factors are shown diagrammatically in Figure 1.3. It seems likely, given the wide range of growth stats that exist in a biofilm, that all of these factors could play a role.

1.3.1 *Pseudomonas aeruginosa*

P. aeruginosa is an aerobic Gram-negative bacterium. It is ubiquitous and can be easily identified due to its pearlescent shape and grape-like odour in *vitro*. Although the optimal metabolism of *P. aeruginosa* is normally aerobic respiration, it can also grow anaerobically while use nitrate as a terminal electron acceptor (Palmer *et al.*, 2007). *P. aeruginosa* can in microaerobic or anaerobic environments in certain circumstances. One common example is *P. aeruginosa* colonization in the lung of cystic fibrosis patients where oxygen concentration is low due to the thick layer of lung mucus.

1.3.1.1 *P. aeruginosa* biofilm formation

P. aeruginosa is one of the model organism to study in the context of biofilms. The process of *P. aeruginosa* biofilm formation is listed in Figure 1.4 (Rasamiravaka *et al.*, 2015). In general, *P. aeruginosa* planktonic cells will first attach to a surface that is covered in a conditioning layer. A conditioning layer is a film of macromolecules that forms on a submerged surface. The flagellar pole is where *P. aeruginosa* attachment

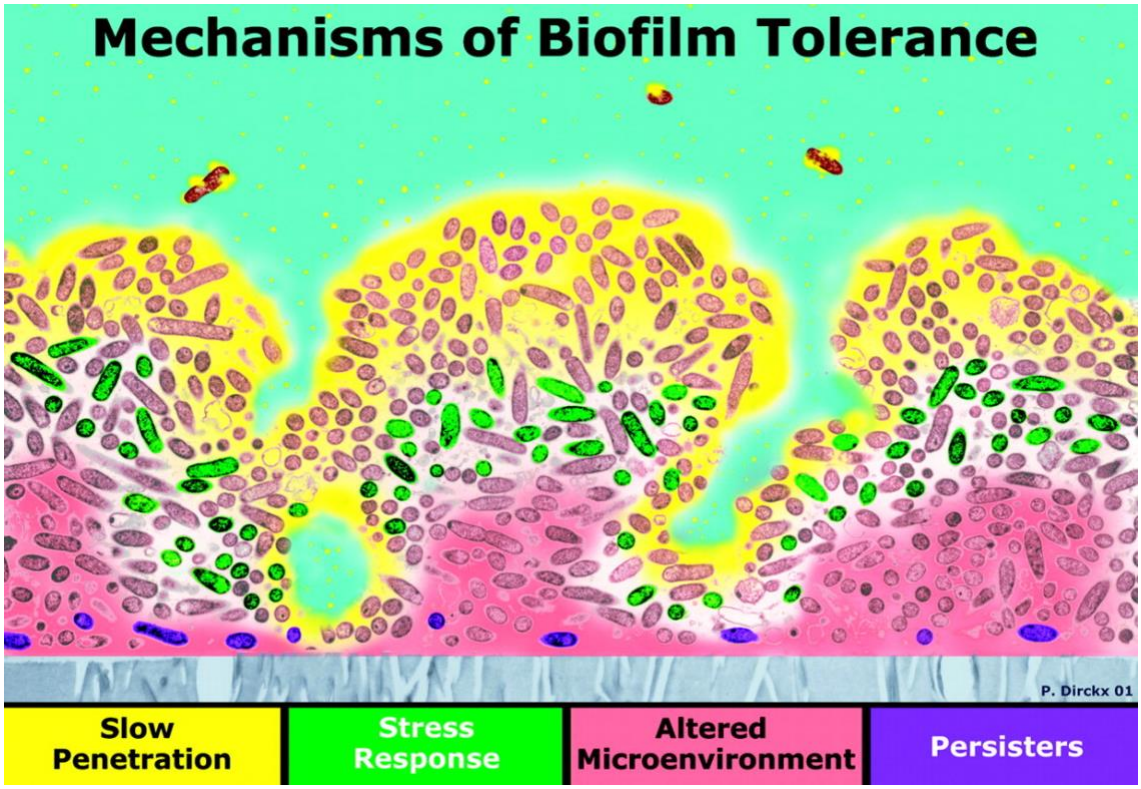


Figure 1.3 Four possible mechanisms of biofilm antibiotic tolerance. This figure is a cross-section of a biofilm with the attachment surface (grey) at the bottom and the aqueous phase containing the antibiotic (yellow) at the top. In zones where there is nutrient depletion (red), antibiotic action may be antagonised. Some bacteria may have active stress responses (green), while others may differentiate into a protected phenotype (purple) (Chambless *et al.*, 2006).

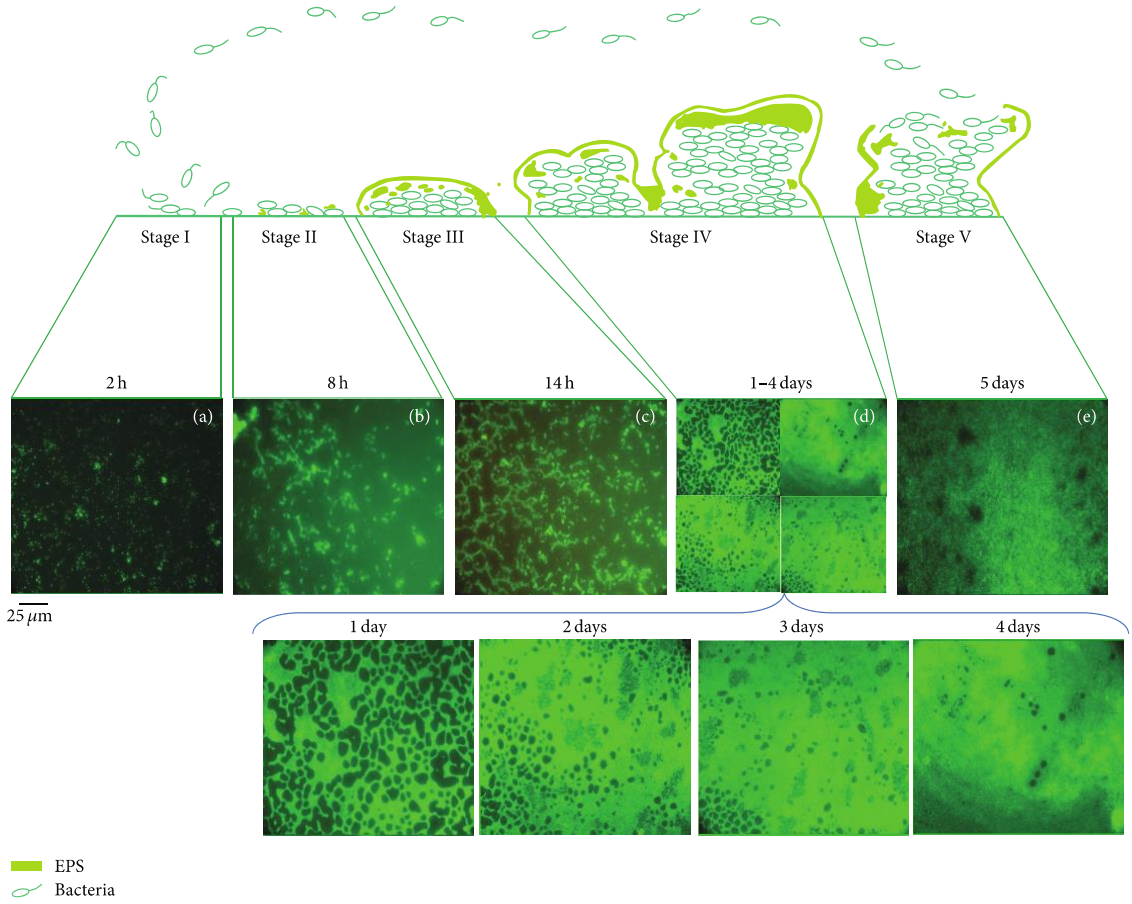


Figure 1.4 Biofilm lifestyle cycle of *P. aeruginosa* PAO1 grown in glucose minimal media. In stage I, planktonic bacteria initiate attachment to an abiotic surface, which becomes irreversible in stage II. Stage III corresponds to microcolony formation. Stage IV corresponds to biofilm maturation and growth of the three-dimensional community. Dispersion occurs in stage V and planktonic bacteria that are released from the biofilm to colonize other sites. The biofilm formation by *P. aeruginosa* PAO1 was revealed with Syto9 and visualized in Leica DM IRE2 inverted fluorescence microscope with 400x magnification at 2 h (Stage I), 8 h (Stage II), 14 h (Stage III), 1 to 4 days (Stage IV), and 5 days (Stage V). Images represent a 250 × 250-μm field (Rasamiravaka *et al.*, 2015).

happens. In static environment, flagella are required for initial surface contact. Flagellar stators are necessary for biofilm formation in *P. aeruginosa* but are not required for swimming motility. Secondly, irreversible attachment is the next stage in *P. aeruginosa* biofilm development. This stage is the change from reversible to irreversible attachment that marks the transition of planktonic to biofilm physiology. One of the most significant components of a biofilm community is the exopolysaccharide matrix (EPS, also termed extracellular polysaccharide or extracellular polymeric substance). A major component of EPS produced by *P. aeruginosa* is alginate, whose biosynthesis is carried out by the products of the *algA*, *C* and *D* genes (Olvera *et al.*, 1999). The increased production of alginate is positively regulated by the sigma factor σ^{22}, which also down-regulates the production of flagellin (Garrett *et al.*, 1999). Although overproduction of alginate during lung infection is known to enhance antibiotic resistance and pathogenesis, it is not thought to be essential for biofilm formation and EPS production in *P. aeruginosa* (Ryder *et al.*, 2007). This finding led to the recent discovery of two additional polysaccharides, the synthesis of which are encoded by the *psl* and *pel* genes. *Psl*, thought to be rich in mannose and galactose, has an important function in the *P. aeruginosa* biofilm EPS, possibly in biofilm maturation or structure differentiation, whereas *Pel* is glucose-rich and functions in pellicle production (Ryder *et al.*, 2007). In *P. aeruginosa* biofilms, extracellular DNA has been shown to be prominent in biofilm stalks, and derived from genomic DNA, possibly in lysis events co-ordinated by quorum-sensing and iron regulation (Allesen-Holm *et al.*, 2006). QS in *P. aeruginosa* occurs through two hierarchical systems which affect swarming (Shrout *et al.*, 2006), DNA release (Allesen-Holm *et al.*, 2006) and matrix production (Sakuragi and Kolter, 2007), ultimately affecting biofilm development and structure, although this may not necessarily be the case in *vivo* (Schaber *et al.*, 2007). In the final stage, *P. aeruginosa* cells are detaching from the biofilm. Co and colleagues discovered mucins, a biological gel produced by epithelial tissue, separate and disperse the cells in *P. aeruginosa* biofilms into suspension (Co *et al.*, 2018).

1.3.1.2 Consequences of biofilm formation in *P. aeruginosa* infections

P. aeruginosa is a pathogenic bacterium that causes opportunistic infections. *P. aeruginosa* can infect numerous kinds of hosts including plants, animals (Rahme *et al.*, 1995), nematodes (Gallagher and Manoil, 2001), fungi (Hogan and Kolter, 2002), and insects (Miyata *et al.*, 2003). In humans, *P. aeruginosa* can result in acute pneumonia, bacteraemia, burn wound infections, meningitis, otitis externa, otitis media, ulcerative keratitis and urinary tract infections (Lyczak *et al.*, 2000, Moore and Flaws, 2011, Sadikot *et al.*, 2005) in addition to Cystic Fibrosis (CF) lung infections.

The role of biofilms in the lungs of CF patients has been widely studied. Because a leading cause of CF-related infection is *P. aeruginosa*, this species is a popular candidate for biofilm research. CF results in the accumulation of mucus in the lungs, in which colonization by opportunistic bacteria can take place. Biofilm formation then occurs, and due to the combination of a decreased antibiotic response, a switch to a mucoid phenotype (alginate production), difficulty of dislodging mucus, and a host response that is more damaging to the host than to the biofilm, a severe chronic infection results (Parad *et al.*, 1999, Post *et al.*, 2004).

A large body of evidence confirmed that *P. aeruginosa* biofilms persist in the lungs of CF patients. The presence of microcolonies and large biofilm-like structures in the airways of CF patients was observed by microscopic examinations on sputum samples and lung tissue sections collected from CF patients (Worlitzsch *et al.*, 2002). These bacteria within the biofilm-like structures were identified as *P. aeruginosa* by fluorescence (Bjarnsholt *et al.*, 2009). In the study by Bjarnsholt *et al.* (2009), they suggest that without sufficient antimicrobial treatment, the existence of these biofilms covers the entire lung; in contrast, in patients who have aggressive antibiotic treatment, biofilms are limited to the conductive region, and not present in the lower airways.

Numerous consequences occur in the CF lung due to the formation of *P. aeruginosa* biofilms. The persistence of *P. aeruginosa* may be due to several characteristics of

biofilms including antibiotic resistance, immune resistance and possibility of bacterial mutants. In addition, Wolfgang *et al.* (2004) have found that the spread of the microorganism was limited by contacting *P. aeruginosa* with airway fluid. Indeed, Govan and Deretic (1996) pointed out bacterial colonizers of the CF lung rarely have systemic spread. So the biofilms have shown some survival advantages because of the colonization of infecting bacteria. However, the precise reason that biofilms are so much more resistant than planktonic cells is still not clear.

1.3.1.3 Current treatments for *P. aeruginosa* infections in CF

Current treatments of *P. aeruginosa* infections of the cystic fibrosis lung include oral and parenteral antibiotics, and especially the inhaled nebulized antibiotics. The aminoglycoside antibiotic tobramycin has the most frequently used and has been most studied (Gibson *et al.*, 2003, Ryan *et al.*, 2011). The European CF society (ECFS) recommended inhaled aztreonam lysine or Colistimethate (2 MU twice daily) as the currently widely used therapy for patients with chronic *P. aeruginosa (Castellani et al., 2018).*

One particular efficient treatment for CF lung infections is antibiotic inhalation therapy. In the study by Ryan *et al.* (2011), the efficacy of several inhaled antibiotics including aztreonam lysine, ceftazidime, cephaloridine, colistin, gentamicin, taurolidine, tobramycin, and a gentamicin/carbenicillin combination were investigated. The utilization of these inhaled antibiotics can strengthen lung function and weaken the frequency of exacerbation when compared to a placebo. The advantages of inhalation therapy for *P. aeruginosa* infections in the CF have been highlighted for over 45 years (Mearns, 1970). This therapy is a highly effective way to treat lung disease especially, because it can maximize the dose at the location of the disease instead of systemic exposure and toxicity to the rest of the body.

Colistin is a member of the polymyxin group. It was initially used therapeutically in the 1950s (Kumazawa and Yagisawa, 2002) but was abandoned due to the reported high

incidence of nephrotoxicity in early 1980s (Brown *et al.*, 1970). However, the emergence of multidrug-resistant Gram-negative bacteria and the shortage of new antimicrobial agents have brought back the interest of using colistin as a valuable therapeutic option. There is increasing research with use of aerosolized colistin in *P. aeruginosa* infections in cystic fibrosis (Jensen *et al.*, 1987, Hodson *et al.*, 2002, Berlana *et al.*, 2005, Herrmann *et al.*, 2010).

1.3.1.4 Antimicrobial resistance

Antimicrobial resistance (AMR) is defined as the resistance of microorganisms to an antimicrobial agent to which they were once sensitive (Jindal *et al.*, 2015). The appearance of antibiotic resistant strains of bacteria has been reported since the first introduction of effective antimicrobials in the 1930s; the use of penicillin in the clinic in 1940 was preceded by the identification of a penicillinase, an enzyme capable of destroying penicillin, in 1928 by the team who had led the discovery of penicillin (Abraham, 1940, Davies and Davies, 2010). Resistance has since hindered the long-term therapeutic use of antimicrobials and in recent years the lack of novel antimicrobial drugs and the global spread of antibiotic resistance genes (ARG) amongst clinically relevant bacteria has resulted in increased hospitalization and high mortality rates amongst patients infected with resistant microorganisms (Berendonk et al., 2015). Antimicrobial resistance has spread rapidly and had already emerged before the use of antimicrobials in the clinic.

There has been a dramatic rise of pathogenic strains of both gram-positive and gram-negative bacteria and bacteria bearing resistance to one or more antimicrobials from at least three different antimicrobial classes are called multidrug-resistant (MDR) bacteria (Bassetti and Righi, 2015). The most frequently reported gram-positive and gram-negative MDR bacterial strains have been called the 'ESKAPE' pathogens and include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacteriaceae* (Boucher *et al.*, 2009) (Rice, 2008). Gram-negative bacteria have shown resistance to many different types of antimicrobials including penicillins, cephalosporins and quinolones (Kanj and Kanafani,

2011). In particular, the emergence of MDR Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) and MDR *P. aeruginosa* have led to the use of wide-spectrum antibiotics (e.g. carbapenems/fluoroquinolones) which in turn have resulted in the emergence of isolates of gram-negative bacteria that are resistant to all antibiotics currently in use, and such microorganisms are termed extreme drug-resistant (XDR) strains (Gupta *et al.*, 2011, Munoz-Price *et al.*, 2013, Kanj and Kanafani, 2011).

The molecular mechanisms of resistance in bacteria have been extensively studied. Emergence of antibiotic-resistant strains of bacterial organisms is primarily due to transfer of ARGs in populations which occur through three main mechanisms including; horizontal gene transfer (HGT-now implicated in the movement of ARGs), proliferation of antibiotic resistant bacteria due to selective pressures imposed by antimicrobial compounds, for example biocides and finally, genetic mutation and recombination events in hyper-mutating bacterial strains (Berendonk *et al.*, 2015, Baquero *et al.*, 2008, Wright, 2010). Perhaps one of the most problematic drug-categories of which resistance is widespread belongs to the β-lactam class of antibiotics, for example penicillin. Resistance to these drugs is afforded by the production of β-lactamases, of which 1,000 have been identified. HGT has played a key role in the transmission of the β-lactam class of antibiotics with the plasmid-encoded β-lactamase, TEM (Davies and Davies, 2010). In addition to HGT, random mutation in the genes encoding β-lactamase enzymes has led to extended-spectrum β-lactamase variants, including CTX-M which has emerged in large subsets of human populations across Europe and now pose a significant threat to human health (Livermore *et al.*, 2007). Alternative anthropogenic reasons for bacterial resistance need to be considered since therapeutic use in humans accounts for less than half of all antibiotics produced commercially (Davies and Davies, 2010). Not only is the resistance to current antimicrobials strengthened by over-prescribed antibiotic use in the clinic, but antibiotic resistant hotspots are now emerging in environments where high bacterial loads are coupled with sub-therapeutic levels of antibiotics, for example in agricultural facilities (Berendonk *et al.*, 2015). Some examples of alternative uses of antibiotics are surprisingly unaccounted for in the emergence of resistance, these include i) therapeutic/prophylactic use in household pets, ii) uses as biocides in toiletries, iii) pest control for plants in agricultural systems, iv) growth promotion/prophylactic use in

animals and finally v) cloning and selection in research and industry (Davies and Davies, 2010). Uses of antibiotics as described above create reserves of resistance in the environment: genetic and genomic studies of organisms isolated in water-treatment plants indicate an environment where bacteria are rich in r genes, usually located on transmissible genetic elements (Schluter *et al.*, 2008, Szczepanowski *et al.*, 2009).

The overuse of antibiotics to treat CF lung infections has led to high levels of antibiotic resistance. Emerson *et al.* (2010) observed that the resistance of a CF lung isolate to a particular antibiotic is relevant to the treatment with that antibiotic. Greater resistance leads to longer therapies, which consequently induces more resistance (Plummer and Wildman, 2011). Report from the UK Cystic Fibrosis Trust Antibiotic Working Group, antibiotic treatment for cystic fibrosis – 3rd edition, showing resistance rates in *P. aeruginosa* infections in the CF in the UK have risen dramatically with about 40% resistant to 2 or more antibiotics. Therefore, novel antimicrobial agents that could replace or complement current therapies are needed to fight chronic infections in CF patients.

1.4 Conclusions and scope of thesis

The potential of using CORMs as novel therapeutic agents has been widely investigated in the past decade. The antimicrobial activities of CORMs against both gram-positive and gram-negative bacteria have confirmed *in vitro*. Furthermore, the effects of CORMs have also been discovered *in vivo* (see section 1.2).

With the growing evidence showing biofilms are resistant to antibiotic treatment, and the serious problems resulted from multi-drug resistant strains, using CORMs to replace or complement current therapies for biofilm related infections is an attractive option.

The work presented in this thesis therefore aims to develop and evaluate methods for the growth of *P. aeruginosa* biofilms, in order to investigate the effect of ruthenium-based CORMs on *P. aeruginosa* biofilms. Although Murray *et al.* studied the effect of CORM-

2 on *P. aeruginosa* biofilm, they found that CORM-2 not only kills *P. aeruginosa* planktonic cells, but also attenuates biofilm maturation and established biofilms (Murray *et al.*, 2012). The disadvantage of CORM-2, insoluble in water, makes the water-soluble carbonyl compound CORM-3 preferable as a model compound to study its antimicrobial activities.

In addition to a study of planktonic *P. aeruginosa*, biofilms formed on both 96-well plate and the Modified Robbins Device were examined to investigate whether ruthenium-based CORMs are effective against *P. aeruginosa* biofilm (Chapter 3). As discussed in section 1.3, biofilm is more resistant against antibiotics compare to its planktonic counterparts, so the hypothesis in Chapter 3 is that *P. aeruginosa* biofilm will be less sensitive to CORMs than its planktonic cells. If this hypothesis is true, the factors related to the resistance of *P. aeruginosa* biofilm against CORMs will also be examined, such as penetration, oxygen limitation and nutrient limitation (Chapter 3).

Although CO inhalation therapy has shown some beneficial effects (see section 1.1.3), accumulation of CO in biological system can have toxic effects. Thus a product that delivers CO by a safer mechanism, (i.e. using CORMs through inhalation or aerosol) is promising. Therefore, the work in this thesis was extended to apply nebulized controlled doses of CORMs to *P. aeruginosa* cells. By monitoring bacterial viability and the uptake of CORMs, we will assess the potential of this nebulise therapy for treating *P. aeruginosa* related infections with CORMs. CORM-3 will be used as a model CORM as it is chemically well characterised and is water soluble, a property of benefit in the nebulizer treatment. Colistin, which is a widely used antibiotic in chronic *P. aeruginosa* infections, was used in order to compare the efficiency between CORM-3 and colistin.

Ruthenium-based CORMs have successfully shown the antimicrobial effects on both Gram-positive and Gram-negative bacteria (see section 1.2.3), but such compounds still have limitations for use as therapeutic agents in biological system. One example is that CO release is not finely controlled. However, a novel CORM with photo-labile CO

ligands may overcame this issue. Therefore, another investigation in this thesis will be given to study the antibacterial activity of a novel manganese-based PhotoCORM ($[Mn(CO)_3(tpa\text{-}\kappa^3N)]^+$) against *P. aeruginosa* planktonic and biofilm cells. This compound is stable in the dark, and only activated upon photoexcitation. Colistin will also be used in order to compare the efficiency of PhotoCORM and antibiotic (Chapter 5).

Chapter 2

Materials and Methods

2.1 Bacteriological techniques

2.1.1 Bacterial strains

The bacterial strain used in this study was wild type *Pseudomonas aeruginosa* PAO1, it was kindly given by Mathieu Desmard from Jorge Boczkowski Lab, University of Paris-Est.

2.1.2 Media

Unless stated otherwise, all reagents were purchased from Sigma Aldrich or BDH. Nutrient agar, tryptone and yeast extract were all purchased from Oxoid.

All media were prepared with distilled-deionised water and sterilized by autoclaving at 121 ℃ and 15 psi for 15 min. All solutions and antibiotics were filter-sterilized as needed with Millipore filters, 0.2 μm pore size.

2.1.2.1 Luria-Bertani medium (LB)

Into 1 l of distilled H_2O was dissolved tryptone (10 g), yeast extract (5 g) and NaCl (10 g). The pH was then adjusted to 7.0 with 10 M NaOH (Sambrook and Russell, 2001).

2.1.2.2 Aerobic M9 medium

For batch growth, into 990 ml of distilled H_2O was dissolved Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g). To this, 10 ml of trace element solution was added. The pH was then brought to 7.4 and the media was autoclaved. Before use, 10 ml l^{-1} of 20% glucose and 1 ml l^{-1} of 1M $MgSO_4$ were added.

For chemostat growth, into 990 ml of distilled H_2O was dissolved Na_2HPO_4 (24 g), KH_2PO_4 (12 g), NaCl (1 g), NH_4Cl (2 g). To this, 20 ml of trace element solution was added. The pH was then brought to 7.5 and the media was autoclaved. Before use, 10 ml l^{-1} of 20% glucose, 2 ml l^{-1} of 1M $MgSO_4$ and 4 ml l^{-1} LB were added.

2.1.2.3 Anaerobic M9 medium

Into 985 ml of distilled H_2O was dissolved Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g), KNO_3 (10 g). To this, 10 ml of trace element solution was added. The pH was then brought to 7.4 and the media was autoclaved. Before use, 10 ml l^{-1} of 20% glucose, 1 ml l^{-1} of 1M $MgSO_4$ and 5 ml l^{-1} LB were added.

2.1.2.4 Trace elements solution for M9 medium

Into 700 ml of distilled H_2O was dissolved EDTA (5 g) and the pH was adjusted to 7.4 with 10 M NaOH before adding $FeCl_3.6H_2O$ (0.5 g), ZnO (50 mg), $CuCl_2.2H_2O$ (10 mg), H_3BO_3 (10 mg), ammonium molybdate (0.12 mg) and sodium selenite (17 mg). The volume was made up to 1 l with distilled H_2O and the medium was filter-sterilised. The solution stored at 4 °C.

2.1.2.5 SOC medium

Into 1 l of distilled H_2O was dissolved bactotryptone (20 g), yeast extract (5 g), NaCl (0.5 g) and 10 ml of a 250 mM solution of KCl. After adjusting the pH to 7 and autoclaving, 10 ml of 1 M $MgCl_2$ and 20 ml of sterile 1 M glucose were added.

2.1.2.6 TB medium

Into 100 ml of distilled H_2O was dissolved bactotryptone (0.8 g) and NaCl (0.5 g). The medium was then autoclaved.

2.1.2.7 TB soft agar

To make TB soft agar, agar (0.7 g) was dissolved in TB medium (100 ml) prior to autoclaving.

2.1.2.8 Nutrient agar (NA)

To make NA, dried nutrient agar (28 g) was dissolved in 1 l H_2O.

2.1.3 Buffers and solutions

2.1.3.1 Phosphate-buffered saline (PBS)

Into 1 l of distilled H_2O was dissolved NaCl (8 g), Na_2HPO_4 (1.15 g), KCl (0.2 g) and K_2HPO_4 (0.2 g). The pH was then adjusted to 7.4 before autoclaving.

2.1.3.2 CO-saturated solution

CO-saturated solution was made by bubbling M9 medium with CO gas from a cylinder (BOC, Guildford) in a 7 ml Bijoux bottle fitted with a Suba-Seal and gas escape needle at room temperature for 15 min. This generates a solution of approximately 1 mM.

2.1.4 Strain storage

2.1.4.1 *P. aeruginosa*

Strains were cultured on NA plates and stored at 4 °C and, when necessary, subcultured onto fresh plates. Glycerol stocks were made by adding 2 ml sterile LB medium containing 25% (v/v) glycerol onto a lawn of bacteria grown on NA. The bacteria were resuspended and removed using a sterile 1 ml pipette. This slurry was dispensed into a cryovial (Nalgene) and stored at - 80 °C.

2.1.4.2 Antibiotics

Kanamycin and colistin stock solutions were prepared in H_2O and stored at 4 ℃. Fresh solutions were made for each experiment.

2.1.5 Culture conditions

2.1.5.1 Cultures for *P. aeruginosa* planktonic cells growth and viability studies under aerobic conditions

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in aerobic M9 medium (10 ml) and a 5% (v/v) inoculum added to 20 ml aerobic M9 medium in side arm conical flasks. The cultures were kept at 37 ℃ and were shaken at 200 rpm. For growth and viability studies, CORM-2 or CORM-3 was added after the OD of the cultures reached ~ 50 Klett units.

2.1.5.2 Cultures for *P. aeruginosa* planktonic cells growth studies under anaerobic conditions

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in anaerobic M9 medium (10 ml) and a 5% (v/v) inoculum added to 8 ml anaerobic M9 medium in Klett-compatible 8.5 ml screw-cap tubes. The cultures were kept at 37 ℃ and were shaken at 200 rpm. For growth studies, CORM-3 was added after the OD of the cultures reached ~ 40 Klett units.

2.1.5.3 Cultures for *P. aeruginosa* energy starved growth studies

P. aeruginosa starter cultures were grown in 20 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in aerobic M9 medium (200 ml) and incubated at 37 °C shaking incubator until the OD_{600} of the cultures reached ~0.5. The aerobic M9 medium were removed by centrifugation and replaced by M9 medium that did not supply with glucose. The cultures were

incubated at 37 ℃ shaking incubator for further 2.5 h. The cultures were centrifuged one more time to replace the medium with PBS (200 ml). CORM-3 was added at this point for further analysis.

2.1.5.4 Cultures for *P. aeruginosa* biofilm cells growth and viability studies on a 96-well plate

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in aerobic M9 medium to an OD_{600} of ~ 0.05. A 200 µl aliquot of the cell suspension was added to each well on a 96-well plate. The plate was incubated in a non-shaking 37°C incubator for 48 h before the crystal violet assay (see 2.2.2) was carried out. For biofilm formation studies, CORM-3 was added to cell cultures before incubation. For established biofilm studies, CORM-3 was added after replacing planktonic cells with fresh aerobic M9 medium after biofilm formed for 48 h.

2.1.5.5 Cultures for *P. aeruginosa* biofilm cells growth and viability studies on a Modified Robbins Device (MRD)

The Modified Robbins Device was constructed in the engineering workshops in the University of Sheffield (Figure 2.1). The device was produced based on (Nickel *et al.*, 1985). The MRD contains twenty-five evenly spaced sampling ports on a 60 cm acrylic block. The removable sampling plugs were attached to each sampling port. The cell cultures could flow through the inner surface of the device without hampering flow characteristics. The biofilms are generated on the surface of each sampling plug.

The MRD was connected to two 1 l reservoirs (Figure 2.2). One reservoir contains bacterial cell cultures held in a 45 ℃ water bath. The increased the temperature 45 ℃ has taken the heat loss into consideration. Another reservoir contains CORM-3 held at room temperature. Cell cultures were pumped from the reservoir through the MRD by a pump with a delivery rate set at 30 ml h^{-1}. The sampling plugs on the MRD could then be aseptically removed from the device for analysis. Before each experiment, the

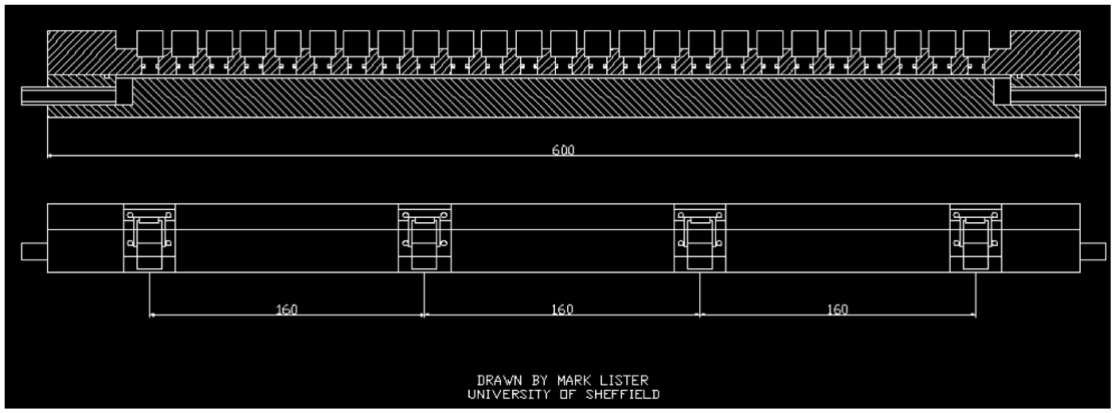


Figure 2.1 The structure of the Modified Robbins Device. Apparatus designed and manufactured by Mark Lister, the University of Sheffield. MRD was produced based from Nickel's paper in 1985 (Nickel *et al.*, 1985). The MRD contains twenty-five evenly spaced sampling ports on a 60 cm acrylic block as shown in the figure above. The removable sampling plugs were attached to each sampling port. The cell cultures could flow through the inner surface of the device without hampering flow characteristics. The biofilms are generated on the surface of each sampling plug.

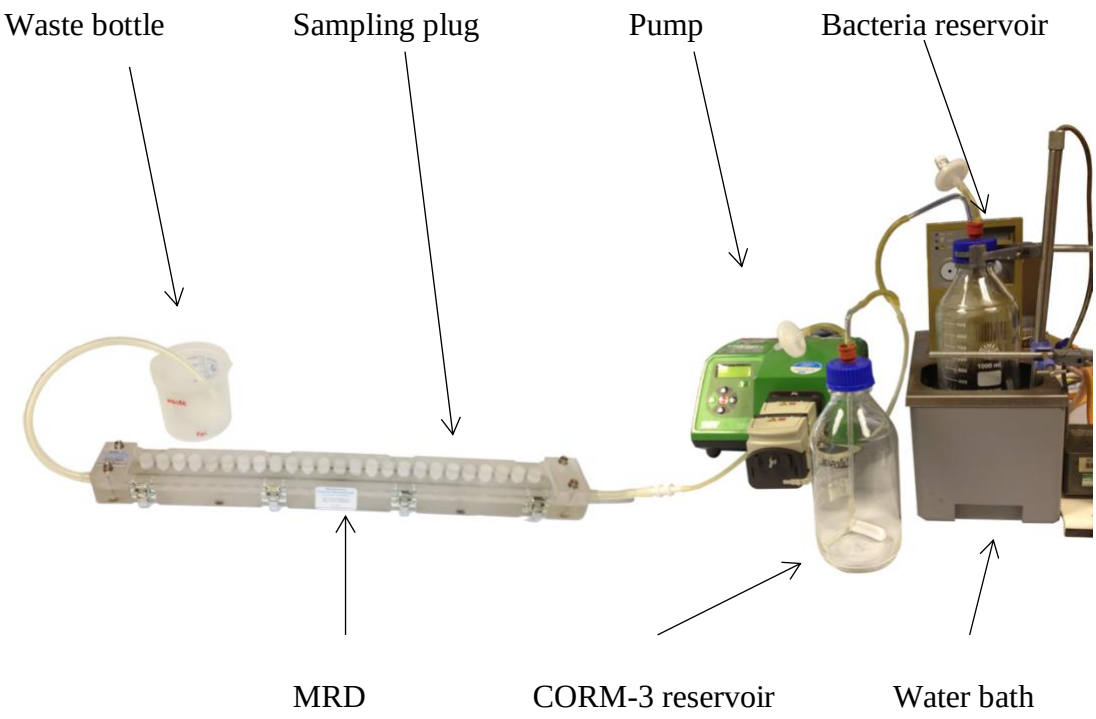


Figure 2.2 The biofilm formation on the Modified Robbins Device. The cell culture was grown in the bacteria reservoir in a 45 °C water bath. A pump was used to deliver the cell culture from bacteria reservoir to MRD. After biofilms generated on the sampling plugs on the MRD, CORM-3 solution was pumped from a CORM-3 reservoir to the MRD to treat the biofilm cells.

entire experimental apparatus was sterilized by wiping ethanol throughout the whole device.

2.1.5.6 Cultivation of *P. aeruginosa* planktonic cells in batch culture

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in aerobic M9 medium (10 ml) and a 4% (v/v) inoculum added to 125 ml aerobic M9 medium in a chemostat vessel. To maintain a culture temperature of 37 °C, a water jacket was constructed from silicon tubing (attached to the outside of a plastic 250 ml beaker within which the chemostat vessel was a snug fit). This tubing was connected to a circulating water bath set at 45 °C (which allowed for loss of heat). Cultures were aerated by employing a vortex (Pirt, 1975), aided by the three baffles of the spinner flasks. To this end, the flask was placed on KMO 2 Basic, IKA®-Werke stirrers (SLS, arbitrary setting of 300), which gave stirring without splashing.

2.1.5.7 Cultivation of *P. aeruginosa* biofilm cells in continuous culture

Biofilms were generated on the MRD from one custom-built chemostat. The apparatus design is based on a Proculture Dynalift spinner flask (Sigma) with 125 ml capacity, which has been modified to include an overflow weir as an outlet for waste and spent media (Figure 2.3). Silicon tubing was connected to the overflow weir to allow waste run into the MRD. One hole was drilled into the plastic screw lid of the flask in which a 17 mm subaseal (Fisher) was fitted. A silicon tube attached to a Hepa-Vent filter (Whatman) was pushed through the seal, which allowed gas transfer.

Cultures were fed with aerobic M9 medium (see section 2.1.2.2), where glucose was the growth-limiting nutrient. This was pumped from a reservoir (3 l conical flask, containing M9 medium) using a Minipuls 2 peristaltic pump (Gilson). Glass tubing was fed through a silicon rubber bung in the neck of the reservoir and attached to silicon tubing. The tubing connected, via a flow-back trap, into the vessel's sidearm through glass tubing inserted through a silicon bung. A second length of tubing attached to a Hepa-Vent filter was

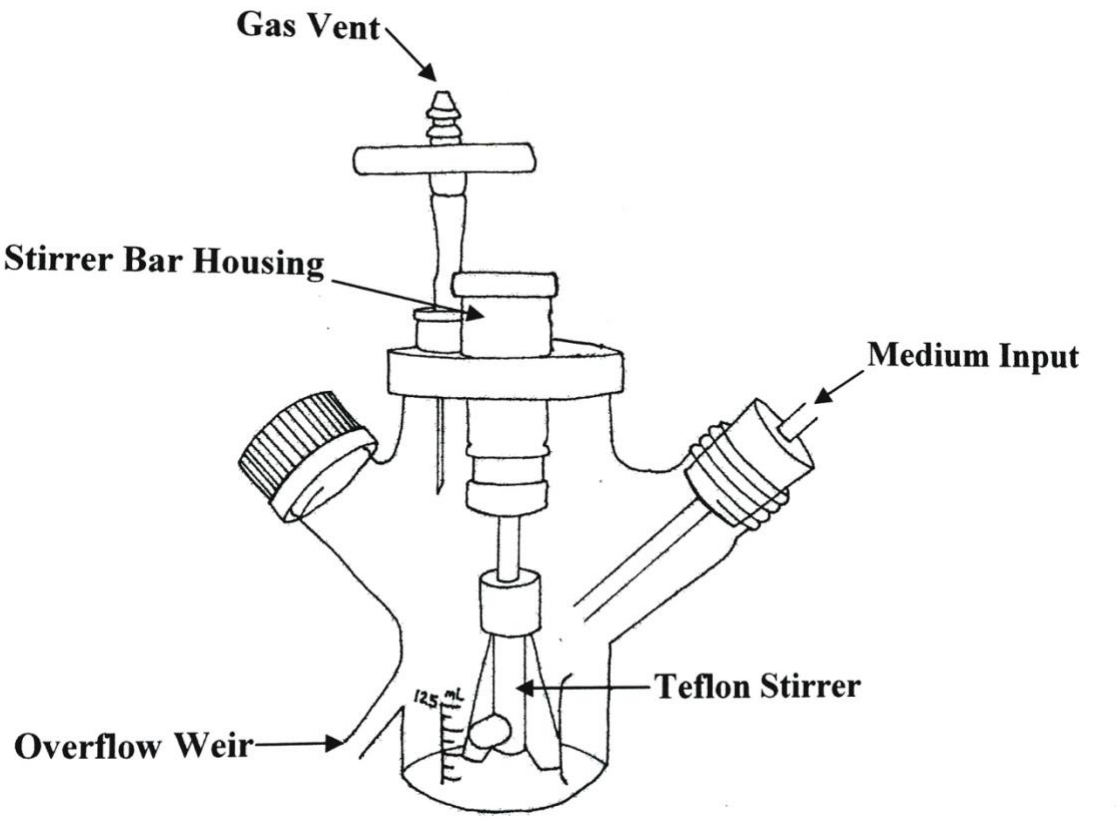


Figure 2.3 Modifications made to a Sigma Proculture Dynalift spinner flask for use as a chemostat vessel.

inserted into the bung in the neck of the reservoir to allow sterile gas exchange. The Minipuls 2 pump was calibrated by timing the transfer of water from a flask to a measuring cylinder.

Growth conditions in the chemostat were kept constant. To maintain a culture temperature of 37 °C, a water jacket was constructed from silicon tubing (attached to the outside of a plastic 250 ml beaker within which the chemostat vessel was a snug fit). This tubing was connected to a circulating water bath set at 45 °C (which allowed for loss of heat). Cultures were aerated by employing a vortex (Pirt, 1975), aided by the three baffles of the spinner flasks. To this end, the flask was placed on KMO 2 Basic, IKA®-Werke stirrers (SLS, arbitrary setting of 300), which gave stirring without splashing.

The whole system was sterilised in one complete piece in an autoclave at 121 °C and 15 p.s.i. for 15 min. After autoclaving, the pump was turned on and the chemostat vessel allowed to fill to maximum volume with the magnetic stirrer switched on. When full, the pump was stopped and, once the water bath had heated the medium to 37 °C, the medium in the vessel was inoculated with a starter culture (1% of the chemostat volume) through the appropriate subaseal. The cultures were allowed to grow in batch conditions for 30 h with a stir rate of 100. After this time, the stir rate was increased to 260 and the medium flow was started at 30 ml h^{-1}. After a further 72 h, aerobically grown biofilm-associated cells were harvested on the MRD and ready for analysis. A 2 ml sample was also taken at the entrance or the end of the MRD to test the OD_{600}, pH and glucose limitation. To check for glucose limitation, culture supernatants were subjected to a glucose assay (see section 2.2.3).

2.1.5.8 Applying CORM-3 and colistin to *P. aeruginosa* cells by an aerosol method

Applying CORM-3 and colistin to *P. aeruginosa* was achieved by a custom-built aerosol system (Figure 2.4). An airway plastic tube attached to the nebuliser device (Figure 2.5) was connected to a pipeline that contains ten evenly spaced sampling ports. Silicon tubing was connected to the other end of the pipeline to allow waste gas run into a waste bottle.

Figure 2.4 Custom-built aerosol system setup.

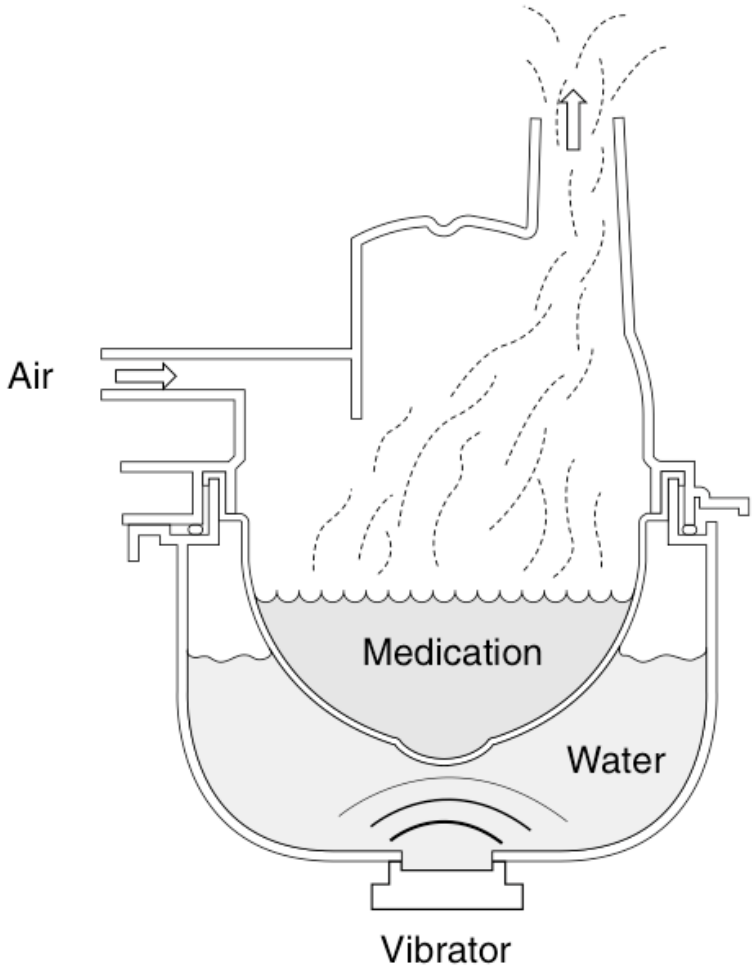


Figure 2.5 The structure of the nebuliser device.

Two holes were drilled into the silicon rubber bung in the neck of the waste bottle. In one, a silicon tube was connected to a vacuum, which pull the gas towards the waste bottle. The other hole was used as gas exchange port.

The nebuliser device was set up as follows nebulisation volume = 9, air-flow volume = 9 (according to machine manufacture guideline), vacuum level was been turned on before inserting cells to the sampling ports. The plugs contain cells which been inserted to this apparatus were either from a uniform plate or the MRD.

For agar plate cells studies, twelve agar plate cell circles were taken with a 4 mm borer from a uniform plate. Ten of these circles were inserted into the sampling ports; the other two were suspended in 1 ml PBS for viability assays. CORM-3 solution or colistin solution was sprayed onto the agar plate cells for 10 min, and then agar plate cells were removed from the sampling ports to the Eppendorf tubes that contain 1 ml PBS for viability assay.

For biofilm cells studies, *P. aeruginosa* biofilm cells were formed on the MRD sampling plugs before being moved to the nebuliser apparatus. CORM-3 solution or colistin solution was sprayed onto these plugs for 10 min, and then biofilm cells were suspended in 10 ml PBS for viability assays.

2.1.5.9 Cultures for growth studies of *P. aeruginosa* planktonic cells with PhotoCORM

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in M9 medium (10 ml) and a 3% (v/v) inoculum added to 2.5 ml M9 medium in bijou bottles. The cultures were stressed with PhotoCORM (100-500 μM), and then irradiated for 6 min at 365 nm to release CO. The cultures were grown aerobically at 37 °C for 24 h. For CO-saturated solution experiments, the starter cells were resuspended in M9 medium to an OD_{600} of ~ 0.05. A portion (200 μl) of the cell suspension was added to each well of a 96-

well plate and incubated on a shaking plate reader for 24 h at 37 °C. PhotoCORM (300 μM) or CO-saturated solution (900 μM) were added after the OD_{595} of the cultures reached ~ 0.5.

2.1.5.10 Cultures for growth and viability studies of *P. aeruginosa* biofilm cells with PhotoCORM

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in aerobic M9 medium to an OD_{600} of ~ 0.05. A 200 μl aliquot of the cell suspension was added to each well on a 96-well plate. The plate was incubated at non-shaking 37°C incubator for 48 h before crystal violet assay was carried out.

For biofilm formation studies, the cultures were stressed with PhotoCORM (200-500 μM), colistin (4-8 μg/ml), CO-saturated solution (1 mM) or H_2O_2 (16-64 mM), and irradiated for 2 min at 365 nm before inoculation into wells of a 96-well plate. The plate was incubated in a 37 °C non-shaking incubator for 48 h. Under these conditions, a biofilm formed at the bottom of the well. Afterwards, both crystal violet assays and viability assays were carried out.

For established biofilm studies, fresh M9 medium or PBS containing PhotoCORM (200-500 μM), colistin (4-8 μg/ml), CO-saturated solution (1 mM) or H_2O_2 (16-64 mM) was added to the plate after 24 h pre-incubation, and then the plate was irradiated for 2 min at 365 nm and incubated in a 37 °C non-shaking incubator for a further 24 h. Afterwards, both crystal violet assays and viability assays were carried out.

2.1.6 Culture turbidity measurements

A Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York) with a number 66 (red) filter was used to measure the growth of a culture. Before measurements were taken, a blank containing the sterile medium was used to zero the

instrument. The cultures were grown in 250 ml side-arm flasks that allowed the optical density reading to be taken without removing any culture from the flask.

2.1.7 Viability assay

Viability in biofilms formed either on the 96-well plate or the MRD were determined by suspending the cells in PBS. Under sterile conditions, cultures were serial diluted (10^{-5} - 10^{-8}) and plated onto nutrient agar plates. The plates were incubated overnight at 37 ℃ to allow the cfu ml^{-1} (colony forming units) to be calculated.

2.1.8 *P. aeruginosa* uniform plate

To make a uniform plate, 1 ml LB medium was gently mixed with 1.5 ml TB soft agar in sterile tube, this mixture was been kept in 50-55 ℃ water bath. 50 µl of *P. aeruginosa* cells from a starter culture was then properly mixed with this super-soft agar. The mixture was poured onto a nutrient agar plate. The top agar was solid after a few minutes. The plate was kept in a 37 ℃ incubator for 6 h.

2.2 Biochemical techniques

2.2.1 Preparation of CORM stock solutions

2.2.1.1 CORM-2

CORM-2 ($[Ru(CO)_3Cl_2]_2$) was purchased from Sigma Aldrich. Fresh stock solutions (10 mM or 50 mM) were made by dissolving in DMSO and wrapped in foil to exclude light.

2.2.1.2 CORM-3

CORM-3 ([$Ru(CO)_3Cl$(glycinate)]) was obtained from Professor Brian Mann (Chemistry Department, University of Sheffield). This compound was synthesised by the published method (Johnson *et al.*, 2007). Stock solutions were freshly made by dissolving the compound in water.

2.2.1.3 PhotoCORM-CN028-31

PhotoCORM-CN028-31 ($[Mn(CO)_3(tpa\text{-}\kappa^3N)]^+$) was provided by Christoph Nagel (Institut fur Anorganische Chemie, Julius-Maximilians-Universitat Wurzburg, Germany) after synthesis following the published method (Nagel *et al.*, 2014). A stock solution (2 mM) was made by dissolving in water and wrapped in foil to exclude light. Stocks were used fresh or on the following day after overnight storage at 4 °C.

2.2.2 Crystal violet assay (CV)

After biofilm formation in a 96-well plate, the planktonic culture inside each well was removed by gently pipetting out. A 200 µl aliquot of 1% (w/v) crystal violet was added to each well and kept at room temperature for 1 min. Unbound crystal violet and any remaining planktonic cells were removed by inverting the plate, draining it briefly and then each well was gently washed with PBS for several times. Biofilm-bound crystal violet was eluted into the wells by adding 200 µl of a solution containing 80% ethanol and 20% acetone. Crystal violet was quantified using the plate reader by measuring absorbance at 595 nm for *P. aeruginosa* studies.

2.2.3 Glucose assay

Glucose was detected using the glucose oxidase (GO) Assay Kit (Sigma Aldrich) according to the manufacturer's instruction. A set of solutions was prepared by mixing glucose standard (0-0.08 ml) with water (1-0.92 ml) in marked tubes. The reaction was started by adding 2 ml of Assay Reagent to each tube and proper mixed for 30 to 60 s. Each tube was then kept at 37 °C water bath for exactly 30 min. 2 ml of 12 N H_2SO_4 was added to each tube and gently mixed to stop the reaction. The absorbance at 540 nm was read for each tube. A standard curve was calculated by plotting absorbance at 540 nm (y axis) *vs.* mg of glucose (x axis) (Figure 2.6). For sample assays, glucose concentrations (mg ml^{-1}) were determined from the standard curve.

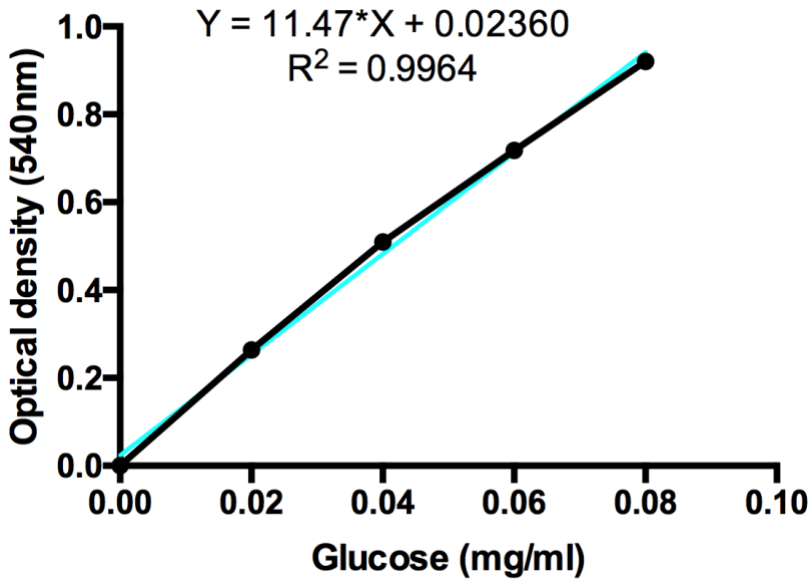


Figure 2.6 Glucose standard curves.

2.2.4 Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in aerobic M9 medium to an OD_{600} of ~ 0.05. Aliquots of 1.2 ml cell cultures were distributed in the marked tubes. Colistin (0.25-128 µg/ml) was added with increasing doubling concentrations. After aerobic incubation for 24 h, at 37 °C and 200 rpm, MIC was determined by reading the OD_{600}. For the MBC determination, 10 µl of each culture was plated on nutrient agar plates and incubated for a further 72 h. The lowest concentration that prevented formation of colonies was considered as the MBC.

2.2.5 Determination of Fractional Inhibition Concentration (FIC)

P. aeruginosa starter cultures were grown in 10 ml LB broth overnight and then the LB removed by centrifugation at 5500 rpm for 5 min. The cells were then resuspended in aerobic M9 medium to an OD_{600} of ~ 0.05. Aliquots of 200 µl cell cultures were distributed in a 96-well plate. A set of solutions was prepared by combining colistin (0-8 µg ml^{-1}) with CORMs (0-240 µm). The solutions were added to the cell cultures on the 96-well plate for 24 h incubation, at 37 °C and 200 rpm. MIC of colistin alone, MIC of CORM alone, MIC of colistin in combination and MIC of CORM in combination were determined by reading the OD_{595}. The summation of FIC was calculated as the summation of (MIC of colistin in combination / MIC of colistin alone) and (MIC of CORM in combination / MIC of CORM alone) (Punam, 2007):

$$FIC\ for\ CORM = \frac{MIC\ of\ CORM\ in\ combination}{MIC\ of\ CORM\ alone}$$

$$FIC\ for\ antibiotic = \frac{MIC\ of\ antibiotic\ in\ combination}{MIC\ of\ antibiotic\ alone}$$

$$\sum FIC = FIC\ for\ CORM + FIC\ for\ antibiotic$$

The interpretation is synergy if the summation of FIC is ≤ 0.5, the interpretation is indifference if the summation of FIC is >0.5 and ≤4, and the interpretation is antagonism if the summation of FIC is >4.

2.2.6 Penetration assay

Penetration assay was conducted by modifying the protocol from Kumon et al (1994) (Figure 2.7). The sodium alginate (Sigma) was suspended in distilled water, then 200 µl of this alginate solution was mixed with 40 µl 0.1 M $CaCl_2$ to form an alginate gel layer on a membrane filter in a culture plate insert (Millicell®-PCF, 0.4 µm, 12 mm in diameter, Millipore). After the gel had set, the insert was held in a fixed position slightly submerged in 15 ml water in a small beaker. A stir bar was placed at the bottom of the beaker to stir the water on a magnetic-stirrer device. A volume of 50 µl of CORM-3 was added to the gel and incubation was done at room temperature for 24 h, after which ruthenium concentration in the water was measured.

2.2.7 Inductively-coupled plasma-mass spectrometer (ICP-MS)

Planktonic and biofilm culture of *P. aeruginosa* were analysed by ICP-MS to assess the abundance of biologically-significant elements. In general, cell cultures treated with CORM-3 were centrifuged at 5500 rpm for 20 min at 4 °C. The pellets were then resuspended in 0.5% nitric acid and centrifuged at 13,000 rpm for 5 min at 4°C. This pellet wash process was repeated three times. The resulting pellets after washes were digested with 1 ml nitric acid (Aristar, 69% v/v) for 60 min and then diluted to 5 ml with 1% nitric acid. Analysis of ruthenium (mass 101) was done using an Agilent 7500CE ICP-MS instrument using rhodium (mass 103) as an internal standard. Calibration standards were made up in 20% nitric acid to match the samples a sample blank was prepared comprising 1 ml nitric acid diluted to 5 ml with 1% nitric acid (Davidge *et al.*, 2009b). This ICP-MS work was all done at the Centre for Analytical Sciences (CAS, University of Sheffield).

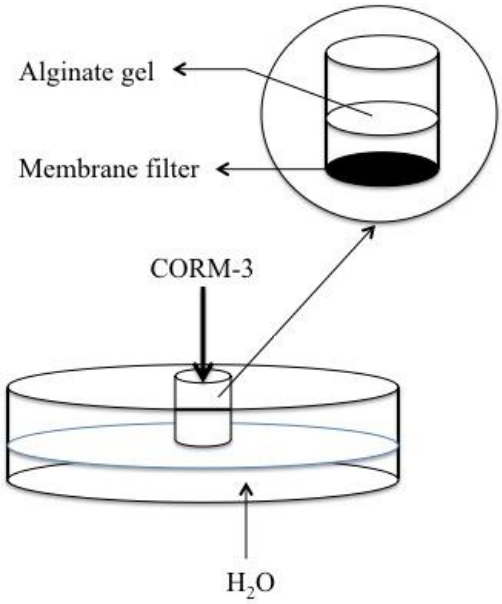


Figure 2.7 A diagram of the penetration assay.

Chapter 3

Interaction of *P. aeruginosa* with ruthenium-based CORMs: CORM-2 and CORM-3

3.1 Introduction

3.1.1 *P. aeruginosa* biofilm

P. aeruginosa is an opportunistic Gram-negative pathogen of animals and plants (Bodey *et al.*, 1983). It has always served as a model organism for biofilm research (Monds and O'Toole, 2009). The clinical interests are widely expressed in therapies that disrupt or prevent *P. aeruginosa* biofilm formation since this pathogen is an important cause of pulmonary disease especially in the context of cystic fibrosis (CF) (Waters and Ratjen, 2015).

Current treatments for CF include oral and parenteral antibiotics, particularly inhaled nebulized antibiotics (Hassett *et al.*, 2010). With the increase in antibiotic-resistant bacteria, it is essential to investigate other bactericidal compounds such as CORMs to replace or complement current therapies to fight chronic infections in CF patients. The reason this resistant to therapeutic agents is the high ability of form biofilm.

The reason that biofilms are so much more resistant to antimicrobial agents than planktonic cells is still not clear, but several hypothesises have been proposed. It may be that penetration of the drug into the biofilm is restricted by the biofilm itself, particularly the extracellular polymeric substance (EPS) component. This hypothesis was supported by Lewis' group, who have shown that diffusion of a molecule through a bacterial aggregate results in a concentration gradient, and cells might be able to degrade or modify antibiotic molecules at these low concentrations before they became inhibitory (Lewis, 2001). There are several other factors that may contribute to resistance, such as nutrient limitation (Anderl *et al.*, 2003), oxygen limitation (Borriello *et al.*, 2004), low metabolic

activity (Walters *et al.*, 2003), adaptive stress responses (Marr *et al.*, 2007), and the formation of persister cells (Lewis, 2007).

3.1.2 The antimicrobial effect of CORMs on *P. aeruginosa*

The therapeutic properties of CORMs led researchers to investigate their effect on *P. aeruginosa* in the past two decades. Desmard and co-workers (2009) have shown that CORM-3 was bactericidal to both a laboratory strain (PAO1) and three clinical isolates of *P. aeruginosa*. Very low concentrations (0.5 – 10 μM) of the compound were needed to inhibit the growth of an antibiotic-resistant strain of *P. aeruginosa in vitro*, and these concentrations were 50-fold lower than concentrations that can cause cytotoxicity to mammalian cells (Desmard *et al.*, 2009). Moreover, CORM-3 (10 μM) was as effective at reducing the bacterial count *in vitro* as the established antibiotics amikacine (50 mg l^{-1}, or 0.8 mM) and ticarcilline (50 mg kg^{-1}).

The same group found that CORM induced bactericidal effects and inhibition of *P. aeruginosa* respiration that might be due to the specific metal carbonyl used. Although bacterial oxygen consumption was similarly affected, ruthenium-based CORM-2 and CORM-3 were more bactericidal than a novel, slow CO-releaser, manganese-based CORM-371. CORM-A1, which does not contain a metal centre, but releases CO at a similar rate to CORM-371, was found to elicit only a bacteriostatic effect and mild perturbations in the respiratory capacity of *P. aeruginosa*. The abolishment of CORM function by NAC was also compound-dependent with the activity of ruthenium-based CORMs being the most affected (Desmard *et al.*, 2012).

Murray and colleagues were the first to evaluate the effects of CORM-2 on *P. aeruginosa* biofilm maturation and surface colonization. They have shown that CORM-2 (25 - 200 μM) prevented biofilm maturation as early as 60 min after addition. CORM-2 (100 μM) also decreased the number of viable bacteria recovered from the biofilm as early as 30 min after treatment, and persisting for a further 6 h. CORM-2 prevented biofilm maturation to a similar extent as tobramycin, an established antibiotic to *P. aeruginosa*

infections, and was more effective when combined with the drug. Additionally, CORM-2 (50 - 200 μM) disrupted *P. aeruginosa* surface colonization of human bronchial epithelial cells, and these concentrations are not cytotoxic to the cells (Murray *et al.*, 2012).

3.1.3 The aim of this work

The work described in this chapter aims to compare the effects of ruthenium-based CORMs, CORM-2 and CORM-3, on *P. aeruginosa* planktonic cells with biofilm cells. It was hypothesised that *P. aeruginosa* planktonic cells would be more sensitive to ruthenium-based CORMs than biofilm cells. Furthermore, the mechanisms behind the activity of CORM-3 resistance from *P. aeruginosa* biofilm cells were also examined.

3.2 Results

3.2.1 The growth of *P. aeruginosa* planktonic cells *in vitro*

Initially, the growth of *P. aeruginosa* planktonic cells was assessed in aerobic M9 medium (Figure 3.1). It was found that the bacterial cells reached stationary phase after 9 h and the doubling time was 1.7 h.

In order to enhance the growth of *P. aeruginosa* planktonic cells, two experiments were conducted in this study. One culture had additional 0.4 % LB broth added to the M9 medium (Figure 3.2A); the other experiment involved using three different concentrations of glucose (Figure 3.2B). The LB broth did provide more cell growth in logarithmic phase. Increasing glucose concentration in the medium increased *P. aeruginosa* cell growth when the culture reached stationary phase.

3.2.2 The effect of CORM-3 on *P. aeruginosa* cells *in vitro*

3.2.2.1 The effect of CORM-3 on *P. aeruginosa* planktonic cell growth and viability

In order to investigate the antimicrobial action of CORM-3, its effect on the growth of *P. aeruginosa* planktonic cells was examined on 20 ml cultures growing in M9 medium. Cultures were grown aerobically at 37 ℃ until 60 Klett units was reached; cultures were then stressed with CORM-3 (10-100 μM). CORM-3 inhibited bacterial growth in a concentration-dependent manner (Figure 3.3A). CORM-3 had a slight deleterious effect on growth at 10 μM, with the negative impact on growth increasing as CORM-3 concentration was increased. Meanwhile, comparing the cell growth of *P. aeruginosa* planktonic cells in the presence of CO-saturated solution (1 mM) or CORM-3 (1 mM) (Figure 3.3B), no effect of CO gas in solution on growth was observed in cells suggesting that the effect on growth by CORM-3 can be attributed to the compound as a whole and not the released CO.

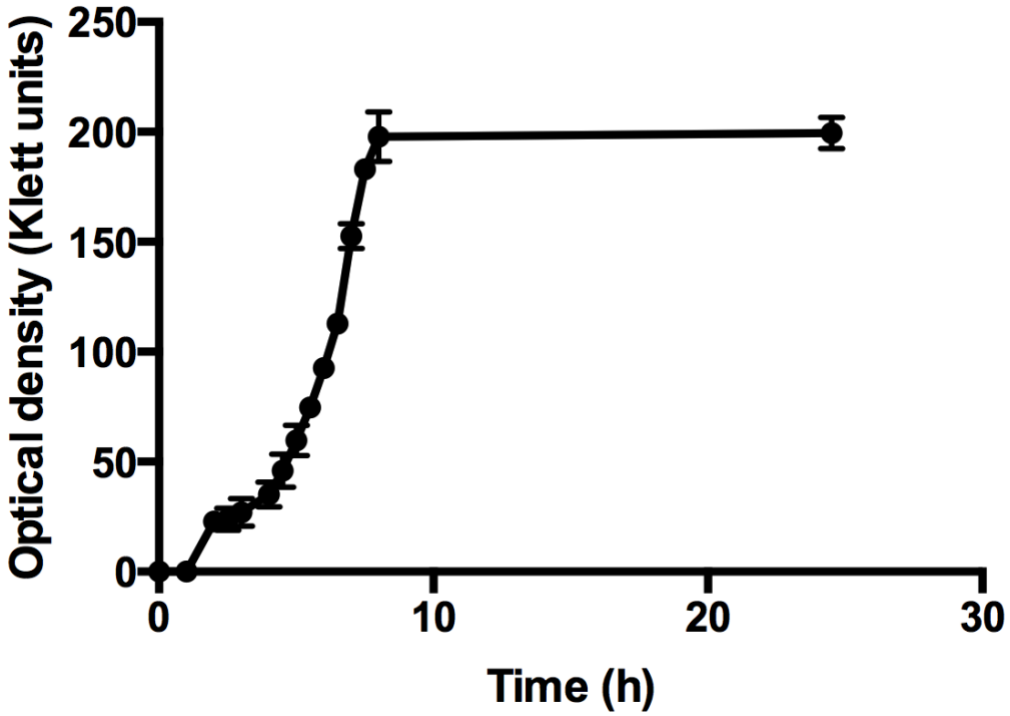
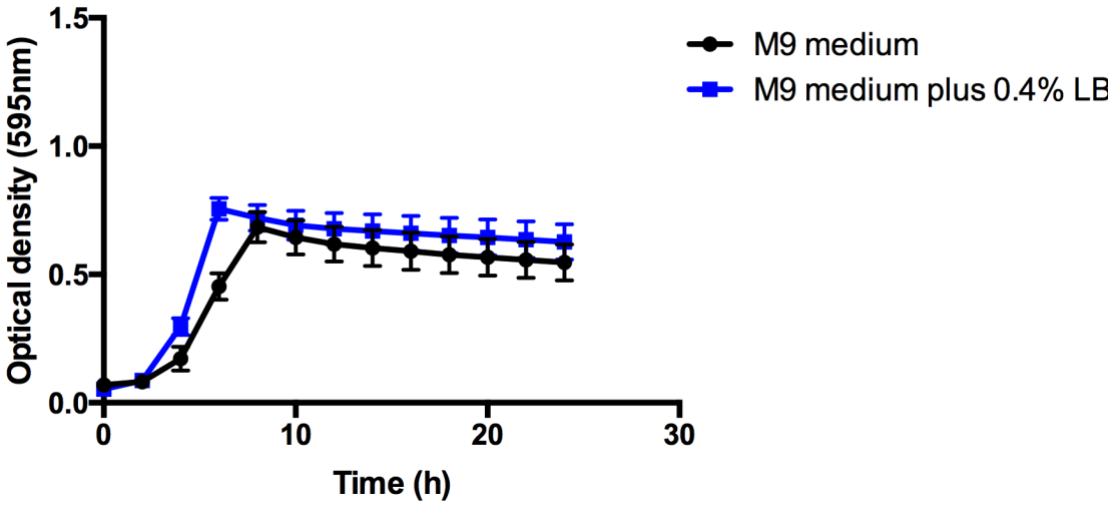


Figure 3.1 The growth of *P. aeruginosa* planktonic cells in M9 medium. *P. aeruginosa* planktonic cells were grown aerobically in M9 medium in side-arm flasks, and the optical densities were recorded at regular intervals by a Klett-Summerson photoelectric colorimeter. Values shown are the mean of at least 3 biological repeats. Error bars show ± S.D.

A



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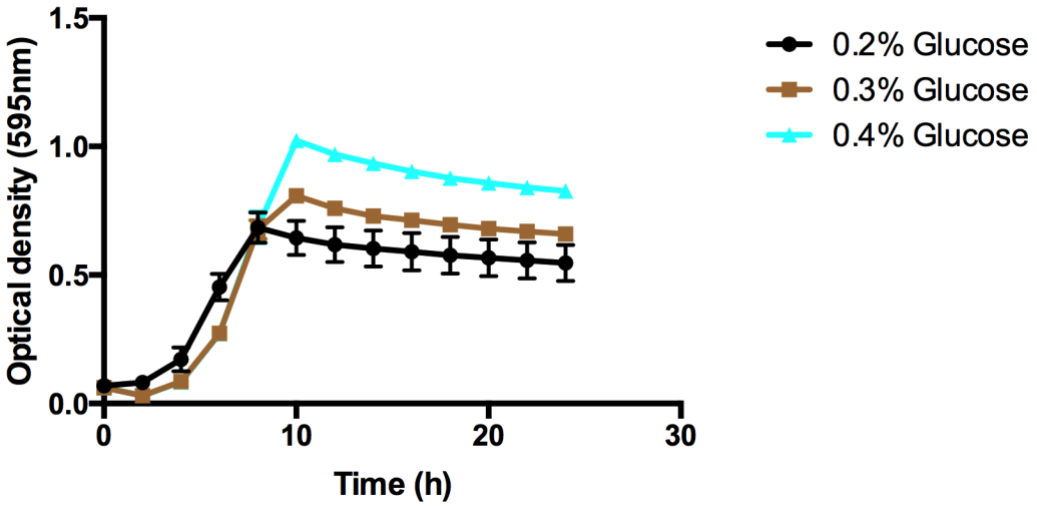
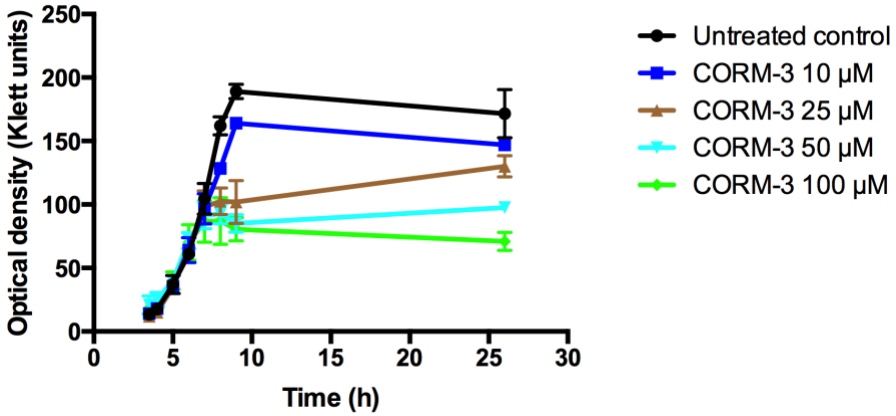


Figure 3.2 The growth of *P. aeruginosa* planktonic cells in different M9 medium. *P. aeruginosa* planktonic cells were grown aerobically in M9 medium supplied with 0.4 % LB broth **(A)** or M9 medium with enhanced glucose **(B)** over 25 h in a plate reader under 37 °C shaking condition. The OD_{595} was recorded at regular intervals. Values shown are the mean of at least 3 biological repeats. Error bars show ± S.D.

A



B

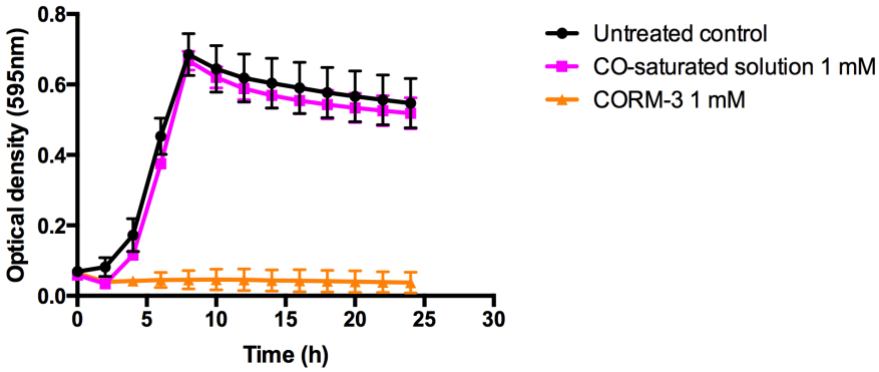


Figure 3.3 The effect of CORM-3 or CO-saturated solution on the growth of *P. aeruginosa* planktonic cells. (A) The growth of *P. aeruginosa* was followed until a Klett OD of ~60 Klett was reached. The cultures were then stressed with CORM-3 (10 - 100 µM) for 24 h. The optical density was recorded over the time course. Values shown are mean of at least 3 repeats. Error bars show ± S.D. **(B)** Cell cultures of *P. aeruginosa* in the presence and absence of CORM-3 (1 mM) or CO gas-saturated solution (1 mM) were added to a 96-well plastic plate. The plate was incubated for 24 h and readings were taken at regular intervals. Data shows the mean and standard deviation from 5 technical repeats.

In addition, the effect of CORM-3 on *P. aeruginosa* planktonic cell viability was investigated. Cells were grown aerobically at 37 ℃ for 6 h. Then CORM-3 (30 μM) was added to the culture. Cell viabilities were measured every half an hour till 2.5 h later (Figure 3.5). CORM-3 (30 μM) completely killed *P. aeruginosa* planktonic cell as early as 30 min, confirmed after 2.5 h.

To find a time period in which CORM-3 can completely kill *P. aeruginosa* planktonic cells, the effect of various concentrations of CORM-3 (30 - 100 μM) was tested. Cells were grown aerobically at 37 ℃ until 30 Klett units was reached; cultures were then stressed with various concentrations of CORM-3 (30 - 100 μM) for 8 h. Cell viabilities were measured at 6 h and 8 h time points (Figure 3.6). 30 μM CORM-3 almost inhibited *P. aeruginosa* planktonic cells growth between 6 and 8 h, which then started to slowly recover (Figure 3.6). Increasing CORM-3 concentration to 100 μM completely killed *P. aeruginosa* planktonic cells after 8 h.

3.2.2.2 The effect of CORM-3 on *P. aeruginosa* biofilm formation

After the initial examination of CORM-3 on *P. aeruginosa* planktonic cells, it was important to investigate whether CORM-3 can impact the growth and cell viability of surface-associated *P. aeruginosa* biofilm cells. The biofilm cells were formed on the inside of a 96-well plate well (Cowan and Fletcher, 1987). In this study, to determine the effect of CORM-3 on *P. aeruginosa* biofilm formation, a 6 h-old pre-matured *P. aeruginosa* biofilm preformed on a 96-well plate, then exposure to CORM-3 (10 - 100 μM) for further 24 h. The biomass was quantified by recording the absorbance of biofilm-bound crystal violet (Figure 3.7). CORM-3 had a significant effect on biofilm formation at 10 μM with only 50% of biomass detected after CORM-3 treatment. Crystal violet biomass staining decreased after CORM-3 exposure in a concentration-dependent manner. Thus CORM-3 directly killed *P. aeruginosa* planktonic cells and reduced their ability to form biofilm.

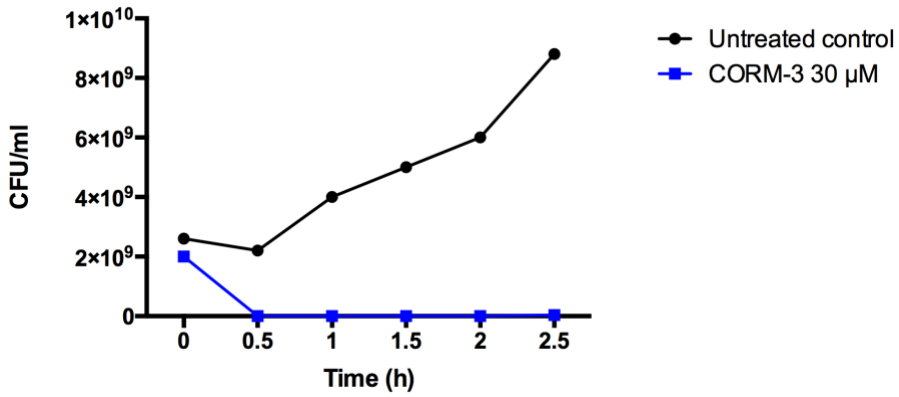


Figure 3.4 The effect of CORM-3 on *P. aeruginosa* planktonic cell viability. *P. aeruginosa* planktonic cell cultures were grown in aerobic M9 medium for 6 h. The cultures were then stressed with CORM-3 (30 µM) for 2.5 h. The number of viable cells was measured every 0.5 h.

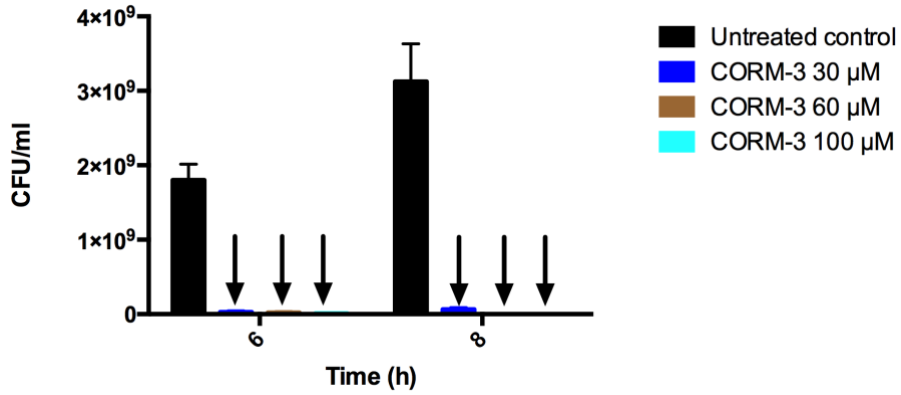


Figure 3.5 The effect of various concentrations of CORM-3 on *P. aeruginosa* planktonic cell viability. *P. aeruginosa* planktonic cell cultures were grown in aerobic M9 medium until a Klett OD of ~30 was reached. The cultures were then stressed with various concentrations of CORM-3 (30 - 100 µM) for 8 h. The number of viable cells was measured at 6 h and 8 h. Data shows the means and standard deviations from 2 biological replicates of which there were 3 technical repeats.

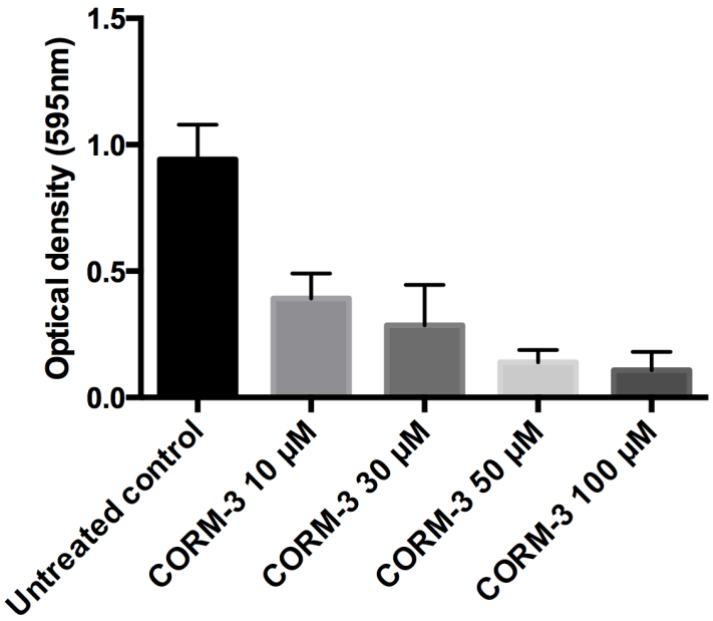


Figure 3.6 The effect of CORM-3 on *P. aeruginosa* biofilm formation. A 6 h P. aeruginosa biofilm was formed on a 96-well plate. The biofilm was then stressed with CORM-3 (30 - 100 µM) for 2.5 h. Crystal violet (CV) biomass staining decreased after CORM-3 exposure in a dose-dependent manner. Each condition was done in triplicate.

3.2.2.3 The effect of CORM-3 on *P. aeruginosa* established biofilms

With the purpose of examining *P. aeruginosa* biofilms, a Modified Robbins Device (MRD) was constructed and used in this study. This device allows the attachment of cells to twenty-five removable plug surfaces before beginning a flow of medium over those surfaces (see Figure 2.1 in section 2.1.5.5). The colonization of adherent bacteria cells was generated on the MRD for 4 h and 24 h before the number of viable sessile bacteria in the biofilm was evaluated by measuring the colony-forming units per cm^2 (Figure 3.7). Thus, the biofilm cells could be easily harvested from the MRD.

The effect of sonication on release of cells and biofilms from the removable plugs was assessed. Test biofilms were prepared, washed and vortexed in PBS, and sonicated for 90 s. The optical densities of the resulting suspensions were measured at 600 nm, and the cell viabilities were recorded upon sonication (Figure 3.8). The effect of sonication on cell release was slight, with most cells being released after 20 s. The optical density of the suspension declined by 27 % with further sonication after 90 s. This is reflected by the viability assay data, in which the number of viable cells was reduced after 90 s. Thus, prolonged sonication probably causes not only release of cells but also their lysis.

The bactericidal effect of CORM-3 on established biofilm that formed on the MRD was examined by allowing 30 μM CORM-3 solution to flow through the MRD for 2.5 h. The treated removable plugs were sterilized with IMS before suspended in 10 ml PBS. After vortexing the suspension and 20 s sonication, the number of viable sessile bacteria in the biofilm was evaluated by measuring the colony-forming units per cm^2 (Figure 3.9). A significant decrease on *P. aeruginosa* biofilm cells viability was observed.

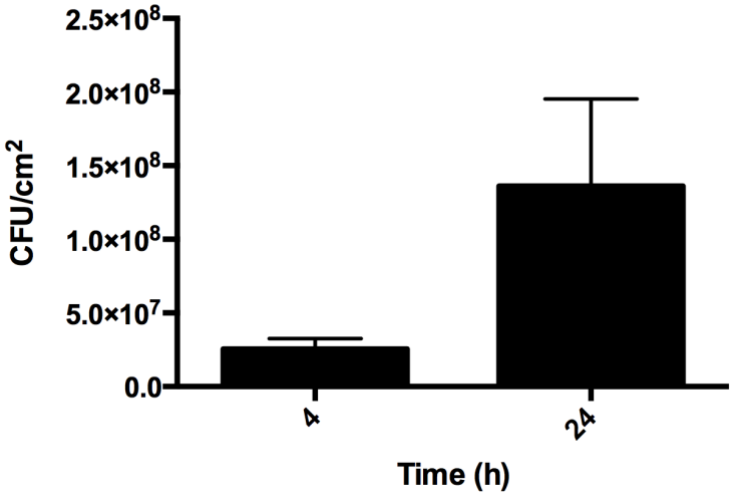


Figure 3.7 The formation of *P. aeruginosa* biofilms on the MRD. *P. aeruginosa* cell cultures were grown in a reservoir that contained aerobic M9 medium and then pumped through the MRD. During the formation of *P. aeruginosa* biofilm on the MRD, two plugs were taken for viability assay at 4 h (left panel) and 24 h (right panel). The bars represent the average of the number of colonies from two different plugs at each time point. Error bars show ± S.D.

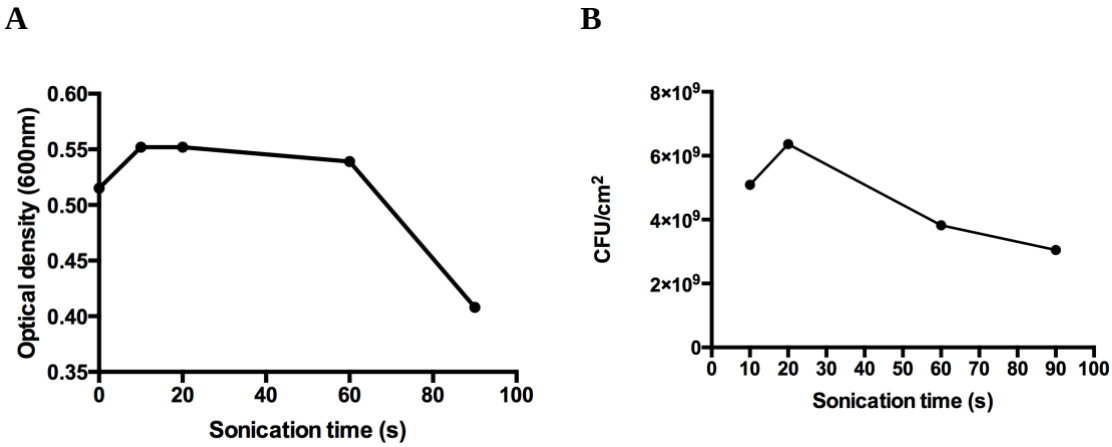


Figure 3.8 Sonication of *P. aeruginosa* established biofilms. A 24 h *P. aeruginosa* biofilm was first formed on the MRD. The biofilm cells were sonicated over the time course. The OD_{600} **(A)** and the viable cells **(B)** were measured during sonication.

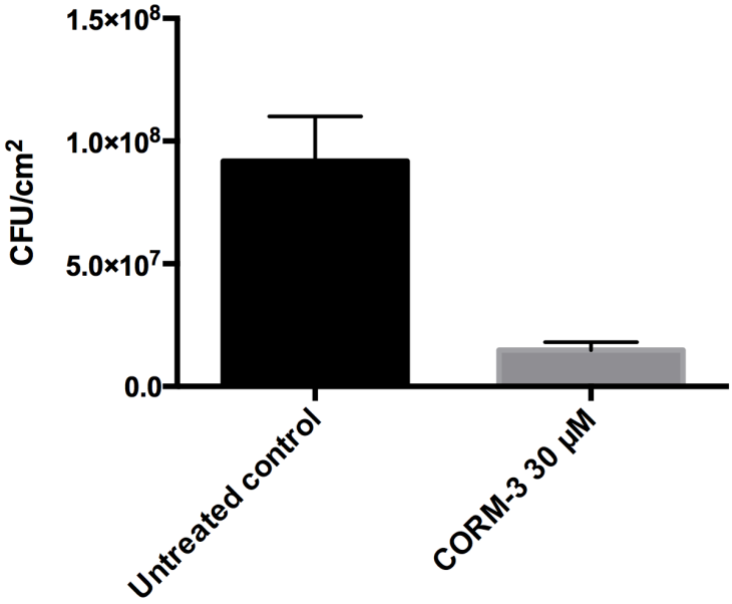


Figure 3.9 The effect of CORM-3 on *P. aeruginosa* established biofilms. A 24 h *P. aeruginosa* biofilm was first formed on the MRD. CORM-3 (30 μM) was then pumped through the MRD for 2.5 h. After 20 s sonication, the number of viable sessile bacteria in the biofilm was counted as the colony-forming units per cm^2. The bars represent the average of the number of colonies from four different plugs at each time point. Error bars show ± S.D.

3.2.3 The effect of CORM-2 on *P. aeruginosa* cells in *vitro*

3.2.3.1 The MIC and MBC of colistin for the *P. aeruginosa* planktonic cells

Colistin is currently widely used as an antibiotic to treat *P. aeruginosa* infections. The toxicity of colistin towards *P. aeruginosa* PAO1 was investigated (see section 2.2.6). This strain showed susceptibility to colistin with an MIC of 4 μg ml^{-1}. Moreover, colistin acted as a bactericidal agent when it reached 8 μg ml^{-1}.

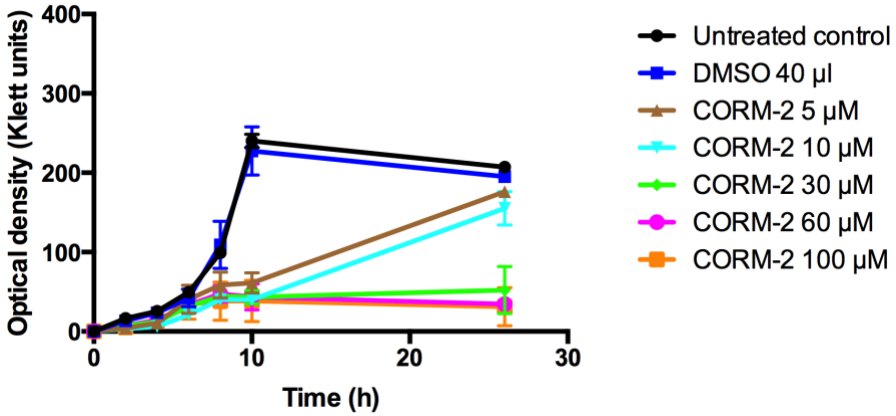
3.2.3.2 The effect of CORM-2 on *P. aeruginosa* planktonic cell growth

To determine the susceptibility of *P. aeruginosa* to CORM-2 and colistin, cells cultured in LB overnight were used to inoculate 20 ml M9 medium in 250 ml side-arm flasks for measurements of optical density with a Klett meter during shaking at 200 rpm and 37 °C. CORM-2 (5 - 100 μM) and colistin (1 - 4 μg ml^{-1}) were added directly to growing cultures in log phase, determined by a Klett OD of ~50. The cell growth was recorded by measuring Klett OD every 2 h for up to 24 h. It is clear that CORM-2 and colistin inhibited bacterial growth in a concentration-dependent manner (Figure 3.10). Addition of 5 μM CORM-2 slowed growth but higher concentrations (30 μM) were required to inhibit growth completely. Colistin (1 μg ml^{-1}) had a slight deleterious effect on growth, with the negative impact on growth increasing as the colistin concentration was increased.

3.2.3.3 The effect of combined CORM-2 and colistin treatment on *P. aeruginosa* planktonic cell growth

To determine if CORM-2 would work as an adjuvant to the currently available antibiotic colistin, the effect of a combination of both CORM-2 and colistin on the growth of the *P. aeruginosa* planktonic cells *in vitro* was examined. The experiment was carried out on 20 ml cultures grown in M9 medium. Cultures were grown aerobically at 37 °C until 50 Klett units were reached and then were stressed by the addition of 2 μg ml^{-1} colistin combined with a sub-lethal dose of CORM-2 (Figure 3.11). The combination of CORM-2 and colistin significantly inhibited cell growth during log phase, however, the cell recovery was observed after 24 h.

A



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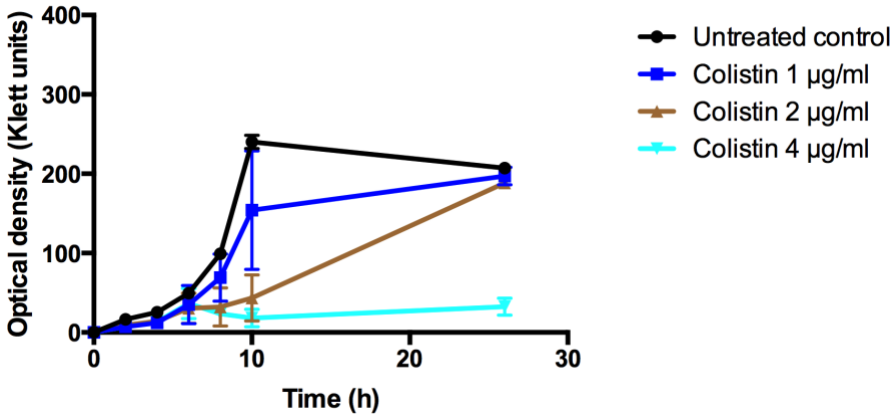


Figure 3.10 The effect of CORM-2 or colistin on *P. aeruginosa* planktonic cell growth. The growth of *P. aeruginosa* was followed until a Klett OD of ~40 was reached. CORM-2 (5 - 100 µM) **(A)** and colistin (1 - 4 µg/ml) **(B)** were then added to the cultures and readings taken at regular intervals. The control was adding DMSO (40 µl) alone to the cultures. Values are an average of two biological samples with the respective SE.

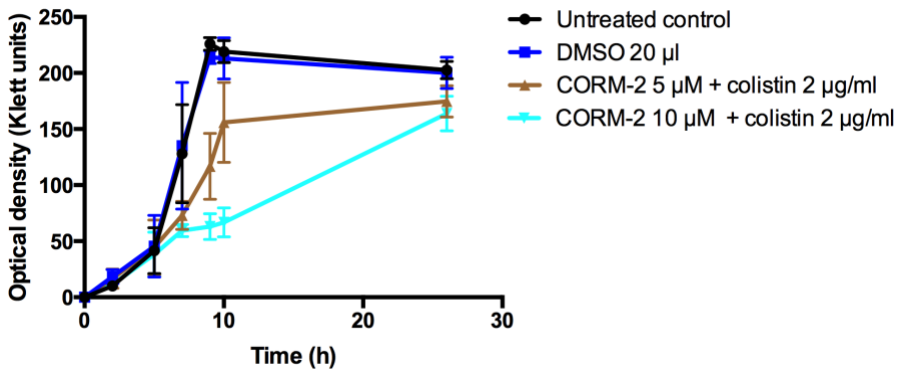


Figure 3.11 The effect of combined colistin and CORM-2 treatment on *P. aeruginosa* planktonic cell growth. The growth of *P. aeruginosa* was followed until a Klett OD of ~40 was reached. The combination of CORM-2 (5 - 10 µM) and colistin (2 µg/ml) was then added to the cultures and readings taken at regular intervals. The control was adding DMSO (40 µl) alone to the cultures. Values are an average of four biological samples with the respective SE.

3.2.3.4 The effect of combined CORM-2 and colistin treatment on *P. aeruginosa* established biofilms

Following the demonstration that CORM-2 or colistin alone inhibits *P. aeruginosa* planktonic cells, it was hypothesized that the combination of two antimicrobial agents may also inhibit the growth of *P. aeruginosa* biofilms. To investigate this, a 24 h mature biofilm was generated on the MRD removable plugs. Plugs containing biofilms from the MRD were moved to a petri dish containing CORM-2 (30 μM) or colistin (4 μg/ml) alone or a combination of two antimicrobial agents. The petri dishes were then incubated in 37 °C non-shaking incubator for 2.5 h. The plugs were removed from petri dishes to 10 ml PBS to evaluate the number of viable sessile bacteria in the biofilm by measuring the colony-forming units per cm^2 (Figure 3.12). The effects of CORM-2 (30 μM) and colistin (4 μg/ml) on *P. aeruginosa* established biofilm cells were not significant ($p>0.05$). Meanwhile, the combination of effects from CORM-2 and colistin was also indifferent against *P. aeruginosa* established biofilm cells. This data clearly demonstrate that CORM-2 does also not enhance the activity of colistin against *P. aeruginosa* biofilm cells.

3.2.3.5 The Interaction between CORM-2 and colistin

To investigate the interaction between CORM-2 and colistin, the FIC was evaluated by a checkerboard method (see section 2.2.6). It is clear that the MIC of CORM-2 alone is 3.84 μg/ml, while MIC of colistin alone is 4 μg/ml. The MIC of CORM-2 in combination was reduced to the 1.92 μg/ml, while the MIC of colistin in combination was reduced to 2 μg/ml (Table 3.1). According to the equations in section 2.2.6, FIC for CORM-2 is calculated as 0.5, FIC for colistin is calculated as 0.5, so the summation of FIC index is calculated as 1, which implies the interaction between CORM-2 and colistin is indifferent.

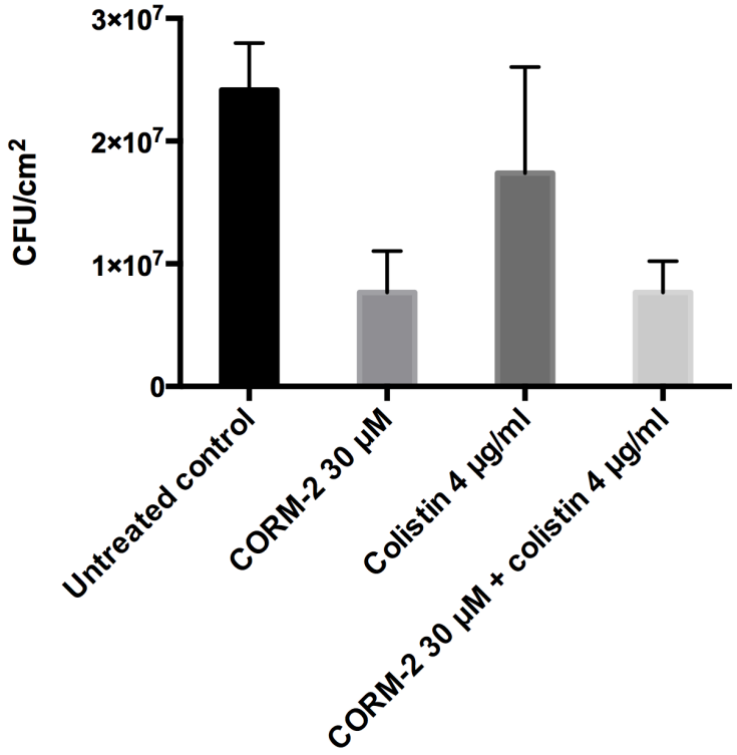


Figure 3.12 The effect of combined colistin and CORM-2 treatment on *P. aeruginosa* established biofilms. A 24 h *P. aeruginosa* biofilm was generated on the MRD. CORM-2 (30 µM), colistin (4 µg/ml) or the combination of two antimicrobial agents was applied directly to the biofilm cells in separate petri dishes for 2.5 h. The number of viable sessile bacteria in the biofilm was evaluated by measuring the colony-forming units per cm^2. Each value shows an average of three biological samples with the respective SE.

Table 3.1 MICs of CORM-2 and colistin to the *P. aeruginosa* PAO1.

Strain	MIC (μg/ml)			
	CORM-2	colistin	CORM-2 in combination	colistin in combination
P. aeruginosa PAO1	3.84	4	1.92	2

$$FIC(CORM-2) = \frac{MIC(CORM-2\ in\ combination)}{MIC(CORM-2\ alone)} = \frac{1.92}{3.84} = 0.5$$

$$FIC(colistin) = \frac{MIC(colistin\ in\ combination)}{MIC(colistin\ alone)} = \frac{2}{4} = 0.5$$

$$\sum FIC = FIC(CORM-2) + FIC(colistin) = 0.5 + 0.5 = 1$$

$$0.5 < \sum FIC < 4\ is\ indifferent$$

3.2.4 The potential protective mechanisms in *P. aeruginosa* biofilms against CORM-3

3.2.4.1 Anaerobic microenvironment

In the biofilm, oxygen concentration is reduced at the base. The presence or absence of oxygen is known to modulate action of the antibiotic aminoglycosides (Tack and Sabath, 1985). Bacteria in an anaerobic region of a biofilm may be differentially protected from these antibiotics, even if they are capable of fermentative growth. To understand how *P. aeruginosa* cells behave under anaerobic conditions, cultures were grown in anaerobic M9 medium. 8 ml cell culture were grown in Klett-compatible 8.5 ml screw-cap tubes at 200 rpm 37 ℃ incubator. CORM-3 was added directly to the growing cultures when a Klett OD of ~40 was reached. The Klett OD was recorded using a Klett meter every 2 h (Figure 3.13). Lower concentrations of CORM-3 (10 - 100 μM) did not stop *P. aeruginosa* planktonic cell growth under anaerobic condition. To completely prevent the growth of *P. aeruginosa* anaerobic culture a higher concentration (200 μM) was required.

3.2.4.2 Starvation-induced tolerance

In the laboratory, bacteria become highly resistant to antibiotics when nutrients are limited in the media (Nguyen *et al.*, 2011). Nguyen and co-workers have found the starvation-induced stringent response is a determinant of biofilm-specific antimicrobial resistance in *P. aeruginosa*, because inactivation of this protective mechanism greatly sensitizes *P. aeruginosa* biofilms to various classes of antibiotics and markedly enhances the efficacy of antibiotic treatment in experimental infections of *P. aeruginosa*.

To estimate if the starvation in the biofilm would affect CORM-3 uptake, a starter culture was grown overnight in 20 ml LB medium and cells pelleted by centrifugation; the pellet was then resuspended in 20 ml aerobic M9 medium with 20% glucose to replace LB. This was used to inoculate a flask containing 180 ml

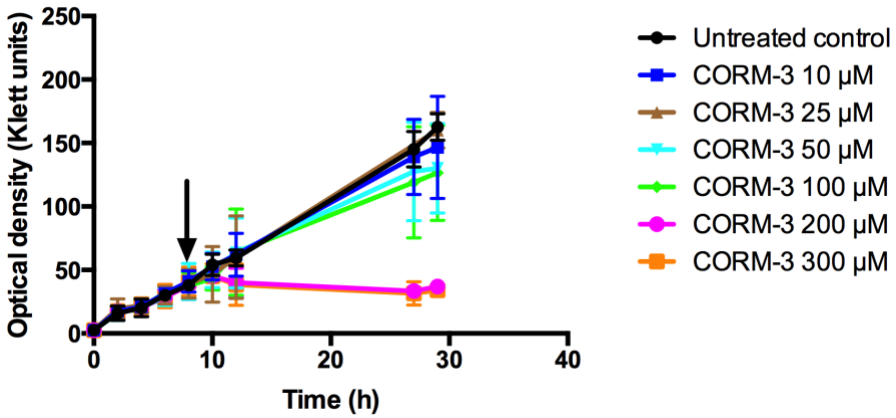


Figure 3.13 The effect of CORM-3 on anaerobic *P. aeruginosa* planktonic cells growth. *P. aeruginosa* anaerobic planktonic cell culture was grown in anaerobic M9 medium in a screw-cap tube. CORM-3 was added to growing culture when a Klett OD of ~40 was reached. Data shows the mean and standard deviation from 2 biological replicates.

fresh M9 medium with 20% glucose. The cells were grown in 37 °C shaking incubator until an OD_{600} of ~0.5 was reached, then stressed with 30 μM CORM-3 for 2 h. The optical densities at 600 nm, cell viability and uptake of ruthenium by *P. aeruginosa* cells were measured over a time-course with samples taken at t=0, 10, 30, 60, 120 minutes after addition of CORM-3 (30μM) (Figure 3.14A, B, C). The cells were actively growing with glucose supplement. Cells were completely killed by CORM-3 (30 μM) after 1 h. Ruthenium was continuously accumulated by cells during 2 h treatment.

To compare with actively growing cells as described above, a starter culture was grown overnight in 20 ml LB medium; it was then centrifuged and replaced with 20 ml M9 medium with 20% glucose before inoculation to a flask containing 180 ml fresh M9 medium with glucose. The cells were grown in 37 °C shaking incubator until an OD_{600} of ~0.5 was reached, then centrifuged and replaced with 200 ml M9 medium in the absence of glucose for 2.5 h. The starved culture was then centrifuged again and replaced with 200 ml PBS containing 30 μM CORM-3 to provide no nutrient to the cells for 2 h. The optical densities at 600 nm, cell viability and uptake of ruthenium by *P. aeruginosa* cells were measured over a time-course with samples taken at t=0, 10, 30, 60, 120 min after addition of CORM-3 (30μM) (Figure 3.14D, E). Cells were energy-starved after 2.5 h treatment without glucose. However, a higher concentration of ruthenium was detected in cells. This may be because starved cells cannot export CORM-3 after accumulating CORM-3 inside. Meanwhile, only a small amount of viable cells ($<10^8$ CFU ml^{-1}) was detected in a viability assay (data not shown).

3.2.4.3 The penetration of CORM-3 through *P. aeruginosa* exopolysaccharide

The biofilm matrix can act as a barrier to delay the diffusion of antibiotics into biofilms because antibiotics may either react chemically with biofilm matrix components or attach to anionic polysaccharides (Stewart, 1996). A major component of exopolysaccharide matrix produced by *P. aeruginosa* is alginate (Olvera *et al.*, 1999). In order to examine the role of exopolysaccharide in CORM-3 penetration, the sodium alginate was used to model the *Pseudomonas* exopolysaccharide in this study. Two different volumes of alginate gel (240 μl and 600 μl) layer were placed on a membrane filter in a culture plate

insert (see Figure 2.5). A volume of 50 µl of CORM-3 (30 µM) was added to the gel and incubation was done at room temperature for 24 h, after which ruthenium concentration in the water was measured (Figure 3.15). A significant reduction in ruthenium concentration was detected with increasing alginate thickness. It is clear alginate gel acted as a protection layer from CORM-3 penetration. This is reflected by the data shown in section 3.2.2, in which *P. aeruginosa* biofilm cells are less sensitive to CORM-3 compared to planktonic cells.

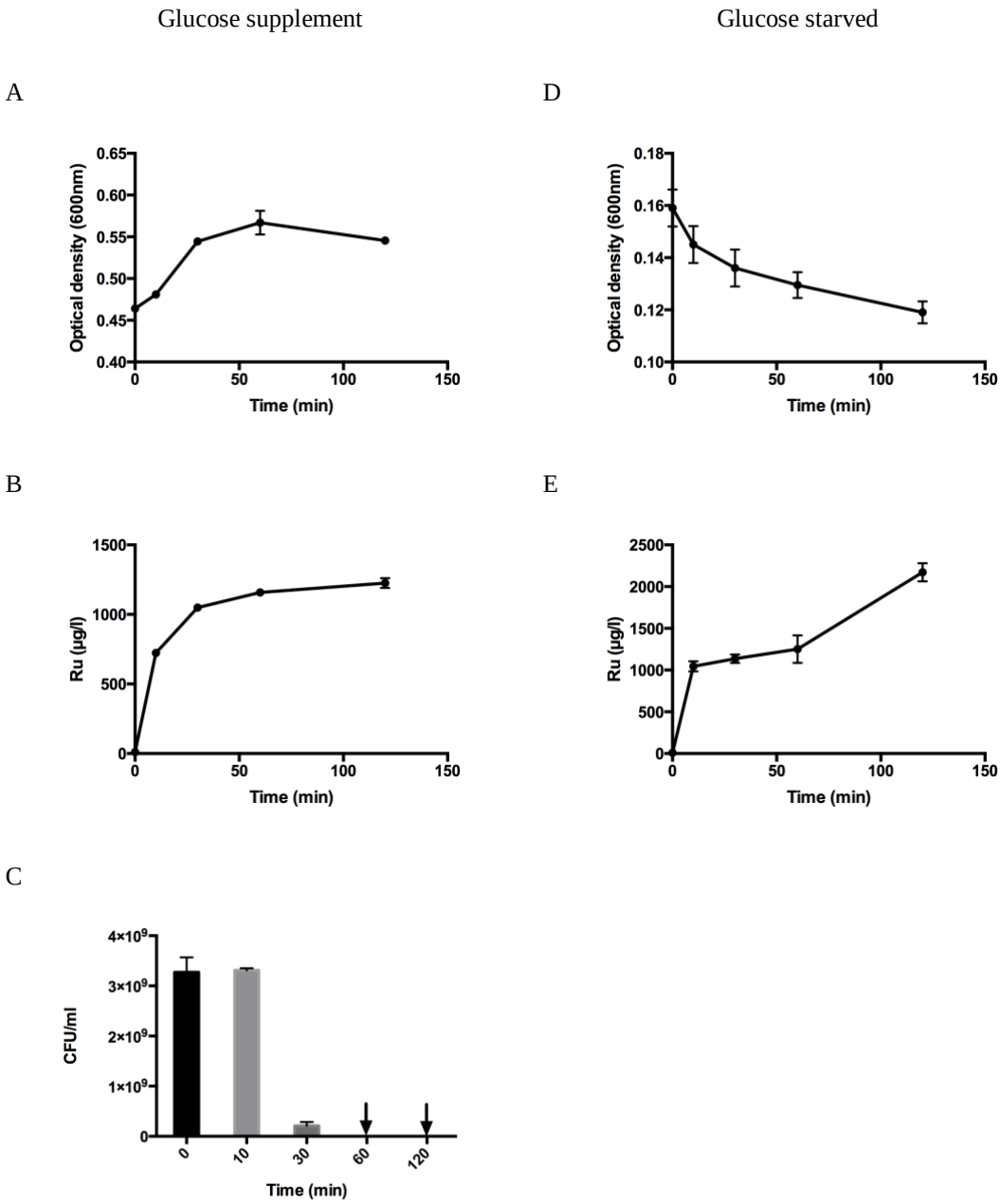


Figure 3.14 The *P. aeruginosa* planktonic cell growth under glucose starvation. Cell growth with glucose supplement was recorded by measuring OD_{600} **(A)**, Ruthenium uptake **(B)** and cell viability **(C)**. Cell growth under glucose starvation was recorded by measuring OD_{600} **(D)** and Ruthenium uptake **(E)**. Data shows the mean and standard deviation from 2 biological replicates.

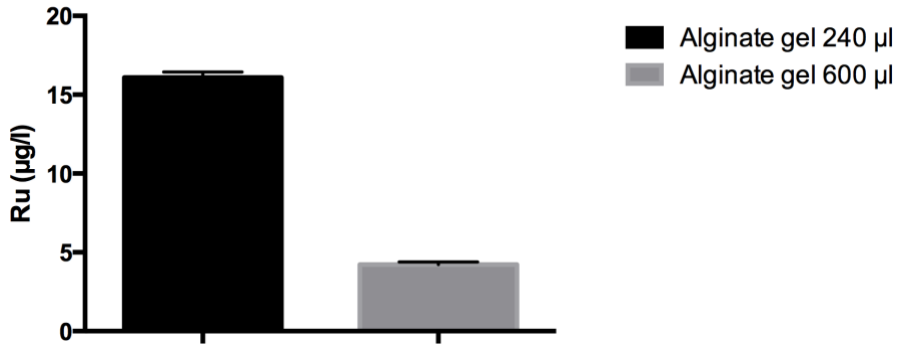


Figure 3.15 Penetration of CORM-3 through two different volumes of alginate gel. CORM-3 (30 µM) was penetrated through two different volumes of alginate gel. Samples were taken after 24 h to measure the ruthenium concentration. Data shows the mean and standard deviation from 2 biological replicates of which there were 4 technical repeats.

3.3 Discussion

The bactericidal effect of ruthenium-based CORMs on numerous bacteria had been reported when the work described in this thesis was undertaken, including *E. coli* (Davidge *et al.*, 2009b, McLean *et al.*, 2013, Nobre *et al.*, 2007, Wilson *et al.*, 2013), *C. jejuni* (Smith *et al.*, 2011), *S. enterica serovar* Typhimurium (Rana *et al.*, 2014) and *P. aeruginosa* (Desmard *et al.*, 2009, Desmard *et al.*, 2012). Although Desmard's group have found CORM-3 to be bactericidal against a laboratory strain of *P. aeruginosa* planktonic cells, *P. aeruginosa* is extremely hard to treat due to this organism's high ability to produce biofilm. Therefore, *P. aeruginosa* biofilm mode of growth is the main focus in current presented work. Murray and his co-workers published the first report on the effect of CORM-2 on *P. aeruginosa* biofilms. In that study, CORM-2 not only killed planktonic cells but also attenuated *P. aeruginosa* biofilm formation and established biofilms. However, the details of the effect of CORM-3 on *P. aeruginosa* and how CORM-3 works on *P. aeruginosa* biofilm remained elusive. Thus, the work presented in this chapter firstly investigated the effect of CORM-3 on *P. aeruginosa* planktonic cells with the aim of learning preliminary information about the laboratory strain used in this study. The possibility of using CORM work as an adjuvant is also discussed in this chapter.

3.3.1 The effect of CORM-3 on *P. aeruginosa* cells *in vitro*

The work presented in this chapter first shows that CORM-3 has an inhibition effect on *P. aeruginosa* planktonic cell growth in a concentration-depended manner (Figure 3.3A). CORM-3 is bactericidal when given to planktonic cells at a concentration of 30 μM (Figure 3.4). The finding that the control solution (CO-saturated solution) does not significantly inhibit growth of *P. aeruginosa* planktonic cells (Figure 3.3B) suggests that the CORM-3 molecule is the agent causing the inhibition, rather than CO alone. This finding was in agreement with Desmard's group (Desmard *et al.*, 2009). As shown in this study and by others, the effect of CORM compounds is not mimicked by equimolar concentrations of CO; CO gas is surprisingly ineffective in inhibiting microbial growth

(Wegiel *et al.*, 2014) and has been shown to be much less effective than CORMs in inhibiting bacterial growth (Nobre *et al.*, 2007).

Furthermore, we have shown that CORM-3 prevents *P. aeruginosa* biofilm maturation and kills cells within the established *P. aeruginosa* biofilm (Figure 3.6 and Figure 3.9). CORM-3 did not completely kill all *P. aeruginosa* biofilm cells at a concentration of 30 μM after 2.5 h treatment. However, in *P. aeruginosa* planktonic cell studies, CORM-3 completely killed *P. aeruginosa* planktonic cells at 30 μM. This indicates biofilm formation does protect *P. aeruginosa* cells from the bactericidal effect of CORM-3.

3.3.2 The effect of CORM-2 on *P. aeruginosa* cells in *vitro*

CORM-2 was examined in these initial experiments to provide more information about the effect of ruthenium-based CORMs on *P. aeruginosa* cells. Murray and co-workers showed that CORM-2 killed *P. aeruginosa* planktonic cells as early as 30 min, with a minimal growth inhibitory concentration of 10 μM (Murray *et al.*, 2012).

In agreement with Murray's report, the planktonic cells studies presented in this chapter shows both CORM-2 and colistin have an antimicrobial effect on *P. aeruginosa* planktonic cells (Figure 3.10). In comparison to CORM-3 studies, CORM-2 was more inhibit on *P. aeruginosa* planktonic cell growth than the same concentration of CORM-3. This finding was also supported by Nobre and co-workers, who found that higher concentrations of CORM-3 than CORM-2 were required to reduce the cell viability in *E. coli* and *S. aureus* cultures (Nobre *et al.*, 2007).

The combination of CORM-2 and colistin study shows CORM-2 does not enhance the activity of colistin against *P. aeruginosa* planktonic cells (Figure 3.11). It is clear that CORM-2 combined with colistin had inhibitory effects on bacterial growth. However, compared with the inhibitory effect of CORM-2 or colistin on *P. aeruginosa* planktonic cells (Figure 3.10), the combination effect of CORM-2 and colistin was not synergistic

against *P. aeruginosa* planktonic cells. Therefore, the addition of CORM-2 did not enhance the activity of colistin. This finding is further confirmed by the FIC result (Table 3.1), which shows the interaction between CORM-2 and colistin is indifferent. Meanwhile, the data presented in Figure 3.12 clearly demonstrate that CORM-2 does also not enhance the activity of colistin against *P. aeruginosa* biofilm cells.

3.3.3 Biofilm growth methods

Two different methods were developed to examine *P. aeruginosa* biofilm growth in this chapter. One is the microtitre plate method, which is a quick and efficient way to evaluate the biofilm growth. The benefit of the microtitre plate assay is that it is possible to grow planktonic and biofilm populations simultaneously (in each well) under different conditions (in each plate) and semi-quantitatively measures their relative growth. However, this method has been criticized because of the inability of crystal violet to differentiate between living and dead cells, and between cells and matrix material (Peeters *et al.*, 2008). Another concern about this method is that there is no standard protocol for this method, and different groups use different concentrations of inoculum, biofilm incubation times, dye concentrations and staining times. The other method used in this chapter is the Modified Robbins Device method, which is a relatively simple way to generate the biofilm, but requires a specialised apparatus. The benefit of this device is that biofilm cells can easily be removed from the device for quantitation or examination after the biofilm formation, and this device is able to study both young and mature biofilm. However, the major issue with this device was that the bactericidal efficiency varied on different plugs.

It is clear that the *P. aeruginosa* biofilms were successfully formed on the surface of the MRD. However, in the Nickel (1985) experiment, the mature biofilms on the catheter material reached around 2.7 x 10^9 CFU/cm^2 after colonization is completed at 12 h. Compared to the result shown in Figure 3.7, Nickel's group has formed about 10-fold more biofilm cells. Therefore, the future improvement for generating more *P. aeruginosa* biofilm on the MRD can be investigated.

3.3.4 The potential protective mechanisms in *P. aeruginosa* biofilms against CORM-3

It is clear that *P. aeruginosa* biofilm cells are more resistant to CORM-3 compared to planktonic cells (See section 3.2.2). In order to understand the mechanisms behind this activity, three hypotheses were assessed.

Firstly, the aerobic microenvironment inside the biofilm was initially considered to be one of the major causes of *P. aeruginosa* biofilm tolerance to CORM-3. Previous data have demonstrated that CORM-3 completely inhibits the growth of *P. aeruginosa* aerobic cultures at a concentration of 100 μM (Figure 3.3 and Figure 3.5). However, to prevent the growth of *P. aeruginosa* anaerobic culture, a higher concentration (200 μM) was needed (Figure 3.13). Therefore, *P. aeruginosa* anaerobic cultures are more resistant to CORM-3 compare to aerobic cultures. It implies the anaerobic microenvironment in the *P. aeruginosa* biofilm could contribute to protect the biofilm cells from CORM-3 bactericidal effect. Hassett's group has found under strict anaerobic conditions, *P. aeruginosa* increased producing alginate, which formed a barrier for it to resistant to antimicrobial agents (Hassett, 1996). Meanwhile, Davidge and co-workers have also reported that anaerobic *E. coli* cultures have been shown to be susceptible to killing by high concentrations of CORM-3 (Davidge *et al.*, 2009b).

Another hypothesis for the resistance seen with CORM-3 on *P. aeruginosa* biofilms is that the starvation in the biofilm could induce the tolerance towards the antimicrobial effect of CORM-3. However, the data presented in Figure 3.14 disproved the original hypothesis. CORM-3 is accumulated to a greater extent in energy-starved cells than actively growing cells. This indicates that starvation stress in the *P. aeruginosa* biofilm does not contribute to CORM-3 tolerance. This may due to the limitation of nutrient, cell membrane are more vulnerable for CORM-3 to permeate into the cells.

The last hypothesis assessed in this chapter is if the *P. aeruginosa* biofilm extracellular matrix could act as a barrier to protect *P. aeruginosa* cells from CORM-3. In order to

understand whether this was the case, the infusion of CORM-3 through *Pseudomonas* exopolysaccharide alginate gel was examine. Less CORM-3 was detected with increasing the volume of alginate gel (Figure 3.15). This finding indicates alginate is able to delay the infusion of CORM-3. Therefore, *P. aeruginosa* biofilm extracellular matrix may contribute to the resistance against CORM-3.

3.3.5 Conclusions

CORM-3 rapidly kills planktonic cells of a *P. aeruginosa* laboratory strain (PAO1). Although CORM-3 is effective at inhibiting surface-associated growth of *P. aeruginosa* by both preventing biofilm maturation and killing bacteria within the established biofilm, cells growing in a biofilm are more resistant to CORM-3 than planktonic cells. Poor penetration and anaerobic microenvironment may contribute to this resistance, but not starvation. Similarly, bactericidal activity of CORM-2 is confirmed in *P. aeruginosa* planktonic cells. Although CORM-2 or colistin kills bacteria within the established biofilm, the combination effect of those two antimicrobial compounds is found to be indifferent against *P. aeruginosa* planktonic cells and biofilm cells.

Chapter 4

Applying CORM-3 and colistin to *P. aeruginosa* cells by an aerosol method

4.1 Introduction

4.1.1 Chemostat

Chapter 3 revealed that *P. aeruginosa* biofilms could be successfully formed on the surface of the MRD. However, in order to conduct nebuliser studies of *P. aeruginosa* biofilms, it is important to be able to harvest a large number of cells. For this reason, this chapter aims to grow cells continuously in a chemostat system rather than under batch conditions.

A chemostat is a culture in which fresh medium is introduced, while a mixture of spent medium and cells is removed at the same rate. Although the practice of adding fresh medium to a growing bacterial culture had been discussed before 1950, it was the work of Jacques Monod that first revealed that, by using a chemostat culture, it is possible to control the growth rate of a bacterial population. Chemostat theory was developed by the ground-breaking theoretical work of John Pirt in the 1970s (Pirt, 1975). The main advantage to the use of a chemostat is that it is possible to maintain the growth of the population at a set rate, i.e. in a steady state. Steady state is defined as the state of the population at which growth rate is equal to the dilution rate. Thus the population is kept at a constant density by virtue of controlling the concentration of a limiting nutrient. The second advantage to using a chemostat is that it is possible to maintain a long-term culture of cells that are growing in relatively defined and controlled conditions.

Chemostat culture is not without its limitations. Growth rate can only be controlled by dilution rate up to the point at which cells are washed out of the system. Additionally

limiting nutrients will result in a population undergoing starvation responses and reduced growth rates, resulting in gene regulation and increased rates of mutation (Ferenci, 2008).

4.1.2 Inhalation therapy for *P. aeruginosa* infections in the CF lung

It was discussed in Chapter 1 that current treatments for *P. aeruginosa* infections in the CF lung include oral and parenteral antibiotics, and especially the inhaled nebulized antibiotics. The advantages of inhalation therapy for *P. aeruginosa* infections in the CF have been highlighted for over 45 years (Mearns, 1970). This therapy is a highly effective way to treat lung disease especially, since it can maximize the dose at the location of the disease instead of systemic exposure, toxic to the rest of the body. In UK, the majority of patients are treated with twice daily colistin or tobramycin solution for inhalation. The latter drug is administered on a one month on/one month off regimen (Castellani *et al*., 2018).

Colistin is a member of the polymyxin group. It was initially used therapeutically in the 1950s (see (Kumazawa and Yagisawa, 2002) for a survey) but was abandoned due to the reported high incidence of nephrotoxicity in the early 1980s (Brown *et al.*, 1970). However, the emergence of multidrug-resistant Gram-negative bacteria and the shortage of new antimicrobial agents have brought back the interest of using colistin as a valuable therapeutic option. There is increasing research with the use of aerosolized colistin in *P. aeruginosa* infections in cystic fibrosis (Jensen *et al.*, 1987, Hodson *et al.*, 2002, Berlana *et al.*, 2005, Herrmann *et al.*, 2010).

With the emergence of bacteria resistant to most commercially available antibiotics and advances in the discovery of novel antibiotic compounds, novel antimicrobial agents that could replace or complement current therapies are needed to fight chronic infections in CF patients. Recently, there have been numerous studies on the therapeutic effects of CORMs (Motterlini and Otterbein, 2010, Romao *et al.*, 2012). However, these researches have only focused on studies using growing cultures of bacteria in liquid culture. The

effectiveness of CORMs when applied as aerosols suitable for treating infections is still unknown.

4.1.3 The aim of this work

This chapter aims to apply nebulized controlled doses of CORMs to *P. aeruginosa* cells. By monitoring bacterial viability and the uptake of CORMs, we will assess the potential of this nebuliser therapy for treating *P. aeruginosa* related infections with CORMs. The work described in this chapter used CORM-3 as a model CORM as it is chemically well characterised and is water-soluble, a property that is important for the nebulizer treatment. Colistin, which is widely used antibiotic in chronic *P. aeruginosa* infections, was used in order to compare the efficacy of CORM-3 and colistin.

4.2 Results

4.2.1 Development of methods to grow biofilm

4.2.1.1 Cultivation of *P. aeruginosa* planktonic cells in batch culture

To determine a suitable time point for further experiments, a time course was initially conducted in which *P. aeruginosa* PAO1 planktonic cells were grown in M9 medium for 48 h in a chemostat vessel (Figure 4.1). The pH changes were detected by a pH meter. The residual glucose was also measured (see section 2.2.3), and the optical density of the planktonic cells growth was also recorded. It was found that the bacterial cells reached stationary phase after 24 h and the doubling time was 2.1 h. To maintain stable pH in the fermenter vessel, the concentration of phosphates in the medium was increased (see section 2.1.2.2). The pH in the fermenter vessel decreased over time. The glucose was effectively used by *P. aeruginosa* planktonic cells during the bacterial growth, and had run out after 24 h. As cells would eventually be grown in a chemostat at steady state by limiting the carbon source, 30 h was selected for trial in the chemostat to start a continuous growth.

4.2.1.2 Cultivation of *P. aeruginosa* biofilm cells in continuous culture

In order to harvest a maximum yield of *P. aeruginosa* biofilm cells, cultures were grown in a chemostat as described in section 2.1.5.7 and Figure 2.3. An overnight starter culture of *P. aeruginosa* PAO1 was inoculated into 125 ml M9 medium (4 % inoculum). *P. aeruginosa* planktonic cells were grown as a batch culture for 30 h until it was switched to a continuous culture. The cell growth was determined by measuring the optical density every hour after switching to a continuous culture (Figure 4.2A). The pH changes in the chemostat system were detected by a pH meter (Figure 4.2B). The glucose assay was used to measure the glucose changes during bacterial growth (Figure 4.2C). It is clear from Figure 4.2 that the optical density of the *P. aeruginosa* planktonic cells decreased after switching to a continuous culture, but seemed to be stable after 48 h. Meanwhile, the pH stayed in an optimal range. Glucose was sufficient in the chemostat vessel during bacterial growth. These measurements suggest the glucose is

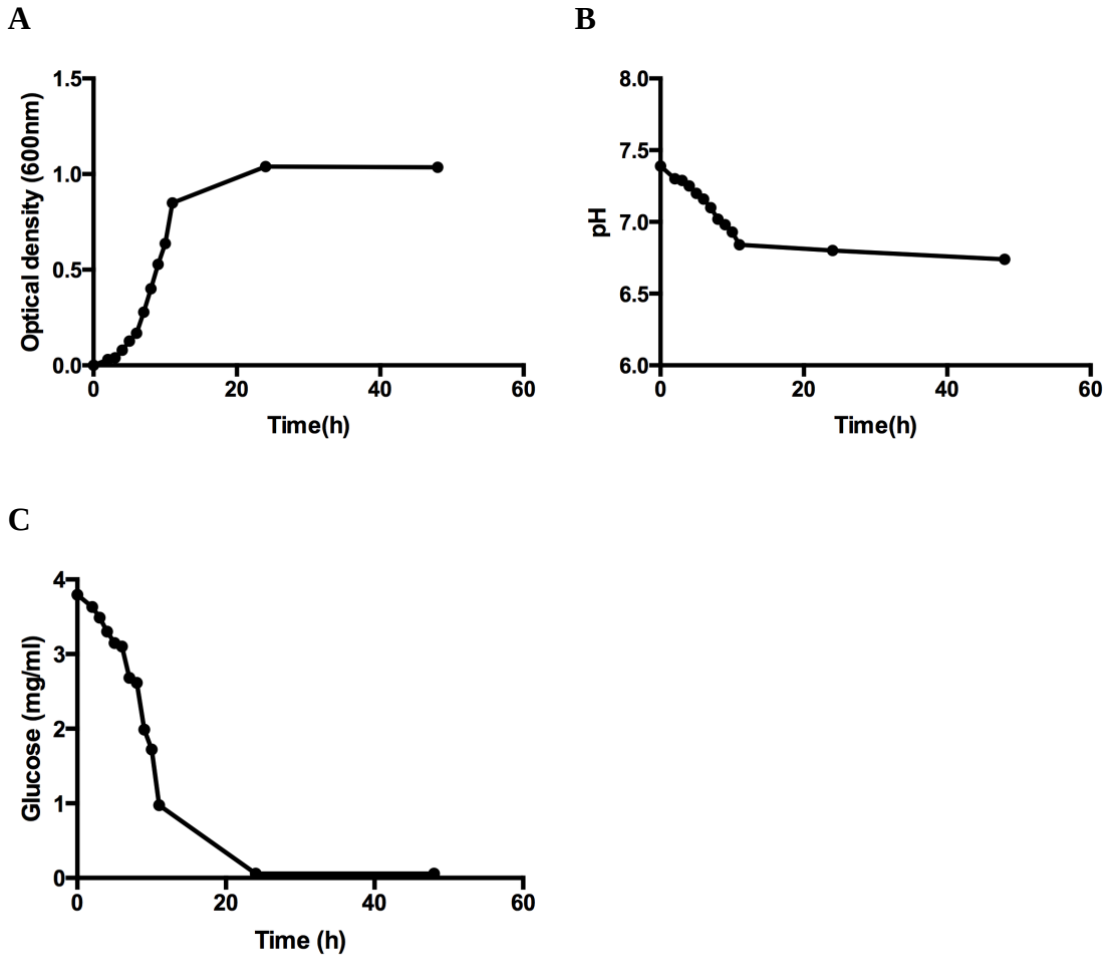


Figure 4.1 Growth of *P. aeruginosa* planktonic cells in the chemostat vessel operated as a batch culture. *P. aeruginosa* was grown in the chemostat vessel containing M9 medium for 48 h. The optical density at 600 nm **(A)**, pH changes **(B)** and glucose changes **(C)** were recorded over time course.

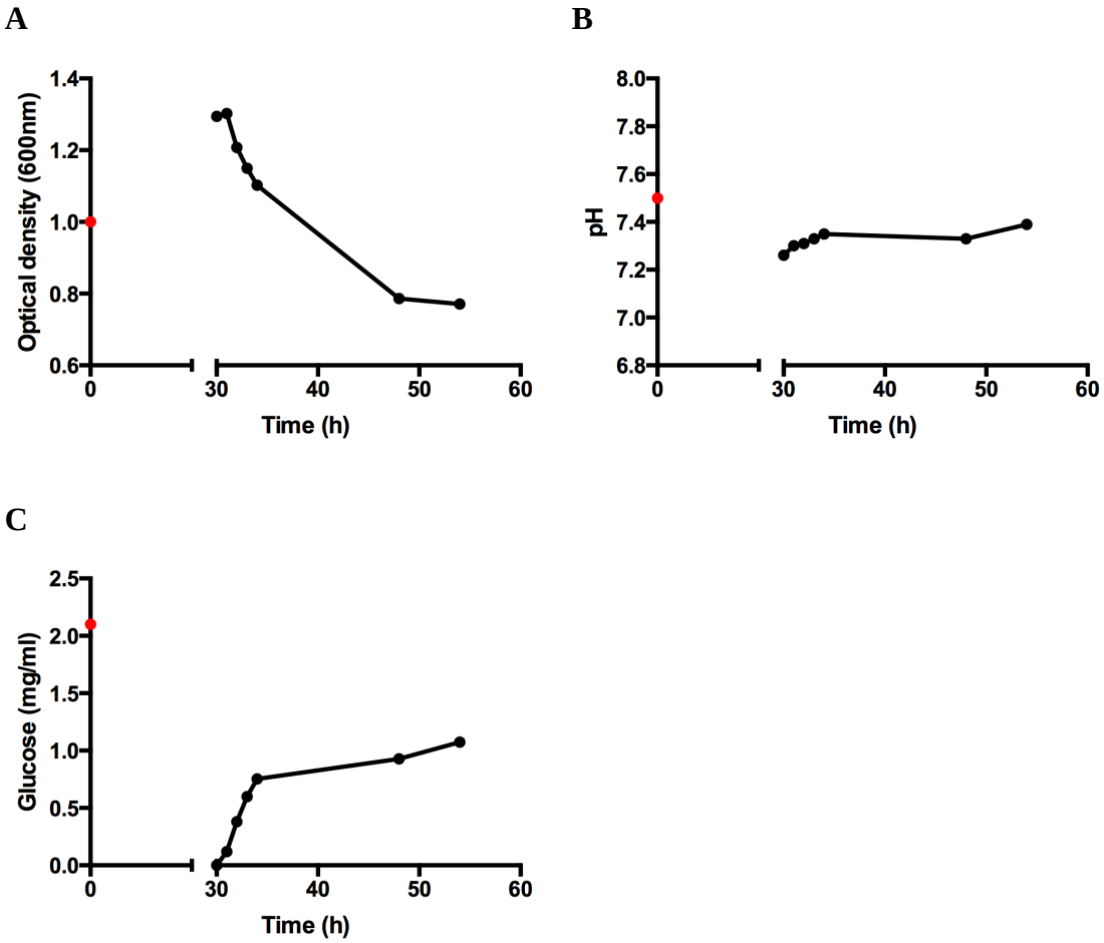


Figure 4.2 Growth of *P. aeruginosa* planktonic cells in the chemostat vessel operated as a continuous culture. *P. aeruginosa* was grown in the chemostat vessel containing M9 medium for 30 h before switching to a continuous culture. The optical density at 600 nm **(A)**, pH changes **(B)** and glucose changes **(C)** were measured at 30 h time point (•). Data were also recorded for a further 24 h.

not the limiting component for growth. However, this is an advantage for the MRD protocol, since the glucose carried over can support an initial phase of growth on the MRD plugs.

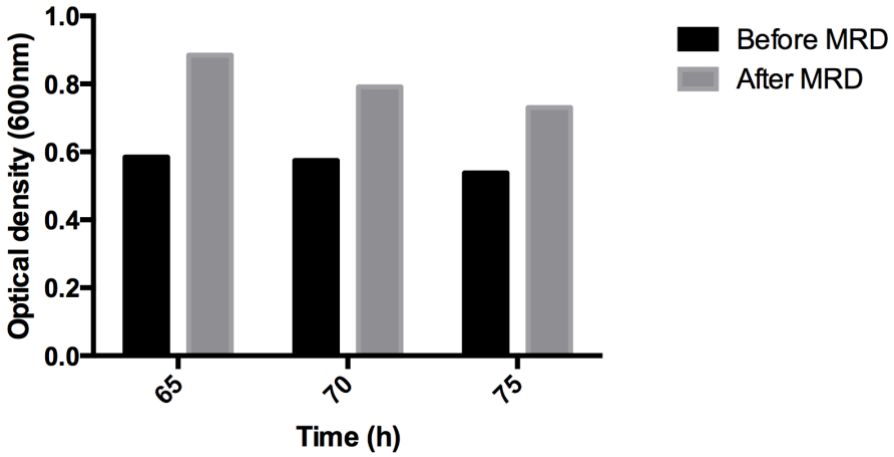
The *P. aeruginosa* biofilm populations were generated on the MRD connected to this continuous culture. The cultures were initially allowed to grow in the chemostat vessel as a batch culture for 30 h with a stir rate of 100. After this time, the stir rate was increased to 260 and the medium flow was started at 30 ml h^{-1}. After the cultures were pumped through the MRD for a further 72 h, aerobically grown biofilm-associated cells were harvested on the MRD and ready for analysis. A 2 ml sample was also taken at the entrance or the end of the MRD to test the OD_{600} and glucose limitation (Figure 4.3). To check for glucose limitation, samples were subjected to a glucose assay. The optical density was increased after cultures flow through the MRD. Meanwhile, the glucose concentration was significantly decreased after cultures flow through the MRD. One additional experiment was conducted by not supplying glucose to fresh medium after switching to continuous culture. The optical densities were the same at the entrance and the end of the MRD over time.

4.2.2 Applying CORM-3 and colistin to *P. aeruginosa* cells by an aerosol method

4.2.2.1 The effect of CORM-3 and colistin administered by the nebuliser on *P. aeruginosa* agar-grown cells

The initial design of the experiment was to convert CORM-3 solution and colistin solution into a mist which can be applied to *P. aeruginosa* cells, as outlined in section 2.1.5.8. The first task was to form a uniform plate contains *P. aeruginosa* cells. The uniform plate was made according to section 2.1.8. Agar plate cell circles were then taken with a 4 mm borer before inserting into the sampling ports. 20 ml of CORM-3 (310 μg/ml) or colistin (500 μg/ml) solution was converted into the mist and sprayed on the agar plate cell circles for 10 min treatment. The viabilities of these cell circles were compared to the circles without the treatment. Treatment with 500 μg/ml colistin reduced the viability of *P. aeruginosa* agar plate cells to 2% of the untreated control

A



B

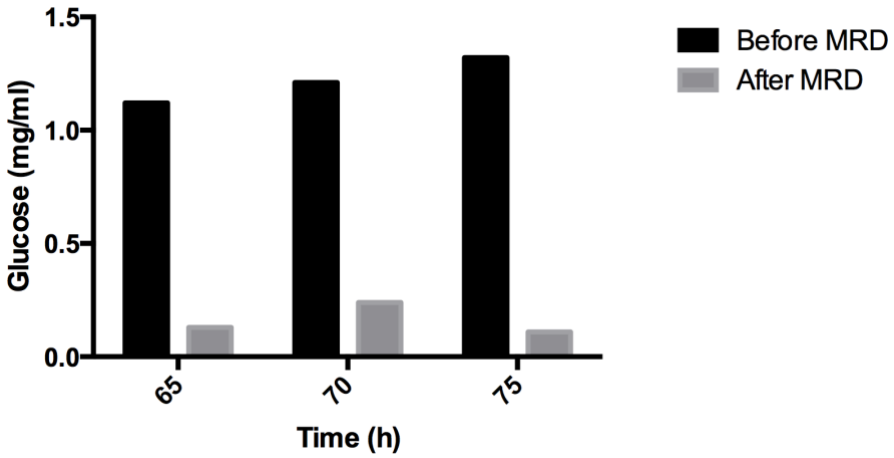


Figure 4.3 Growth of *P. aeruginosa* biofilm cells on the MRD that connected to a continuous culture. *P. aeruginosa* was grown aerobically as batch-culture for 30 h in the chemostat vessel. It was then switched to continuous-culture and pumped through the MRD for biofilm formation. Samples were taken at the entrance and the end of the MRD for measuring the optical density changes **(A)** and the glucose changes **(B)**.

after 10 min (Figure 4.4). Similarly, treatment with 310 μg/ml CORM-3 decreased the viability of *P. aeruginosa* agar plate cells to 42% of the untreated control after 10 min (Figure 4.5).

4.2.2.2 The effect of CORM-3 and colistin administered by the nebuliser on *P. aeruginosa* biofilm cells

In order to compare the effect of aerosolized CORM-3 on *P. aeruginosa* biofilm cells with aerosolized colistin, biofilm cells were formed on the MRD connected to a continuous culture, as described in section 4.2.1.2. After 72 h generation, *P. aeruginosa* biofilm cells were formed on the sampling plugs on the MRD. These removable sampling plugs were easily detached from the MRD and inserted into the pipeline in the aerosol system (Figure 2.5). Solutions (20 ml) of CORM-3 (310 μg/ml) or colistin (500 μg/ml) solution were converted into mist and sprayed on the biofilm cell plugs for 10 min. The viabilities of cells on these plugs were compared to the plugs without the treatment. Treatment with 500 μg/ml colistin reduced the viability of *P. aeruginosa* biofilm cells to 30% of the untreated control after 10 min (Figure 4.6). Similarly, treatment with 310 μg/ml CORM-3 decreased the viability of *P. aeruginosa* biofilm cells to 35% of the untreated control after 10 min (Figure 4.7). The large standard deviation in these data highlights the high variability in biofilm grown on the MRD.

4.2.2.3 Analysis of CORM-3 concentration in *P. aeruginosa* biofilm cells after treatment

In order to demonstrate the uptake of aerosolised CORM-3 by the *P. aeruginosa* biofilm cells, assays for the presence of ruthenium were conducted using ICP-MS (see section 2.3.1). In brief, this technique relies on mass spectrometry to detect levels of trace metals in samples.

P. aeruginosa biofilm cells were formed on the MRD, then stressed with CORM-3 (310 μg/ml) for 10 min in the aerosol system as described in the section above. The treated *P. aeruginosa* biofilm cells were collected from the treated sampling plugs into PBS. After

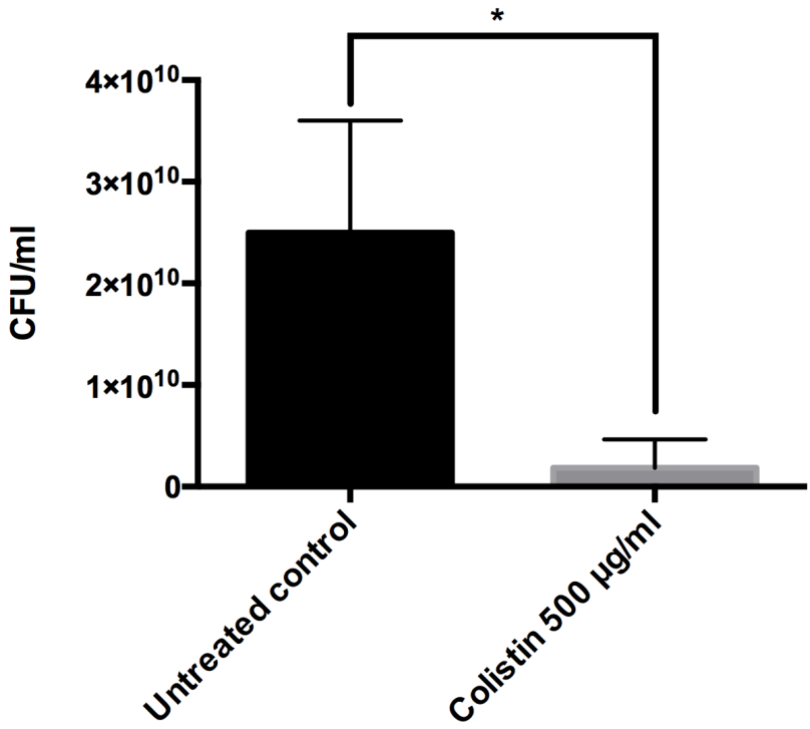


Figure 4.4 Effect of aerosolized colistin on the *P. aeruginosa* agar plate cells. *P. aeruginosa* agar plate cells were formed on a uniform plate. The cell circles were inserted into the aerosol system. Colistin (500 µg ml^{-1}) was sprayed on the cells for 10 min before counting the number of viable cells. The bars represent the cell viability before and after treatment with colistin. Data shows the mean and standard deviation from 5 technical repeats (* means $p<0.05$).

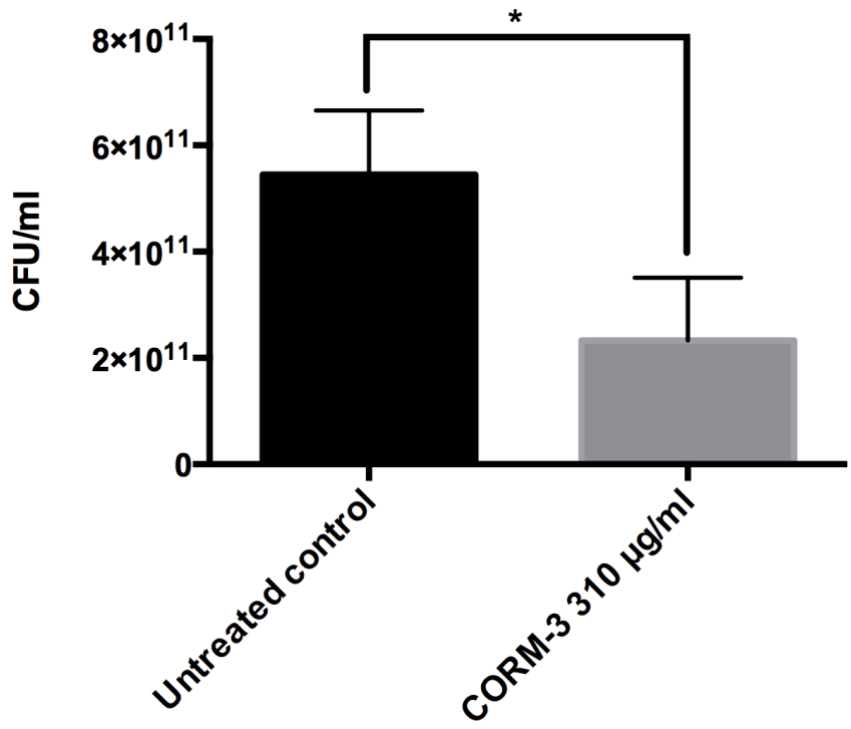


Figure 4.5 Effect of aerosolized CORM-3 on the *P. aeruginosa* agar plate cells. *P. aeruginosa* agar plate cells were formed on a uniform plate. The cell circles were inserted into the aerosol system. CORM-3 (310 µg ml^{-1}) was sprayed on the cells for 10 min before counting the number of viable cells. The bars represent the cell viability before and after treatment with CORM-3. Data shows the mean and standard deviation from 5 technical repeats (* means $p<0.05$).

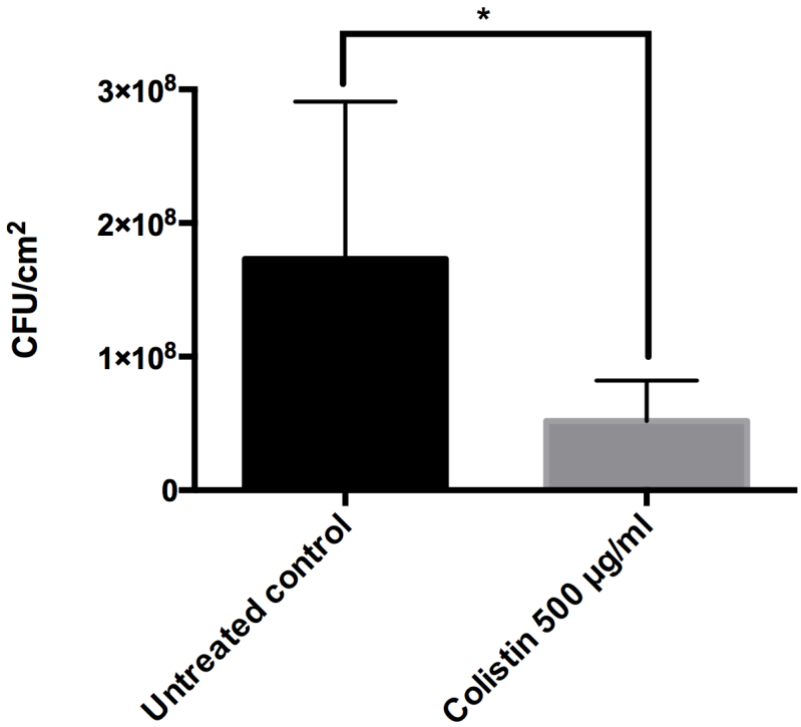


Figure 4.6 Effect of aerosolized colistin on the *P. aeruginosa* biofilm cells. *P. aeruginosa* biofilm cells were generated on the MRD before moving to the aerosol system. Colistin (510 µg ml^{-1}) was sprayed on the cells for 10 min before counting the number of viable cells. The bars represent the cell viability before and after treatment with colistin. Data shows the mean and standard deviation from 5 technical repeats (* means $p<0.05$).

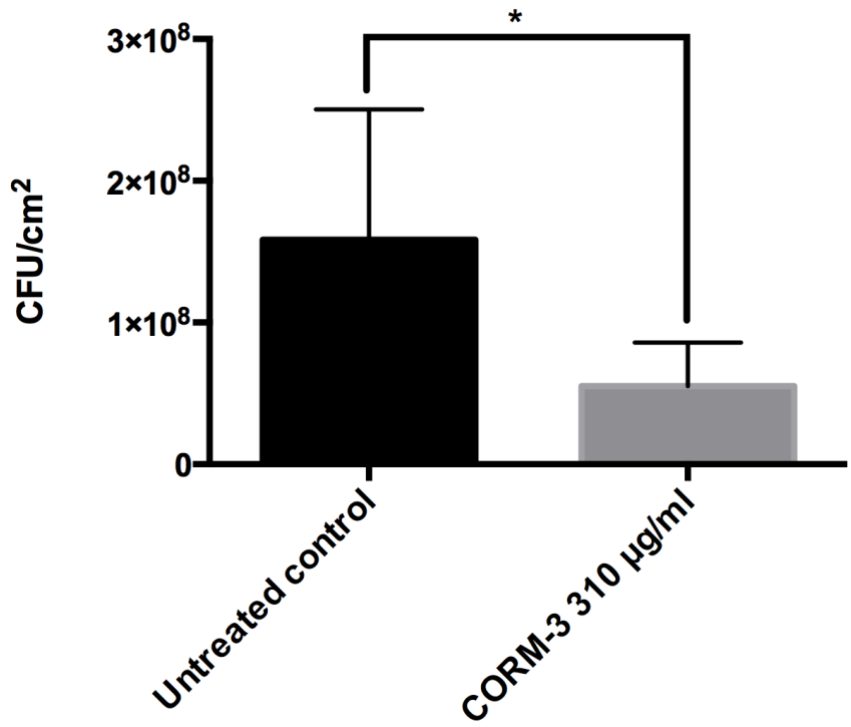


Figure 4.7 Effect of aerosolized CORM-3 on the *P. aeruginosa* biofilm cells. *P. aeruginosa* biofilm cells were generated on the MRD before moving to the aerosol system. CORM-3 (310 µg ml^{-1}) was sprayed on the cells for 10 min before counting the number of viable cells. The bars represent the cell viability before and after treatment with CORM-3. Data shows the means and standard deviations from 5 technical repeats (* means $p<0.05$).

centrifuging the cell mixtures, the cell pellets were washed with dilute HNO3 three times prior to digestion with concentrated acid and subjected to an ICP-MS assay. Raw values for ruthenium levels were obtained, and intracellular concentrations were calculated (Table 4.1). There was a mean of 0.99 μg ruthenium detectable inside the P. aeruginosa biofilm cell pellets from two biological repeats.

4.2.2.4 The interaction between CORM-3 and colistin

To investigate the interaction between CORM-3 and colistin, the FIC was evaluated by a checkerboard method (see section 2.2.6). It is clear the MIC of CORM-3 alone is 4.65 μg/ml, while MIC of colistin alone is 4 μg/ml. The MIC of CORM-3 in combination was reduced to 2.3 μg/ml, while the MIC of colistin in combination was reduced to 2 μg/ml (Table 4.2). According to the equations in section 2.2.6, FIC for CORM-3 is calculated as 0.5, FIC for colistin is calculated as 0.5, so the summation of FIC index is calculated as 1, which implies the interaction between CORM-3 and colistin is indifferent.

Table 4.1 Results of two biological repeats from ICP-MS analysis.

	Measured Ruthenium amount (µg)				
	PBS supernatant	**1st wash**	**2nd wash**	**3rd wash**	**Cell pellet**
Repeat 1	6.13	0.28	0.11	0.044	1.41
Repeat 2	4.32	0.164	0.058	0.0329	0.575

Table 4.2 MICs of CORM-3 and colistin to the *P. aeruginosa* PAO1.

Strain	MIC (μg ml^{-1})			
	CORM-3	Colistin	CORM-3 in combination	Colistin in combination
P. aeruginosa PAO1	4.65	4	2.325	2

4.3 Discussion

4.3.1 Use of custom built chemostat

The presented work revealed the difference between grown *P. aeruginosa* cells in a batch culture and continuously in a chemostat system. In the batch culture, glucose was effectively used by *P. aeruginosa* cells and ran out after 24 h. Since glucose is the sole carbon source in the medium, the yield of *P. aeruginosa* cells could not be maximized. In the chemostat culture, dilution rate, nutrient limitation and pH were all controlled or monitored. However, one major problem that appeared in this experiment was the reduction of the optical density in the initial 24 h flow period. The reasons are still unknown. Factors might include correct flow rate, stable culture temperature and oxygen level etc. Firstly, the correct flow rate was checked through a calculation. The specific growth rate (μ) was measured from Figure 4.1 as 0.3 h^{-1}; to keep a steady growth rate, the dilution rate (D) should be equal to the required growth rate, D is therefore equal to 0.3 h^{-1}. From this, the flow rate (F) can be calculated for a given vessel volume (V, which is 125 ml) and is 37.5 ml h^{-1} ($F = D \times V$). The actual flow rate was used in this experiment is 30 ml h^{-1}, which is optimal. Secondly, the temperature was monitored by a real-time thermometer, which showed to be in 37-38 °C. Oxygen level inside the chemostat vessel was not measured.

The importance of glucose in *P. aeruginosa* biofilm growth was determined in section 4.2.1.2. *P. aeruginosa* biofilm cells used most glucose in the medium during biofilm formation (Figure 4.3). When glucose was not supplied to the medium, *P. aeruginosa* biofilm cells stopped forming.

4.3.2 Applying CORM-3 and colistin to *P. aeruginosa* cells by an aerosol method

With the development of inhaler therapy, which allows medicines to be delivered in the form of a mist through the lung, it is interesting to test if CORM-3 could be successfully applied to *P. aeruginosa* cells by an aerosol method. We have shown that aerosolized CORM-3 had a significant effect on the viability of both *P. aeruginosa* agar plate cells

and biofilm cells (Figure 4.6 and Figure 4.8). In comparison to colistin, a commonly used nebulized antibiotic for CF lung infections, aerosolized colistin also caused a significant decrease on *P. aeruginosa* cell viability.

The ICP-MS results confirmed CORM-3 was taken up by *P. aeruginosa* biofilm cells. Although the mechanism of CORM-3 bactericidal activity against *P. aeruginosa* biofilms is still unknown, but with this finding, *P. aeruginosa* biofilm cells accumulate CORM-3 inside may be a key factor. Recent evidences have shown the antimicrobial effects of CORM-3 is not only because the delivery of CO, but also the toxicity of the ruthenium co-ligand fragment.

4.3.3 Conclusions

It is more favourable to grow *P. aeruginosa* cells continuously in a chemostat system rather than under batch condition. Glucose is essential in the growth of *P. aeruginosa* biofilm. Aerosolized CORM-3 treatment has a similar bactericidal effect to aerosolized colistin. Although CORM-3 or colistin kills *P. aeruginosa* cells, the combination effect of those two antimicrobial compounds is found to be indifferent against *P. aeruginosa* cells.

Chapter 5

Interaction of *P. aeruginosa* with PhotoCORM

5.1 Introduction

5.1.1 The antimicrobial effect of PhotoCORM

Numerous CORMs have been studied against bacteria. In previous chapters, ruthenium-based CORMs were studied with *P. aeruginosa* planktonic and biofilm cells. It was revealed that although CORM-3 inhibits surface-associated growth of *P. aeruginosa* by both preventing biofilm maturation and killing bacteria within the established biofilm, cells growing in a biofilm are more resistant to CORM-3 than planktonic cells. Recent evidence has shown that the ruthenium co-ligand fragment of the CORMs exerts significant biological impacts. Therefore, newer CORMs that lack ruthenium may be desirable for future studies.

One class of particular interest are CORMs that release CO when exposed to a specific wavelength and intensity of light, called PhotoCORMs (photoactivatable carbon monoxide-releasing molecules) (Schatzschneider, 2011). PhotoCORM must be stable in the dark in order for the compound to accumulate in target tissues prior to photoexcitation, after which CO will be released, and ideally will not rebind to a high degree, but rather bind a solvent molecule in order to stabilise the remaining molecule. Recently, a manganese-based PhotoCORM has been described that has therapeutic potential (Ward *et al.*, 2012). Particular advantages of this CORM are that it releases 3 CO molecules from each CORM molecule, is activated by light from a LED (light emitting diode) at 400 nm, and is not toxic to RAW 264.7 murine macrophages before and after irradiation.

The compound used in this chapter is a novel water-soluble PhotoCORM ($[Mn(CO)_3(tpa\text{-}\kappa^3N)]Br$), which is stable in the dark whereas releases CO to myoglobin only on illumination at 365 nm (Nagel *et al.*, 2014). Antimicrobial activity of this compound was

shown against *E. coli* K-12 only after irradiation. Pronounced inhibition of cultures growing in the presence of a non-fermentable carbon source upon activation of the PhotoCORM *in situ*, together with the demonstration of CO binding to *E. coli* terminal oxidases suggested that the mechanism of action of PhotoCORM is related to the inhibition of respiration more than the toxicity exerted by the metal-coordinated backbone (Nagel *et al.*, 2014).

5.1.2 The aim of the work

The advance of PhotoCORM-CN028-31 is using manganese as the CO carrier. In compare to ruthenium, manganese is more biological favourable. Manganese is a naturally occurring element in biology and so it is not toxic at moderate concentrations to bacteria. This chapter aims to investigate the antibacterial activity of this new Mn-based PhotoCORM ($[Mn(CO)_3(tpa\text{-}\kappa^3N)]^+$) against *P. aeruginosa* planktonic and biofilm cells. It was hypothesised that *P. aeruginosa* planktonic cells would be more sensitive to Mn-based CORMs than biofilm cells.

5.2 Results

5.2.1 Effect of PhotoCORM and CO-saturated solution on the growth of *P. aeruginosa* planktonic cell

To investigate the antimicrobial action of PhotoCORM on *P. aeruginosa* PAO1, planktonic cultures of *P. aeruginosa* PAO1 growing in M9 medium were stressed with increasing concentrations of PhotoCORM prior to the exposure to 6 min UV light and incubated for 24 h, as described in section 2.1.5.9. Addition of 100 μM PhotoCORM failed to produce inhibitory effects during the first 10 h of growth. Slight inhibition of the growth was evident in cultures containing 200 μM activated PhotoCORM while higher concentrations (up to 500 μM) caused severe retardation of the growth for ~ 10 h followed by partial recovery (Figure 5.1). On the other hand, cultures treated with 500 μM PhotoCORM but not exposed to UV grew similarly to cultures exposed to UV light in the absence of PhotoCORM. Therefore UV exposure, and presumably CO release, was essential for growth inhibition.

In addition, a CO-saturated solution (see section 2.1.3.2) was used as a control solution to test the effect of CO alone on the growth of *P. aeruginosa* planktonic cells. The cell cultures were treated with PhotoCORM (0, 300 μM) or CO-saturated solution (1 mM CO) followed by 2 min exposure to UV light (365 nm). No inhibition effects were observed in cultures stressed with PhotoCORM (300 μM) or CO-saturated solution without exposure to UV when compared to cultures exposed to UV light in the absence of PhotoCORM (Figure 5.2).

5.2.2 Effect of PhotoCORM and CO-saturated solution on *P. aeruginosa* biofilm formation

Since UV light itself has antimicrobial activity, an experiment was conducted to determine the maximum UV exposure that failed to cause detrimental effects on the biofilm formation of *P. aeruginosa*. Plates containing 200 μl PAO-1 cultures were exposed to UV light for increasing periods of time and incubated statically for 48 h at

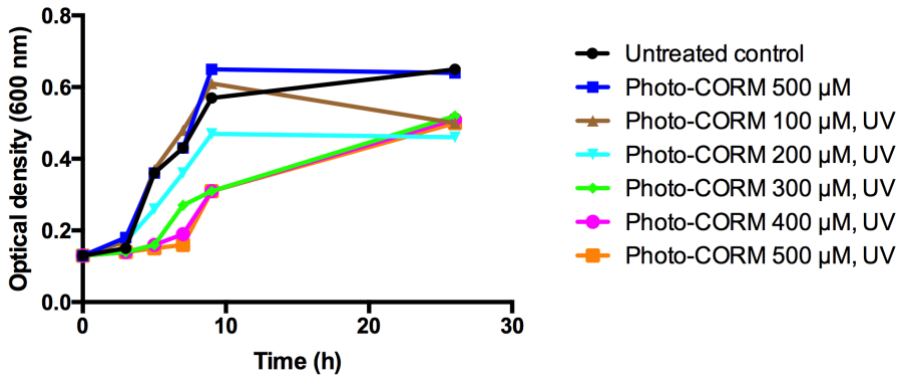


Figure 5.1 Effect of PhotoCORM in the growth of *P. aeruginosa* planktonic cells. Cultures were grown in M9 medium at 37 °C, 200 rpm. A range of PhotoCORM (0–500 µM) was added to cultures (2.5 ml) followed by 6 min exposure to UV light (365 nm). The control was cultures added with 500 µM PhotoCORM and kept in the dark.

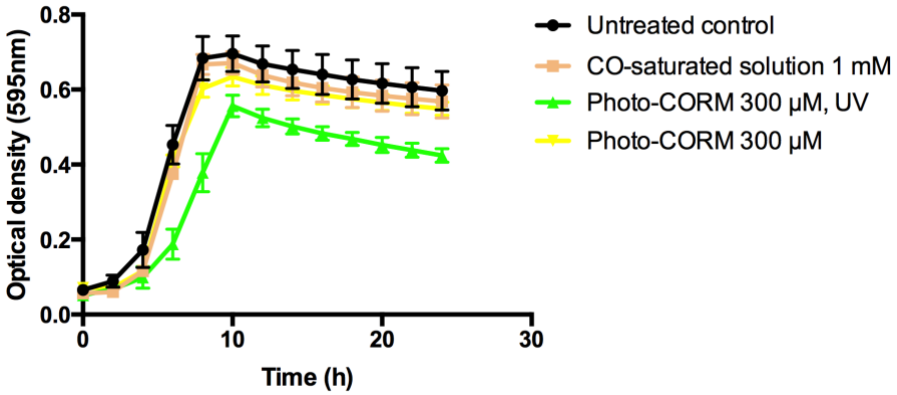


Figure 5.2 Effect of PhotoCORM or CO-saturated solution on *P. aeruginosa* planktonic cells. PhotoCORM (0, 300 µM) or CO-saturated solution (1 mM) was added to cultures (200 µl) followed by 2 min exposure to UV light (365 nm). The control was cultures treated with 300 µM PhotoCORM and kept in the dark. Data shows the means and standard deviations from 5 technical repeats.

37 °C to promote biofilm formation and biofilm biomass was determined by the crystal violet assay. Cultures exposed to UV light for up to 2 min showed no differences with respect to unexposed controls while 3 min or more slightly diminished the biomass (Figure 5.3). Therefore, an illumination time of 2 min was chosen for further biofilm studies.

The biofilm formation from PAO-1 cultures was severely compromised by the presence of 200 μM PhotoCORM and only small differences were observed with further increased concentrations of the compound (Figure 5.4A). The toxicity was clearly related to the activation of the PhotoCORM by exposure to UV, since cultures containing unexposed PhotoCORM (500 μM) and untreated control cultures produced comparable amounts of biomass (Figure 5.4A). Interestingly, the negative effect of activated PhotoCORM on the PAO-1 biofilm formation was similar to that observed by the presence of 8 μg/ml colistin (MIC of colistin for PAO-1 cells is 4 μg ml^{-1}, as seen in Chapter 3), and the antibiotic known by its ability to effectively damage *P. aeruginosa* biofilms (Figure 5.4A). In addition, biofilm formation was not affected by the presence of CO-saturated solution (1 mM), which indicated it is the compound that causes inhibition (Figure 5.4B). Intriguingly, the viability of biofilm-forming cells was slightly decreased by the presence of either activated and no activated PhotoCORM. However, differences were not significant compared to the untreated control (Figure 5.5). The slight effect might be related to direct damage to initial planktonic cells in early biofilm formation phase, and this would consequently impair their ability to produce the biofilm. Corresponding to the data shown in Figure 5.4, the clear effect of activated PhotoCORM on preventing the formation of biofilm may be caused not only by killing initial planktonic cells but also interrupting biofilm formation.

5.2.3 Effects of PhotoCORM and CO-saturated solution on *P. aeruginosa* biofilm maturation

To further explore whether activated PhotoCORM had any direct effects on damaging the biofilm structure, the maturation of early stage pre-formed PAO-1 biofilms (24 h) in

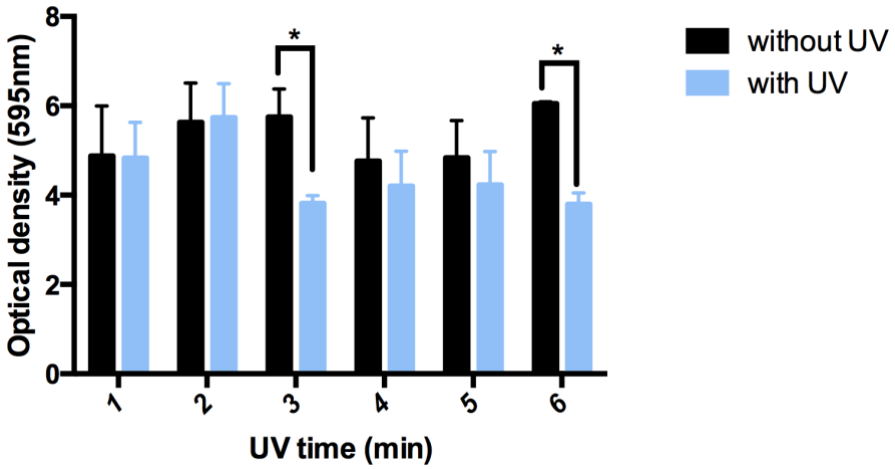
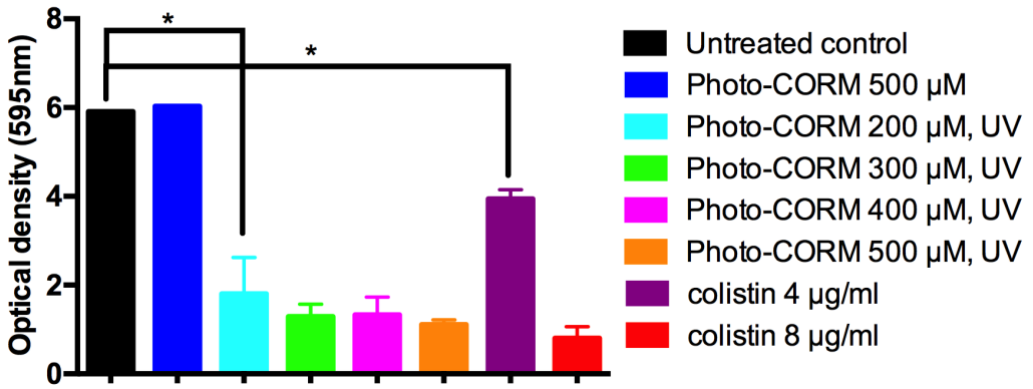


Figure 5.3 Effect of exposure to UV light on *P. aeruginosa* biofilm formation. A liquid culture grown overnight was aliquot (200 µl each) into the wells of a 96-well plate. Each plate was irradiated with UV light for different period of time. After 48 h incubation at 37 °C non-shaking incubator, the biofilm biomass was measured with a crystal violet assay. Bars represent the means and standard deviations of five technical repeats (* means $p<0.05$).

A



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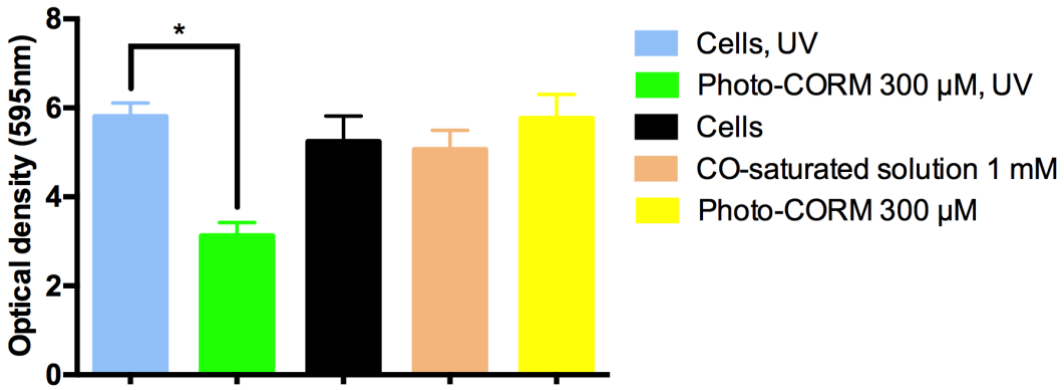


Figure 5.4 Effect of PhotoCORM on the *P. aeruginosa* biofilm formation. Cultures were grown on a 96-well plate at 37 °C non-shaking incubator. After 48 h incubation, the cell biomass was measured with crystal violet assay. In **(A)**, a range of PhotoCORM (0–500 µM) and colistin (4-8 µg/ml) were added to cultures followed by 2 min exposure to UV light (365 nm) before inoculated on the plate. The control was cultures added with 500 µM PhotoCORM and kept in the dark. Data shows the means and standard deviations from 5 technical repeats. In **(B)**, PhotoCORM (0, 300 µM) and CO-saturated solution (1 mM) were added to culture followed by 2 min exposure to UV light (365 nm) before inoculated on the plate. The control was cultures treated with 300 µM PhotoCORM and kept in the dark. Data shows the means and standard deviations from 2 biological replicates of which there were 5 technical repeats (* means $p<0.05$).

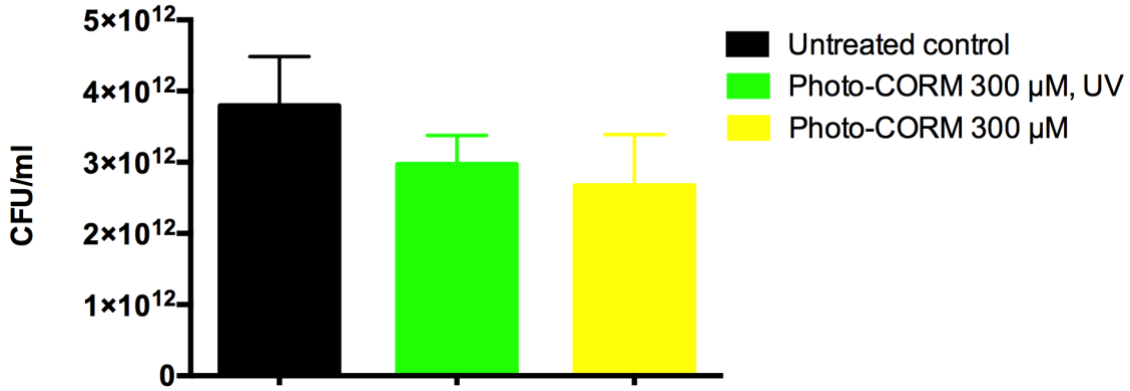


Figure 5.5 Effect of PhotoCORM on the viability of *P. aeruginosa* biofilms. Cultures were grown on a 96-well plate at 37 °C non-shaking incubator. A liquid culture grown overnight was aliquot (200 µl each) into the wells of a 96-well plate. PhotoCORM (0, 300 µM) was added to cultures followed by 2 min exposure to UV light (365 nm). The control was a culture treated with 300 µM PhotoCORM and kept in the dark. After 48 h incubation, the number of viable cells was counted as CFU ml^{-1}. Data shows the means and standard deviations from 3 technical repeats.

the presence of PhotoCORM was investigated (Figure 5.6). After promoting the formation of immature biofilms by 24 h static incubation at 37 °C, the culture medium was removed from the plates and replaced with fresh medium. Then, biofilms were exposed to UV for 2 min upon addition of increasing concentration of PhotoCORM and incubated for an additional 24 h to promote biofilm maturation (Figure 5.6A). With supplementation of sufficient nutrients from fresh medium, the biofilm maturation was successful which resulted in doubling the amount of biofilm at 48 h time point. Activation of 200 μM PhotoCORM significantly inhibited the biofilm maturation to the same extent as colistin (8 μg ml^{-1}), while 300 μM PhotoCORM or more not only produced inhibition but also effectively reduced the biomass of the immature biofilm (Figure 5.7A). Again, addition of PhotoCORM without UV activation, UV exposure in the absence of PhotoCORM or addition of CO gas failed to produce any detrimental effects (Figure 5.7B). Activation of 300 μM PhotoCORM significantly reduced the amount of preformed biofilm compared to the biomass at 24 h time point, which is in agreement with data shown in Figure 5.4, activated PhotoCORM interrupted the biofilm formation. Meanwhile, the viability of biofilm cells was significantly decreased by the presence of activated PhotoCORM after 24 h when compared to either medium contains inactivated PhotoCORM or the untreated control as early as 1 h (Figure 5.8). This contributed to the inhibition of activated PhotoCORM in biofilm maturation as well.

In a separate experiment, to further investigate the direct effect of activated PhotoCORM on PAO-1 established biofilm, after promoting the formation of immature biofilms by 24 h static incubation at 37 °C, the culture medium was replaced with PBS buffer (instead of fresh medium), which could stop the growth and more clearly determine the disruption of the biofilm. Then, biofilms were exposed to UV for 2 min upon addition of increasing concentration of PhotoCORM and incubated for additional 24 h to interact with established biofilm (Figure 5.6B). With supplement pre-formed biofilm with PBS stopped the biofilm maturation during 24 h treatment. Loss of the biomass upon 24 h incubation in the presence of activated PhotoCORM was still observed (Figure 5.9A). This suggests activated PhotoCORM directly disrupt the PAO-1 biofilm structure. However, this effect was less pronounced (compare Figure 5.7A with 5.9A).

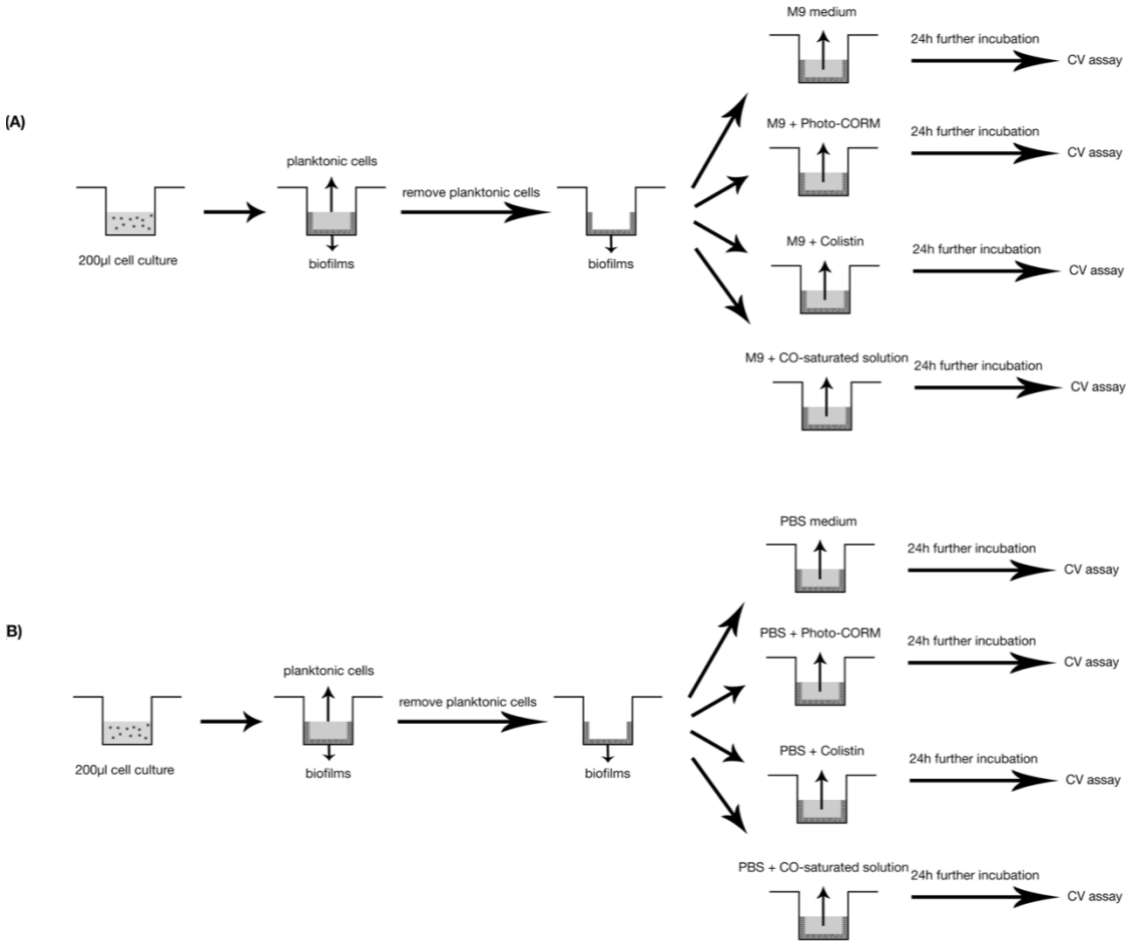
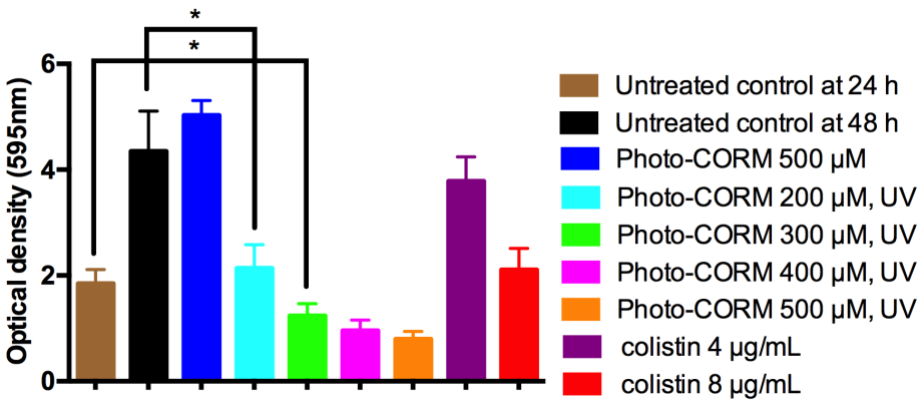


Figure 5.6 Process of investigating the effect of PhotoCORM on biofilm maturation. Cultures were grown on a 96-well plate at 37 °C non-shaking incubator for 24 h. In (A), M9 medium contains PhotoCORM, colistin or CO-saturated solution was added to cultures followed by 2 min exposure to UV light (365 nm) at 24 h time point. The control was cultures treated with PhotoCORM and kept in the dark; In (B), PBS containing PhotoCORM, colistin or CO-saturated solution was added to cultures followed by 2 min exposure to UV light (365 nm) at 24 h. The control was cultures treated with PhotoCORM and kept in the dark. In both experiments, after a further 24 h incubation, the cell biomass was measured with a crystal violet assay.

A



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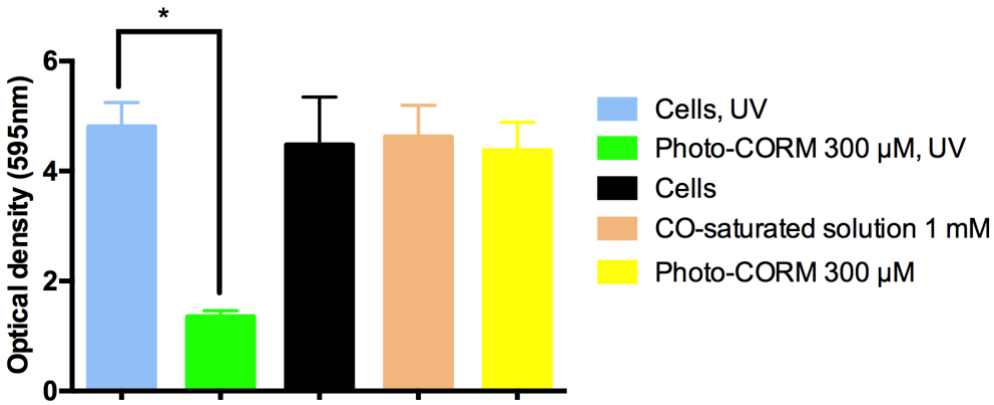

Figure 5.7 Effect of PhotoCORM on the *P. aeruginosa* biofilm maturation. Cultures were grown on a 96-well plate at 37 °C non-shaking incubator for 24 h. In **(A)**, M9 medium contained a range of PhotoCORM (0–500 µM) or colistin (4-8 µg/ml) was added to cultures followed by 2 min exposure to UV light (365 nm) at 24 h. The control was wells treated with 500 µM PhotoCORM and kept in the dark. In **(B)**, M9 medium contain PhotoCORM (0, 300 µM) or CO-saturated solution (1 mM) was added to culture followed by 2 min exposure to UV light (365 nm) at 24 h time point. The control was cultures treated with 300 µM PhotoCORM and kept in the dark. In both experiments, after a further 24 h incubation, the cell biomass was measured with a crystal violet assay. Data shows the means and standard deviations from 2 biological replicates of which there were 5 technical repeats (* means $p<0.05$).

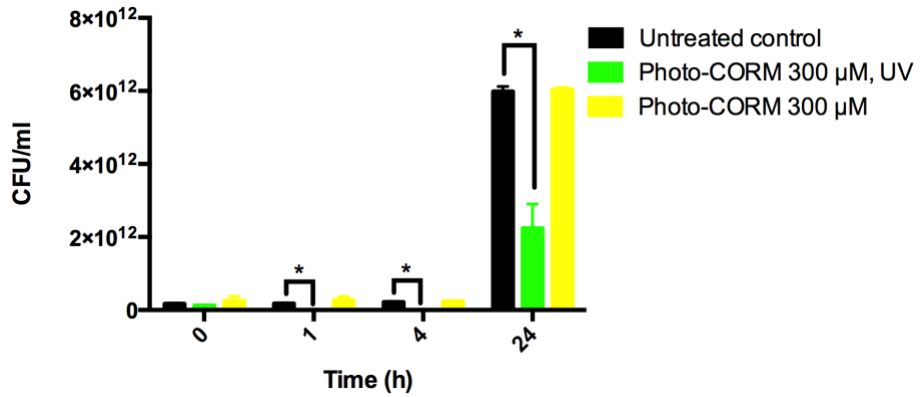

Figure 5.8 Effect of PhotoCORM on the viability of established *P. aeruginosa* biofilms. Cultures were grown on a 96-well plate at 37 °C non-shaking incubator for 24 h. M9 medium contains PhotoCORM (0, 300 µM) was added to cultures followed by 2 min exposure to UV light (365 nm) at 24 h time point. The control was cultures added with 300 µM PhotoCORM and kept in the dark. After a further 24 h incubation was carried out. The number of viable cells was recorded as CFU ml^{-1} at regular time intervals. Data shows the means and standard deviations from 3 technical repeats (* means $p<0.05$).

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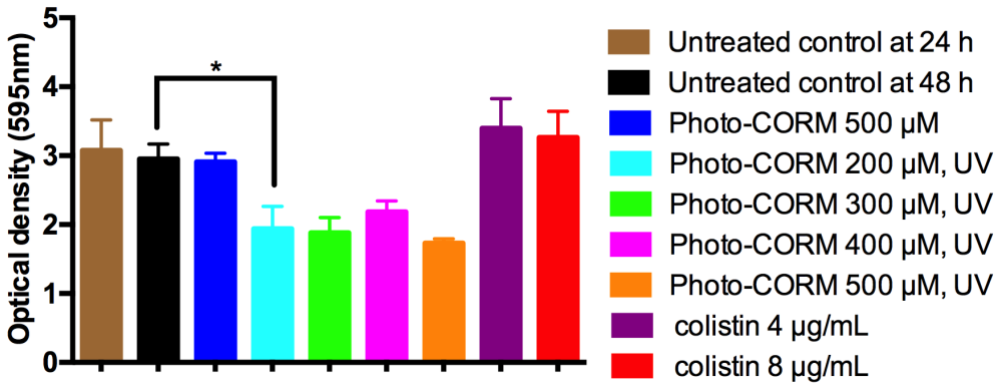

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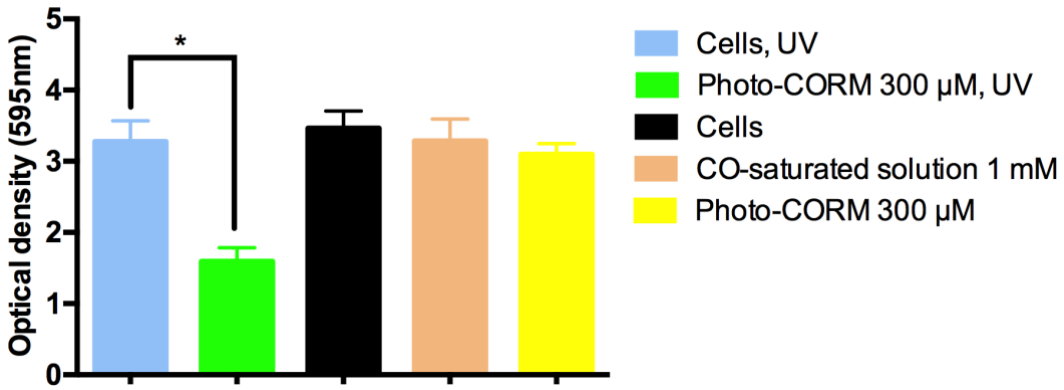

Figure 5.9 Effect of PhotoCORM on the established *P. aeruginosa* biofilm in PBS. Cultures were grown on a 96-well plate at 37 °C non-shaking incubator for 24 h. In **(A)**, PBS contains a range of PhotoCORM (0–500 µM) or colistin (4-8 µg ml^{-1}) was added to cultures followed by 2 min exposure to UV light (365 nm) at 24 h time point. The control was cultures added with 500 µM PhotoCORM and kept in the dark; In **(B)**, PBS contains PhotoCORM (0, 300 µM) or CO-saturated solution (1 mM) was added to culture followed by 2 min exposure to UV light (365 nm) at 24 h time point. The control was cultures added with 300 µM PhotoCORM and kept in the dark. In both experiments, after a further 24 h incubation, the cell biomass was measured with crystal violet assay. Data shows the means and standard deviations from 2 biological replicates of which there were 5 technical repeats (* means $p<0.05$).

5.3 Discussion

5.3.1 Summary of this study

The work in this chapter constitutes the first investigation of the effect of this novel PhotoCORM ($[Mn(CO)_3(tpa\text{-}\kappa^3N)]^+$) on any biofilm. PhotoCORM is a water-soluble CORM that releases CO only upon illumination at 365 nm. In addition, Mn was used as the metal core of this compound, which makes it more favourable in biological systems than Ru.

In this study we demonstrate that activated PhotoCORM has only a slight effect on planktonic cultures of *P. aeruginosa* (Figure 5.1A). In comparison with previous ruthenium-based CORM studies (Figure 3.2 and Figure 3.9), the effect of activated PhotoCORM on the growth is mild. In addition, we have shown that activated PhotoCORM causes a loss of *P. aeruginosa* biofilm biomass during biofilm formation (Figure 5.3A). However, activated PhotoCORM is not bactericidal against *P. aeruginosa* biofilm cells during the formation (Figure 5.4). This suggests activated PhotoCORM inhibits the formation of *P. aeruginosa* biofilm without directly killing *P. aeruginosa* cells.

Furthermore, it was shown that activated PhotoCORM inhibits the biofilm maturation and disrupts pre-formed biofilm on conditions where cells are metabolically active (Figure 5.5A and Figure 5.7A). However, PhotoCORM has only a discrete effect on the disruption of biofilm during starvation. Activated PhotoCORM is more potent to *P. aeruginosa* biofilm cells than planktonic cell, which makes this compound an ideal candidate for further studies of treating infections related to *P. aeruginosa* biofilms. The effect of activated PhotoCORM on *P. aeruginosa* biofilms shows that it is more effective on *P. aeruginosa* biofilms than colistin. The finding that CO-saturated solution does not inhibit the growth of *P. aeruginosa* planktonic cultures and biofilm cultures (Figure 5.1B, 5.3B, 5.5B and 5.7B) suggests that the effect on growth of PhotoCORM can be attributed to the compound as a whole and not CO alone.

5.3.2 The benefit of Manganese in PhotoCORM

The PhotoCORM used in this study is a Mn based compound. It has not been investigated in this study whether the manganese from PhotoCORM has a protective effect on cells. However, the possibility that manganese may play a protective role in cells has reported by several research groups. It was first arose in studies of *Lactobacillus plantarum* which survives on Mn-rich fermenting plant materials (Archibald and Duong, 1984). Not only was it shown that this bacterium accumulated manganese to exceedingly high levels, it was also shown that the bacterium exhibited a Mn(II)-dependent superoxide dismutase activity that was inhibitable by EDTA (Archibald and Fridovich, 1982). Additionally, in *Deinococcus radiodurans*, a bacterium known for its resistance to ionizing radiation (Cox and Battista, 2005, Slade and Radman, 2011), accumulation of manganese is tolerated to levels as high as 30 mM (Daly *et al.*, 2004). It is thought that the high manganese content of cells is associated with non-enzymatic reactions that protect the organism from reactive oxygen species (Slade and Radman, 2011, Horsburgh *et al.*, 2002). For example, such activities include Mn(II) carbonate which exhibits catalase-like activity (Berlett *et al.*, 1990) and Mn(II) phosphate which has been shown to dismutate superoxide (Barnese *et al.*, 2012).

5.3.3 Conclusion

In summary, activated PhotoCORM exhibits only slight inhibition of the growth of *P. aeruginosa* planktonic cultures. However, activated PhotoCORM prevents and disrupts biofilm formation of *P. aeruginosa* but non-illuminated PhotoCORM is without effect. Activated PhotoCORM inhibits biofilm maturation and disrupts pre-formed biofilms. Activated PhotoCORM, but not CO gas, produces toxic effects in both planktonic cultures and biofilms of *P. aeruginosa*.

Chapter 6

General Discussion

6.1 Summary

The beneficial effects of CO have led the research to CORMs. The therapeutic properties of CORMs then led researchers to investigate their effect on *P. aeruginosa* in the past two decades. Before the work was examined in this thesis, CORMs were mainly applied to *P. aeruginosa* planktonic cells. In planktonic culture, CORM-3 was bactericidal to both a laboratory strain (PAO1) and three clinical isolates of *P. aeruginosa* (Desmard *et al.*, 2009). Murray and colleagues were the first to evaluate the effects of CORM-2 on *P. aeruginosa* biofilm maturation and surface colonization. They have shown that CORM-2 (25 - 200 μM) prevented biofilm maturation as early as 60 min after addition. CORM-2 (100 μM) also decreased the number of viable bacteria recovered from the biofilm as early as 30 min after treatment, and persisting for a further 6 h. With the development of CORMs, the work presented in this thesis therefore sought to dissect the effect of both CORM-2 and CORM-3 on *P. aeruginosa* planktonic cells with biofilm cells (see Chapter 3). Bactericidal activity of both CORM-2 and CORM-3 were confirmed in *P. aeruginosa* planktonic cells. Although CORM-3 was effective at inhibiting surface-associated growth of *P. aeruginosa* by both preventing biofilm maturation and killing bacteria within the established biofilm, cells growing in a biofilm were more resistant to CORM-3 than planktonic cells. Poor penetration and anaerobic microenvironment may contribute to this resistance, but not starvation. In study of CORM-2 as potential adjuvant compound with colistin, the combination effect of those two antimicrobial compounds was found to be indifferent against *P. aeruginosa* planktonic cells and biofilm cells.

In order to harvest enough *P. aeruginosa* biofilm cells to establish the role of CORM-3 in biofilm cell killing, grew cells continuously in a chemostat system rather than under batch conditions was carried out (see Chapter 4). In our studies, it was more favourable to grow *P. aeruginosa* cells continuously in a chemostat system rather than under batch

condition. Interestingly, glucose was detected to be a key factor in the growth of *P. aeruginosa* biofilm. Furthermore, aerosolized CORM-3 treatment had a similar bactericidal effect to aerosolized colistin on *P. aeruginosa* cells. Although CORM-3 or colistin killed *P. aeruginosa* cells separately, the combination effect of those two antimicrobial compounds was found to be indifferent against *P. aeruginosa* cells.

A final avenue of exploration in this thesis was studying a new arising CORM: PhotoCORM. To date, little work has been done on the effect of PhotoCORM on bacteria, yet antimicrobial activity of PhotoCORM against a pathogenic *E.coli* has been already been shown by our group (Tinajero-Trejo *et al.*, 2016). The research presented in this thesis for the first time highlighted the effect of PhotoCORM on *P. aeruginosa* cells (see Chapter 5). The activated compound proved to be as effective at bacterial killing as previously used ruthenium CORMs (such as CORM-2 and CORM-3). Overall, activated PhotoCORM prevented and disrupted biofilm formation of *P. aeruginosa* but non-illuminated PhotoCORM was without effect. Activated PhotoCORM inhibited biofilm maturation and disrupted pre-formed biofilms. Activated PhotoCORM, but not CO gas, produced toxic effects in both planktonic cultures and biofilms of *P. aeruginosa*.

6.2 Further scope and investigation

6.2.1 Chemostat

In this *P. aeruginosa* biofilm study, the chemostat is a valuable tool for bacterial cultivation (Chapter 3). However, it is impossible to grow biofilms in a completely steady state, an advantage of biofilm cultivation in a chemostat is long-term control of growth conditions, medium input rate and any extraneous variable to which the researcher may wish to profile a response. As fresh medium is constantly introduced, the chemostat allows the growth of mature biofilms, providing sufficient material for global analysis. It is also possible to use any growth surface, providing it can be attached to the inside of the chemostat, and allow growth of biofilm at the medium/air interface. The biofilm is not subject to a constant unidirectional laminar flow, and approximate shear forces can be

increased without the need to change the medium input rate. A disadvantage is that it is not possible to make direct observations of the biofilm *in situ*.

6.2.2 Transport of CORMs

A challenging, but essential area of research is the transport of CORMs into bacterial cells. Robert's group has proposed a ''Trojan Horse'' mechanism for the antimicrobial actions of CO-RMs to explain the potent effects on respiration that cannot be mimicked by CO gas (Figure 6.1). In brief, a CORM may lose its CO permanently in the extracellular milieu through, for example, the presence of a species to capture the CO or a change in the CO-RM after CO loss. Alternatively, the CORM is transported into the cell and concentrated carrying its CO cargo (Trojan Horse effect), and CO dissociation is promoted by a reaction with intracellular ligands that include sulphite. Thus, CO is immediately accessible at relatively high concentrations to membrane-bound heme targets, to which it binds. Respiration is inhibited in a light-reversible manner, and viability is drastically reduced (Wilson *et al.*, 2013).

The movement of CORMs into cells in this study is measured indirectly through the detection of the metal species within cells using ICP-MS (details see section 2.2.7). However, it is more difficult to determine the fate of the CORM sub-cellularly once it has been taken up into cells. One method that may help to overcome this issue is radioisotope-labelling the metal species of CORMs. Not only could radiolabelling help to generate accurate uptake kinetics but may also reveal the breakdown products of the metal co-ligand species of the compound or where the co-ligand species may bind to other proteins. Another method that may show sub-cellular localization of the compounds is through electron microscopy imaging. If the heavy metal is localized to one place within the cell, heavy metal deposits will be visible after staining, but if the metal species is distributed throughout the cell it may be more challenging to see.

6.2.3 CORMs as potential adjuvant compounds

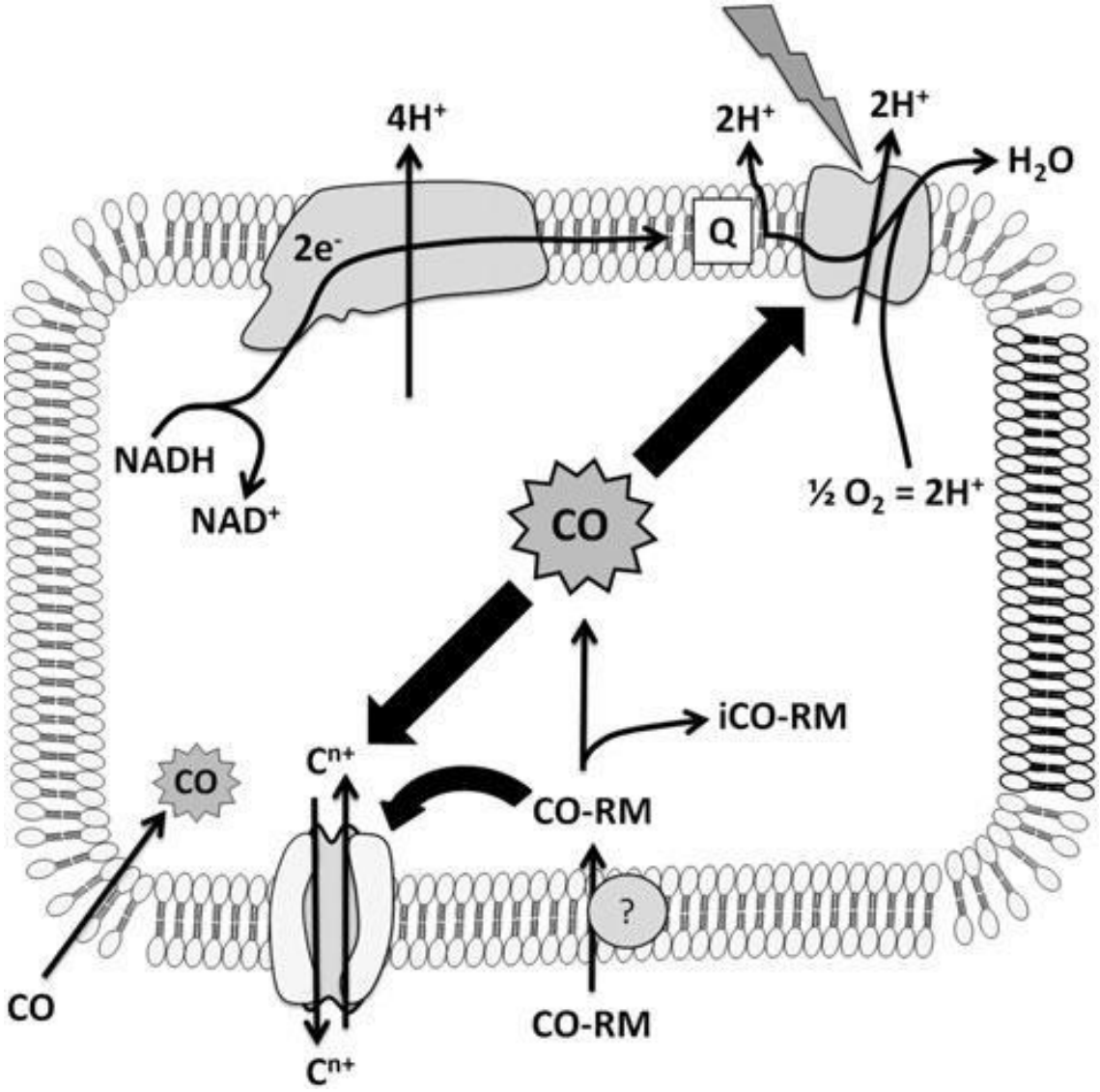

Figure 6.1 Trojan Horse hypothesis. It is postulated that CO- RMs carry their CO ‘‘toxic cargo’’ into the cell interior by unknown mechanisms. Intracellular accumulation of CO- RMs and subsequent release of CO leads to CO reaching concentrations (center of cell) that exceed those which can be attained by passive diffusion of CO gas from the cell exterior (bottom left). The major targets of CORM-3 identified in this work are respiratory electron transfer to O2, catalyzed by terminal quinol oxidases, and cation transport. Inhibition of respiration and growth inhibition is reversed by white light at ambient temperatures (top right). The scheme excludes the bacterial outer membrane and periplasm and the representation of the respiratory chain, terminating in a single oxidase, is greatly simplified (Wilson *et al.*, 2013).

Concerns over the inevitable spread of established antibiotics to treat antibiotic-resistant 'superbugs' has been widely explored. Meanwhile, advances in the discovery of novel antibiotic compounds has been slow. A solution to these problems could be to develop CORMs as adjuvants to existing antibiotic compounds. It has currently been counseled that combination therapy involving non-antibiotic adjuvants should offer a more realistic, long-term approach in controlling rising drug resistance (Brooks and Brooks, 2014). CORMs have already proven promise in combination therapy in a range of animal models. Murray and colleagues were the first to find CORM-2 acts as an adjuvant to tobramycin against *P. aeruginosa* biofilms (Murray *et al.*, 2012). Work published by Tavares's group showed sub-lethal doses of CORM-2 were combined with metronidazole, amoxicillin and clarithromycin and found strongly reducing the ability of *H. pylori* to infect macrophages, and also reducing the viability of cells(Tavares *et al.*, 2013). Wareham and co-workers determined the MICs of three well- established antibiotics (TR, DC, and CT) where CORM-2 was used in agar plates. In their study, CORM-2 led to dramatic reduction in MICs of those three antibiotics (Wareham *et al.*, 2016). A novel manganese CORM, CORM-401, in combination with cefotaxime and trimethoprim was showed to reduce bacterial growth of *E. coli* later around by the same group (Wareham *et al.*, 2018).

References

Abid, S., Houssaïni, A., Mouraret, N., Marcos, E., Amsellem, V., Wan, F., Dubois-Randé, J. L., Derumeaux, G., Boczkowski, J. & Motterlini, R. 2014. p21-Dependent protective effects of a carbon monoxide–releasing molecule-3 in pulmonary hypertension. *Arteriosclerosis, thrombosis, and vascular biology,* 34**,** 304-312.

Abraham, E. C., E 1940. An enzyme from bacteria able to destroy penicillin. *Nature,* 146**,** 837.

Akamatsu, Y., Haga, M., Tyagi, S., Yamashita, K., Graca-Souza, A. V., Ollinger, R., Czismadia, E., May, G. A., Ifedigbo, E., Otterbein, L. E., Bach, F. H. & Soares, M. P. 2004. Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. *Federation of American Societies for Experimental Biology Journal,* 18**,** 771-2.

Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M. & Tolker-Nielsen, T. 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Molecular Microbiology,* 59**,** 1114-28.

Anderl, J. N., Zahller, J., Roe, F. & Stewart, P. S. 2003. Role of nutrient limitation and stationary-phase existence in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial agents and chemotherapy,* 47**,** 1251-6.

Aono, S., Nakajima, H., Saito, K. & Okada, M. 1996. A novel heme protein that acts as a carbon monoxide-dependent transcriptional activator in Rhodospirillum rubrum. *Biochemical and Biophysical Research Communications,* 228**,** 752-6.

Archibald, F. S. & Duong, M.-N. 1984. Manganese acquisition by *Lactobacillus plantarum*. *Journal of Bacteriology,* 158**,** 1-8.

Archibald, F. S. & Fridovich, I. 1982. Investigations of the state of the manganese in *Lactobacillus plantarum*. *Arch Biochem Biophys,* 215**,** 589-96.

Bang, C. S., Kruse, R., Demirel, I., Onnberg, A., Soderquist, B. & Persson, K. 2014. Multiresistant uropathogenic extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* are susceptible to the carbon monoxide releasing molecule-2 (CORM-2). *Microbial Pathogenesis,* 66**,** 29-35.

Bani-Hani, M. G., Greenstein, D., Mann, B. E., Green, C. J. & Motterlini, R. 2006. Modulation of thrombin-induced neuroinflammation in BV-2 microglia by carbon monoxide-releasing molecule 3. *Journal of Pharmacology and Experimental Therapeutics,* 318**,** 1315-1322.

Bannenberg, G. L. & Vieira, H. L. 2009. Therapeutic applications of the gaseous mediators carbon monoxide and hydrogen sulfide. *Expert Opinion on Therapeutic Patents,* 19**,** 663-82.

Baquero, F., Martinez, J. L. & Canton, R. 2008. Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol,* 19**,** 260-5.

Barnese, K., Gralla, E. B., Valentine, J. S. & Cabelli, D. E. 2012. Biologically relevant mechanism for catalytic superoxide removal by simple manganese compounds. *Proc Natl Acad Sci U S A,* 109**,** 6892-7.

Bassetti, M. & Righi, E. 2015. Development of novel antibacterial drugs to combat multiple resistant organisms. *Langenbecks Arch Surg,* 400**,** 153-65.

Beloin, C., Roux, A. & Ghigo, J. M. 2008. *Escherichia coli* biofilms. *Current Topics in Microbiology and Immunology,* 322**,** 249-89.

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M. N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F. & Martinez, J. L. 2015. Tackling antibiotic resistance: the environmental framework. *Nat Rev Microbiol,* 13**,** 310-7.

Berlana, D., Llop, J. M., Fort, E., Badia, M. B. & Jodar, R. 2005. Use of colistin in the treatment of multiple-drug-resistant gram-negative infections. *American Journal of Health-System Pharmacy,* 62**,** 39-47.

Berlett, B. S., Chock, P. B., Yim, M. B. & Stadtman, E. R. 1990. Manganese(II) catalyzes the bicarbonate-dependent oxidation of amino acids by hydrogen peroxide and the amino acid-facilitated dismutation of hydrogen peroxide. *Proc Natl Acad Sci U S A,* 87**,** 389-93.

Bjarnsholt, T., Jensen, P. O., Fiandaca, M. J., Pedersen, J., Hansen, C. R., Andersen, C. B., Pressler, T., Givskov, M. & Hoiby, N. 2009. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatric pulmonology,* 44**,** 547-58.

Boczkowski, J., Poderoso, J. J. & Motterlini, R. 2006. CO-metal interaction: vital signaling from a lethal gas. *Trends in Biochemical Sciences,* 31**,** 614-621.

Bodey, G. P., Bolivar, R., Fainstein, V. & Jadeja, L. 1983. Infections caused by *Pseudomonas aeruginosa*. *Reviews of infectious diseases,* 5**,** 279-313.

Bonam, D., Lehman, L., Roberts, G. P. & Ludden, P. W. 1989. Regulation of carbon monoxide dehydrogenase and hydrogenase in Rhodospirillum rubrum: effects of CO and oxygen on synthesis and activity. *Journal of Bacteriology,* 171**,** 3102-7.

Borriello, G., Werner, E., Roe, F., Kim, A. M., Ehrlich, G. D. & Stewart, P. S. 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrobial agents and chemotherapy,* 48**,** 2659-64.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B. & Bartlett, J. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis,* 48**,** 1-12.

Brooks, B. D. & Brooks, A. E. 2014. Therapeutic strategies to combat antibiotic resistance. *Advanced Drug Delivery Reviews,* 78**,** 14-27.

Brown, J. M., Dorman, D. C. & Roy, L. P. 1970. Acute renal failure due to overdosage of colistin. *The Medical jounal of Australia,* 2**,** 923-4.

Castellani, C., Duff, A. J. A., Bell, S. C., Heijerman, H. G. M., Munck, A., Ratjen, F., Sermet-Gaudelus, I., Southern, K. W., Barben, J., Flume, P. A., Hodkova, P., Kashirskaya, N., Kirszenbaum, M. N., Madge, S., Oxley, H., Plant, B., Schwarzenberg, S. J., Smyth, A. R., Taccetti, G., Wagner, T. O. F., Wolfe, S. P. & Drevinek, P. 2018. ECFS best practice guidelines: the 2018 revision. *J Cyst Fibros,* 17**,** 153-178.

Caumartin, Y., Stephen, J., Deng, J. P., Lian, D., Lan, Z., Liu, W., Garcia, B., Jevnikar, A. M., Wang, H., Cepinskas, G. & Luke, P. P. 2011. Carbon monoxide-releasing molecules protect against ischemia-reperfusion injury during kidney transplantation. *Kidney International,* 79**,** 1080-9.

Cepinskas, G., Katada, K., Bihari, A. & Potter, R. F. 2008. Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. *American Journal of Physiology Gastrointestinal and Liver Physiology,* 294**,** G184-G191.

Chambless, J. D., Hunt, S. M. & Stewart, P. S. 2006. A three-dimensional computer model of four hypothetical mechanisms protecting biofilms from antimicrobials. *Applied and Environmental Microbiology,* 72**,** 2005-13.

Chlopicki, S., Olszanecki, R., Marcinkiewicz, E., Lomnicka, M. & Motterlini, R. 2006. Carbon monoxide released by CORM-3 inhibits human platelets by a mechanism independent of soluble guanylate cyclase. *Cardiovascular Research,* 71**,** 393-401.

Clark, J. E., Naughton, P., Shurey, S., Green, C. J., Johnson, T. R., Mann, B. E., Foresti, R. & Motterlini, R. 2003. Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Circulation research,* 93**,** e2-8.

Co, J. Y., Carcamo-Oyarce, G., Billings, N., Wheeler, K. M., Grindy, S. C., Holten-Andersen, N. & Ribbeck, K. 2018. Mucins trigger dispersal of *Pseudomonas aeruginosa* biofilms. *NPJ Biofilms Microbiomes,* 4**,** 23.

Costerton, J. W., Irvin, R. T. & Cheng, K. J. 1981. The bacterial glycocalyx in nature and disease. *Annual review of microbiology,* 35**,** 299-324.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. 1995. Microbial biofilms. *Annual Review of Microbiology,* 49**,** 711-45.

Costerton, J. W., Montanaro, L. & Arciola, C. R. 2007. Bacterial communications in implant infections: a target for an intelligence war. *The International Journal of Artificial Organs,* 30**,** 757-63.

Cowan, M. M. & Fletcher, M. 1987. Rapid screening method for detection of bacterial mutants with altered adhesion abilities. *Journal of Microbiological Methods,* 7**,** 241-249.

Cox, M. M. & Battista, J. R. 2005. *Deinococcus radiodurans* - the consummate survivor. *Nat Rev Microbiol,* 3**,** 882-92.

Crook, S. H., Mann, B. E., Meijer, A. J., Adams, H., Sawle, P., Scapens, D. & Motterlini, R. 2011. [Mn(CO)4{S2CNMe(CH2CO2H)}], a new water-soluble CO-releasing molecule. *Dalton Transactions,* 40**,** 4230-5.

Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Venkateswaran, A., Hess, M., Omelchenko, M. V., Kostandarithes, H. M., Makarova, K. S., Wackett, L. P., Fredrickson, J. K. & Ghosal, D. 2004. Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. *Science,* 306**,** 1025-8.

Davidge, K. S., Motterlini, R., Mann, B. E., Wilson, J. L. & Poole, R. K. 2009a. Carbon monoxide in biology and microbiology: surprising roles for the "Detroit perfume". *Advances in Microbial Physiology,* 56**,** 85-167.

Davidge, K. S., Sanguinetti, G., Yee, C. H., Cox, A. G., Mcleod, C. W., Monk, C. E., Mann, B. E., Motterlini, R. & Poole, R. K. 2009b. Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. *Journal of Biological Chemistry,* 284**,** 4516-24.

Davies, J. & Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev,* 74**,** 417-33.

Desmard, M., Davidge, K. S., Bouvet, O., Morin, D., Roux, D., Foresti, R., Ricard, J. D., Denamur, E., Poole, R. K., Montravers, P., Motterlini, R. & Boczkowski, J. 2009. A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against *Pseudomonas aeruginosa* and improves survival in an animal model of bacteraemia. *Federation of American Societies for Experimental Biology Journal,* 23**,** 1023-31.

Desmard, M., Foresti, R., Morin, D., Dagouassat, M., Berdeaux, A., Denamur, E., Crook, S. H., Mann, B. E., Scapens, D., Montravers, P., Boczkowski, J. & Motterlini, R. 2012. Differential antibacterial activity against *Pseudomonas aeruginosa* by carbon monoxide-releasing molecules. *Antioxidants & redox signaling,* 16**,** 153-63.

Donlan, R. M. 2002. Biofilms: microbial life on surfaces. *Emerging Infectious Diseases,* 8**,** 881-90.

Donlan, R. M. & Costerton, J. W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews,* 15**,** 167-93.

Dordelmann, G., Pfeiffer, H., Birkner, A. & Schatzschneider, U. 2011. Silicium dioxide nanoparticles as carriers for photoactivatable CO-releasing molecules (PhotoCORMs). *Inorganic chemistry,* 50**,** 4362-7.

Emerson, J., Mcnamara, S., Buccat, A. M., Worrell, K. & Burns, J. L. 2010. Changes in cystic fibrosis sputum microbiology in the United States between 1995 and 2008. *Pediatric pulmonology,* 45**,** 363-70.

Ferenci, T. 2008. Bacterial physiology, regulation and mutational adaptation in a chemostat environment. *Advances in Microbial Physiology,* 53**,** 169-229.

Ferrandiz, M. L., Maicas, N., Garcia-Amandis, I., Terencio, M. C., Motterlini, R., Devesa, I., Joosten, L. a. B., Van Den Berg, W. B. & Alcaraz, M. J. 2008. Treatment with a CO-releasing molecule (CORM-3) reduces joint inflammation and erosion in murine collagen-induced arthritis. *Annals of the Rheumatic Diseases,* 67**,** 1211-1217.

Foresti, R., Hammad, J., Clark, J. E., Johnson, T. R., Mann, B. E., Friebe, A., Green, C. J. & Motterlini, R. 2004. Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. *British Journal of Pharmacology,* 142**,** 453-460.

Furchgott, R. F. & Jothianandan, D. 1991. Endothelium-dependent and endothelium-independent vasodilation involving cyclic GMP- Relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels,* 28**,** 52-61.

Gallagher, L. A. & Manoil, C. 2001. *Pseudomonas aeruginosa* PAO1 kills Caenorhabditis elegans by cyanide poisoning. *Journal of Bacteriology,* 183**,** 6207-14.

Gibson, R. L., Burns, J. L. & Ramsey, B. W. 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. *American journal of respiratory and critical care medicine,* 168**,** 918-51.

Gilles-Gonzalez, M. A., Ditta, G. S. & Helinski, D. R. 1991. A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. *Nature,* 350**,** 170-172.

Goldbaum, L. R., Ramirez, R. G. & Absalon, K. B. 1975. What is the mechanism of carbon monoxide toxicity? *Aviation, Space, and Environmental Medicine,* 46**,** 1289-91.

Govan, J. R. & Deretic, V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological Reviews,* 60**,** 539-74.

Guo, Y., Stein, A. B., Wu, W. J., Tan, W., Zhu, X., Li, Q. H., Dawn, B., Motterlini, R. & Bolli, R. 2004. Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. *American journal of physiology,* 286**,** H1649-53.

Gupta, N., Limbago, B. M., Patel, J. B. & Kallen, A. J. 2011. Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. *Clin Infect Dis,* 53**,** 60-7.

Harmsen, M., Yang, L., Pamp, S. J. & Tolker-Nielsen, T. 2010. An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunology and Medical Microbiology,* 59**,** 253-68.

Hartley, C. L., Neumann, C. S. & Richmond, M. H. 1979. Adhesion of commensal bacteria to the large intestine wall in humans. *Infection and Immunity,* 23**,** 128-32.

Hassett, D. J. 1996. Anaerobic production of alginate by *Pseudomonas aeruginosa*: alginate restricts diffusion of oxygen. *J Bacteriol,* 178**,** 7322-5.

Hassett, D. J., Korfhagen, T. R., Irvin, R. T., Schurr, M. J., Sauer, K., Lau, G. W., Sutton, M. D., Yu, H. & Hoiby, N. 2010. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. *Expert opinion on therapeutic targets,* 14**,** 117-30.

Herrmann, G., Yang, L., Wu, H., Song, Z., Wang, H., Hoiby, N., Ulrich, M., Molin, S., Riethmuller, J. & Doring, G. 2010. Colistin-tobramycin combinations are superior

to monotherapy concerning the killing of biofilm *Pseudomonas aeruginosa*. *The Journal of Infectious Diseases,* 202**,** 1585-92.

Hodson, M. E., Gallagher, C. G. & Govan, J. R. 2002. A randomised clinical trial of nebulised tobramycin or colistin in cystic fibrosis. *European Respiratory Journal,* 20**,** 658-64.

Hogan, D. A. & Kolter, R. 2002. Pseudomonas-Candida interactions: an ecological role for virulence factors. *Science,* 296**,** 2229-32.

Horsburgh, M. J., Wharton, S. J., Karavolos, M. & Foster, S. J. 2002. Manganese: elemental defence for a life with oxygen. *Trends Microbiol,* 10**,** 496-501.

Hou, S. B., Larsen, R. W., Boudko, D., Riley, C. W., Karatan, E., Zimmer, M., Ordal, G. W. & Alam, M. 2000. Myoglobin-like aerotaxis transducers in Archaea and Bacteria. *Nature,* 403**,** 540-544.

Jensen, T., Pedersen, S. S., Garne, S., Heilmann, C., Hoiby, N. & Koch, C. 1987. Colistin inhalation therapy in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. *Journal of Antimicrobial Chemotherapy,* 19**,** 831-8.

Jindal, A. K., Pandya, K. & Khan, I. D. 2015. Antimicrobial resistance: A public health challenge. *Medical Journal, Armed Forces India,* 71**,** 178-81.

Johnson, T. R., Mann, B. E., Clark, J. E., Foresti, R., Green, C. J. & Motterlini, R. 2003. Metal carbonyls: a new class of pharmaceuticals? *Angewandte Chemie International Edition in English,* 42**,** 3722-9.

Johnson, T. R., Mann, B. E., Teasdale, I. P., Adams, H., Foresti, R., Green, C. J. & Motterlini, R. 2007. Metal carbonyls as pharmaceuticals? [Ru(CO)3Cl(glycinate)], a CO-releasing molecule with an extensive aqueous solution chemistry. *Dalton Transactions***,** 1500-8.

Juhna, T., Birzniece, D. & Rubulis, J. 2007. Effect of Phosphorus on survival of *Escherichia coli* in drinking water biofilms. *Applied and Environmental Microbiology,* 73**,** 3755-8.

Kanj, S. S. & Kanafani, Z. A. 2011. Current concepts in antimicrobial therapy against resistant gram-negative organisms: extended-spectrum beta-lactamase-producing Enterobacteriaceae, carbapenem-resistant Enterobacteriaceae, and multidrug-resistant *Pseudomonas aeruginosa*. *Mayo Clin Proc,* 86**,** 250-9.

Kerby, R. L., Youn, H. & Roberts, G. P. 2008. RcoM: A new single-component transcriptional regulator of CO metabolism in bacteria. *Journal of Bacteriology,* 190**,** 3336-3343.

King, G. M. & Weber, C. F. 2007. Distribution, diversity and ecology of aerobic CO-oxidizing bacteria. *Nature Reviews Microbiology,* 5**,** 107-118.

Kostakioti, M., Hadjifrangiskou, M. & Hultgren, S. J. 2013. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harbor Perspectives in Medicine,* 3**,** a010306.

Kumazawa, J. & Yagisawa, M. 2002. The history of antibiotics: the Japanese story. *Journal of Infection and Chemotherapy,* 8**,** 125-33.

Kumon, H., Tomochika, K., Matunaga, T., Ogawa, M. & Ohmori, H. 1994. A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. *Microbiology and Immunology,* 38**,** 615-9.

Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrobial agents and chemotherapy,* 45**,** 999-1007.

Lewis, K. 2007. Persister cells, dormancy and infectious disease. *Nature reviews. Microbiology,* 5**,** 48-56.

Li, L., Hsu, A. & Moore, P. K. 2009. Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation--a tale of three gases! *Pharmacology & Therapeutics,* 123**,** 386-400.

Livermore, D. M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G. M., Arlet, G., Ayala, J., Coque, T. M., Kern-Zdanowicz, I., Luzzaro, F., Poirel, L. & Woodford, N. 2007. CTX-M: changing the face of ESBLs in Europe. *J Antimicrob Chemother,* 59**,** 165-74.

Lyczak, J. B., Cannon, C. L. & Pier, G. B. 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes and Infection,* 2**,** 1051-60.

Madigan, M. T. 2012. *Brock biology of microorganisms,* San Francisco, Benjamin Cummings.

Mann, B. E. 2012. CO-Releasing Molecules: A Personal View. *Organometallics,* 31**,** 5728-5735.

Marr, A. K., Overhage, J., Bains, M. & Hancock, R. E. 2007. The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. *Microbiology,* 153**,** 474-82.

Mclaughlin-Borlace, L., Stapleton, F., Matheson, M. & Dart, J. K. 1998. Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis. *Journal of Applied Microbiology,* 84**,** 827-38.

Mclean, S., Begg, R., Jesse, H. E., Mann, B. E., Sanguinetti, G. & Poole, R. K. 2013. Analysis of the bacterial response to $Ru(CO)_3Cl$(glycinate) (CORM-3) and the inactivated compound identifies the role played by the ruthenium compound and reveals sulfur-containing species as a major target of CORM-3 action. *Antioxidants & redox signaling*.

Mclean, S., Mann, B. E. & Poole, R. K. 2012. Sulfite species enhance carbon monoxide release from CO-releasing molecules: implications for the deoxymyoglobin assay of activity. *Analytical Biochemistry,* 427**,** 36-40.

Mearns, M. B. 1970. Aerosol therapy in cystic fibrosis. *Archives of Disease in Childhood,* 45**,** 605-7.

Megias, J., Busserolles, J. & Alcaraz, M. J. 2007. The carbon monoxide-releasing molecule CORM-2 inhibits the inflammatory response induced by cytokines in Caco-2 cells. *British Journal of Pharmacology*.

Meister, K., Niesel, J., Schatzschneider, U., Metzler-Nolte, N., Schmidt, D. A. & Havenith, M. 2010. Label-free imaging of metal-carbonyl complexes in live cells by Raman microspectroscopy. *Angewandte Chemie International Edition in English,* 49**,** 3310-2.

Melchior, M. B., Vaarkamp, H. & Fink-Gremmels, J. 2006. Biofilms: a role in recurrent mastitis infections? *Journal of Veterinary Science,* 171**,** 398-407.

Miyata, S., Casey, M., Frank, D. W., Ausubel, F. M. & Drenkard, E. 2003. Use of the Galleria mellonella caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. *Infection and Immunity,* 71**,** 2404-13.

Monds, R. D. & O'toole, G. A. 2009. The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends in microbiology,* 17**,** 73-87.

Moore, N. M. & Flaws, M. L. 2011. Epidemiology and pathogenesis of *Pseudomonas aeruginosa* infections. *Clinical laboratory science,* 24**,** 43-6.

Motterlini, R., Clark, J. E., Foresti, R., Sarathchandra, P., Mann, B. E. & Green, C. J. 2002. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circulation research,* 90**,** E17-24.

Motterlini, R. & Otterbein, L. E. 2010. The therapeutic potential of carbon monoxide. *Nature reviews. Drug discovery,* 9**,** 728-43.

Motterlini, R., Sawle, P., Hammad, J., Bains, S., Alberto, R., Foresti, R. & Green, C. J. 2005. CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *FASEB Journal,* 19**,** 284-6.

Munoz-Price, L. S., Poirel, L., Bonomo, R. A., Schwaber, M. J., Daikos, G. L., Cormican, M., Cornaglia, G., Garau, J., Gniadkowski, M., Hayden, M. K., Kumarasamy, K., Livermore, D. M., Maya, J. J., Nordmann, P., Patel, J. B., Paterson, D. L., Pitout, J., Villegas, M. V., Wang, H., Woodford, N. & Quinn, J. P. 2013. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect Dis,* 13**,** 785-96.

Murray, T. S., Okegbe, C., Gao, Y., Kazmierczak, B. I., Motterlini, R., Dietrich, L. E. & Bruscia, E. M. 2012. The carbon monoxide releasing molecule CORM-2 attenuates *Pseudomonas aeruginosa* biofilm formation. *PLoS One,* 7**,** e35499.

Musameh, M. D., Green, C. J., Mann, B. E., Fuller, B. J. & Motterlini, R. 2007. Improved myocardial function after cold storage with preservation solution supplemented with a carbon monoxide-releasing molecule (CORM-3). *Journal of Heart and Lung Transplantation,* 26**,** 1192-1198.

Nagel, C., Mclean, S., Poole, R. K., Braunschweig, H., Kramer, T. & Schatzschneider, U. 2014. Introducing [Mn(CO)3(tpa-κ3N)]+ as a novel photoactivatable CO-releasing molecule with well-defined iCORM intermediates - synthesis, spectroscopy, and antibacterial activity. *Dalton Transactions,* 43**,** 9986-97.

Nakao, A., Choi, A. M. & Murase, N. 2006a. Protective effect of carbon monoxide in transplantation. *Journal of cellular and molecular medicine,* 10**,** 650-71.

Nakao, A., Faleo, G., Shimizu, H., Nakahira, K., Kohmoto, J., Sugimoto, R., Choi, A. M., Mccurry, K. R., Takahashi, T. & Murase, N. 2008. *Ex vivo* carbon monoxide prevents cytochrome P450 degradation and ischemia/reperfusion injury of kidney grafts. *Kidney International,* 74**,** 1009-1016.

Nakao, A., Kimizuka, K., Stolz, D. B., Neto, J. S., Kaizu, T., Choi, A. M., Uchiyama, T., Zuckerbraun, B. S., Nalesnik, M. A., Otterbein, L. E. & Murase, N. 2003. Carbon monoxide inhalation protects rat intestinal grafts from ischemia/reperfusion injury. *American Journal of Pathology,* 163**,** 1587-98.

Nakao, A., Toyokawa, H., Tsung, A., Nalesnik, M. A., Stolz, D. B., Kohmoto, J., Ikeda, A., Tomiyama, K., Harada, T., Takahashi, T., Yang, R., Fink, M. P., Morita, K., Choi, A. M. & Murase, N. 2006b. *Ex vivo* application of carbon monoxide in University of Wisconsin solution to prevent intestinal cold ischemia/reperfusion injury. *American journal of transplantation,* 6**,** 2243-55.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., Mckay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B. E. & Singh, P. K. 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science,* 334**,** 982-6.

Nickel, J. C., Ruseska, I., Wright, J. B. & Costerton, J. W. 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrobial agents and chemotherapy,* 27**,** 619-24.

Niesel, J., Pinto, A., Peindy N'dongo, H. W., Merz, K., Ott, I., Gust, R. & Schatzschneider, U. 2008. Photoinduced CO release, cellular uptake and cytotoxicity of a tris(pyrazolyl)methane (tpm) manganese tricarbonyl complex. *Chemical Communications (Cambridge, England)***,** 1798-800.

Nobre, L. S., Al-Shahrour, F., Dopazo, J. & Saraiva, L. M. 2009. Exploring the antimicrobial action of a carbon monoxide-releasing compound through whole-genome transcription profiling of *Escherichia coli*. *Microbiology,* 155**,** 813-24.

Nobre, L. S., Seixas, J. D., Romao, C. C. & Saraiva, L. M. 2007. Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrobial agents and chemotherapy,* 51**,** 4303-7.

Oelgeschlager, E. & Rother, M. 2008. Carbon monoxide-dependent energy metabolism in anaerobic bacteria and archaea. *Archives of Microbiology,* 190**,** 257-269.

Olvera, C., Goldberg, J. B., Sanchez, R. & Soberon-Chavez, G. 1999. The *Pseudomonas aeruginosa* algC gene product participates in rhamnolipid biosynthesis. *FEMS Microbiology Letters,* 179**,** 85-90.

Otterbein, L. E., Bach, F. H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R. J., Flavell, R. A. & Choi, A. M. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nature medicine,* 6**,** 422-8.

Pal, Z., Urban, E., Dosa, E., Pal, A. & Nagy, E. 2005. Biofilm formation on intrauterine devices in relation to duration of use. *Journal of Medical Microbiology,* 54**,** 1199-203.

Palmer, K. L., Brown, S. A. & Whiteley, M. 2007. Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum. *Journal of Bacteriology,* 189**,** 4449-55.

Parad, R. B., Gerard, C. J., Zurakowski, D., Nichols, D. P. & Pier, G. B. 1999. Pulmonary outcome in cystic fibrosis is influenced primarily by mucoid *Pseudomonas aeruginosa* infection and immune status and only modestly by genotype. *Infection and Immunity,* 67**,** 4744-50.

Parr, S. R., Wilson, M. T. & Greenwood, C. 1975. The reaction of *Pseudomonas aeruginosa* cytochrome *c* oxidase with carbon monoxide. *Biochem J,* 151**,** 51-9.

Peeters, E., Nelis, H. J. & Coenye, T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of microbiological methods,* 72**,** 157-65.

Pfeiffer, H., Rojas, A., Niesel, J. & Schatzschneider, U. 2009. Sonogashira and "Click" reactions for the N-terminal and side-chain functionalization of peptides with $[Mn(CO)_3(tpm)](+)$-based CO releasing molecules (tpm = tris(pyrazolyl)methane). *Dalton Transactions***,** 4292-4298.

Piantadosi, C. A. 2002. Biological chemistry of carbon monoxide. *Antioxidants & redox signaling,* 4**,** 259-70.

Pirt, S. J. 1975. *Principles of microbe and cell cultivation,* New York, Wiley.

Pizarro, M. D., Rodriguez, J. V., Mamprin, M. E., Fuller, B. J. & Motterlini, R. 2009. Protective effects of a carbon monoxide-releasing molecule (CORM-3) during hepatic cold preservation. *Cryobiology,* 58**,** 248-255.

Plummer, A. & Wildman, M. 2011. Duration of intravenous antibiotic therapy in people with cystic fibrosis. *Cochrane database of systematic reviews***,** CD006682.

Post, J. C., Stoodley, P., Hall-Stoodley, L. & Ehrlich, G. D. 2004. The role of biofilms in otolaryngologic infections. *Current Opinion in Otolaryngology & Head and Neck Surgery,* 12**,** 185-90.

Punam, V. 2007. Methods for Determining Bactericidal Activity and Antimicrobial Interactions. *Antimicrobial Susceptibility Testing Protocols.* CRC Press.

Qiu, X., Shen, W., Wang, X., Qin, W. & Sun, B. 2015. A novel strategy for antimicrobial agent: role of exogenous carbon monoxide on suppressing *Escherichia coli* vitality and toxicity. *Pakistan Journal of Pharmaceutical Sciences,* 28**,** 281-92.

Ragsdale, S. W. 2004. Life with carbon monoxide. *Critical Reviews in Biochemistry and Molecular Biology,* 39**,** 165-195.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. *Science,* 268**,** 1899-902.

Rana, N., Mclean, S., Mann, B. E. & Poole, R. K. 2014. Interaction of the carbon monoxide-releasing molecule $Ru(CO)_3Cl$(glycinate) (CORM-3) with Salmonella enterica serovar Typhimurium: in situ measurements of carbon monoxide binding by integrating cavity dual-beam spectrophotometry. *Microbiology,* 160**,** 2771-9.

Rasamiravaka, T., Labtani, Q., Duez, P. & El Jaziri, M. 2015. The formation of biofilms by *Pseudomonas aeruginosa*: a review of the natural and synthetic compounds interfering with control mechanisms. *BioMed Research International,* 2015**,** 759348.

Rice, L. B. 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis,* 197**,** 1079-81.

Rimmer, R. D., Richter, H. & Ford, P. C. 2010. A photochemical precursor for carbon monoxide release in aerated aqueous media. *Inorganic chemistry,* 49**,** 1180-5.

Rodgers, K. R. & Lukat-Rodgers, G. S. 2005. Insights into heme-based O-2 sensing from structure-function relationships in the FixL proteins. *Journal of Inorganic Biochemistry,* 99**,** 963-977.

Romao, C. C., Blattler, W. A., Seixas, J. D. & Bernardes, G. J. 2012. Developing drug molecules for therapy with carbon monoxide. *Chemical Society Reviews,* 41**,** 3571-83.

Ryan, G., Singh, M. & Dwan, K. 2011. Inhaled antibiotics for long-term therapy in cystic fibrosis. *Cochrane database of systematic reviews***,** CD001021.

Sadikot, R. T., Blackwell, T. S., Christman, J. W. & Prince, A. S. 2005. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *American Journal of Respiratory and Critical Care Medicine,* 171**,** 1209-23.

Sakuragi, Y. & Kolter, R. 2007. Quorum-sensing regulation of the biofilm matrix genes (pel) of *Pseudomonas aeruginosa*. *Journal of Bacteriology,* 189**,** 5383-6.

Sambrook, J. & Russell, D. W. 2001. *Molecular cloning : a laboratory manual,* Cold Spring Harbor (N.Y.), Cold Spring Harbor Laboratory Press.

Sandouka, A., Fuller, B. J., Mann, B. E., Green, C. J., Foresti, R. & Motterlini, R. 2006. Treatment with carbon monoxide-releasing molecules (CO-RMs) during cold storage improves renal function at reperfusion. *Kidney International,* 69**,** 239-247.

Sawle, P., Foresti, R., Mann, B. E., Johnson, T. R., Green, C. J. & Motterlini, R. 2005. Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *British Journal of Pharmacology,* 145**,** 800-810.

Schaber, J. A., Triffo, W. J., Suh, S. J., Oliver, J. W., Hastert, M. C., Griswold, J. A., Auer, M., Hamood, A. N. & Rumbaugh, K. P. 2007. *Pseudomonas aeruginosa* forms biofilms in acute infection independent of cell-to-cell signaling. *Infection and Immunity,* 75**,** 3715-21.

Schatzschneider, U. 2011. PhotoCORMs: Light-triggered release of carbon monoxide from the coordination sphere of transition metal complexes for biological applications. *Inorganica Chimica Acta,* 374**,** 19-23.

Schluter, A., Krause, L., Szczepanowski, R., Goesmann, A. & Puhler, A. 2008. Genetic diversity and composition of a plasmid metagenome from a wastewater treatment plant. *J Biotechnol,* 136**,** 65-76.

Scragg, J. L., Dallas, M. L., Wilkinson, J. A., Varadi, G. & Peers, C. 2008. Carbon monoxide inhibits L-type Ca^{2+} channels via redox modulation of key cysteine

residues by mitochondrial reactive oxygen species. *Journal of Biological Chemistry,* 283**,** 24412-24419.

Shelver, D., Kerby, R. L., He, Y. & Roberts, G. P. 1995. Carbon monoxide-induced activation of gene expression in Rhodospirillum rubrum requires the product of cooA, a member of the cyclic AMP receptor protein family of transcriptional regulators. *Journal of Bacteriology,* 177**,** 2157-63.

Shrout, J. D., Chopp, D. L., Just, C. L., Hentzer, M., Givskov, M. & Parsek, M. R. 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Molecular Microbiology,* 62**,** 1264-77.

Slade, D. & Radman, M. 2011. Oxidative stress resistance in *Deinococcus radiodurans*. *Microbiol Mol Biol Rev,* 75**,** 133-91.

Smith, H., Mann, B. E., Motterlini, R. & Poole, R. K. 2011. The carbon monoxide-releasing molecule, CORM-3 ($Ru(CO)_3Cl(Glycinate)$), targets respiration and oxidases in *Campylobacter jejuni*, generating hydrogen peroxide. *IUBMB Life,* 63**,** 363-371.

Southam, H. M., Smith, T. W., Lyon, R. L., Liao, C., Trevitt, C. R., Middlemiss, L. A., Cox, F. L., Chapman, J. A., El-Khamisy, S. F., Hippler, M., Williamson, M. P., Henderson, P. J. F. & Poole, R. K. 2018. A Thiol-Reactive Ru(II) Ion, not CO Release, Underlies the Potent Antimicrobial and Cytotoxic Properties of CO-Releasing Molecule-3. *Redox Biology*.

Stewart, P. S. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial agents and chemotherapy,* 40**,** 2517-22.

Stewart, P. S. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology,* 292**,** 107-13.

Szabo, C. 2010. Gaseotransmitters: new frontiers for translational science. *Science Translational Medicine,* 2**,** 59ps54.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gutzkow, T., Eichler, W., Puhler, A. & Schluter, A. 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology,* 155**,** 2306-19.

Tack, K. J. & Sabath, L. D. 1985. Increased minimum inhibitory concentrations with anaerobiasis for tobramycin, gentamicin, and amikacin, compared to latamoxef, piperacillin, chloramphenicol, and clindamycin. *Chemotherapy,* 31**,** 204-10.

Taille, C., El-Benna, J., Lanone, S., Boczkowski, J. & Motterlini, R. 2005. Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. *Journal of Biological Chemistry,* 280**,** 25350-60.

Tavares, A. F., Parente, M. R., Justino, M. C., Oleastro, M., Nobre, L. S. & Saraiva, L. M. 2013. The bactericidal activity of carbon monoxide-releasing molecules against *Helicobacter pylori*. *PLoS One,* 8**,** e83157.

Tavares, A. F., Teixeira, M., Romao, C. C., Seixas, J. D., Nobre, L. S. & Saraiva, L. M. 2011. Reactive oxygen species mediate bactericidal killing elicited by carbon monoxide-releasing molecules. *Journal of Biological Chemistry,* 286**,** 26708-17.

Tenhunen, R., Marver, H. S. & Schmid, R. 1969. The enzymatic conversion of hemoglobin to bilirubin. *Transactions of the Association of American Physicians,* 82**,** 363-71.

Tinajero-Trejo, M., Jesse, H. E. & Poole, R. K. 2013. Gasotransmitters, poisons, and antimicrobials: it's a gas, gas, gas! *F1000Prime Reports,* 5**,** 28.

Tinajero-Trejo, M., Rana, N., Nagel, C., Jesse, H. E., Smith, T. W., Wareham, L. K., Hippler, M., Schatzschneider, U. & Poole, R. K. 2016. Antimicrobial Activity of the Manganese Photoactivated Carbon Monoxide-Releasing Molecule [Mn(CO)3(tpa-kappa(3)N)](+) Against a Pathogenic Escherichia coli that Causes Urinary Infections. *Antioxidants & redox signaling,* 24**,** 765-80.

Trautner, B. W. & Darouiche, R. O. 2004. Role of biofilm in catheter-associated urinary tract infection. *American Journal of Infection Control,* 32**,** 177-83.

Vadori, M., Seveso, M., Besenzon, F., Bosio, E., Tognato, E., Fante, F., Boldrin, M., Gavasso, S., Ravarotto, L., Mann, B. E., Simioni, P., Ancona, E., Motterlini, R. & Cozzi, E. 2009. *In vitro* and *in vivo* effects of the carbon monoxide-releasing molecule, CORM-3, in the xenogeneic pig-to-primate context. *Xenotransplantation,* 16**,** 99-114.

Van Bijsterveld, O. P. & Jager, G. V. 1996. Infectious diseases of the conjunctiva and cornea. *Current Opinion in Ophthalmology,* 7**,** 65-70.

Vera, T., Henegar, J. R., Drummond, H. A., Rimoldi, J. M. & Stec, D. E. 2005. Protective effect of carbon monoxide-releasing compounds in ischemia-induced acute renal failure. *Journal of the American Society of Nephrology,* 16**,** 950-958.

Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V. & Snyder, S. H. 1993. Carbon monoxide - A putative neural messenger. *Science,* 259**,** 381-384.

Walters, M. C., 3rd, Roe, F., Bugnicourt, A., Franklin, M. J. & Stewart, P. S. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrobial agents and chemotherapy,* 47**,** 317-23.

Ward, J. S., Lynam, J. M., Moir, J. W., Sanin, D. E., Mountford, A. P. & Fairlamb, I. J. 2012. A therapeutically viable photo-activated manganese-based CO-releasing molecule (photo-CO-RM). *Dalton Transactions,* 41**,** 10514-7.

Wareham, L. K., Begg, R., Jesse, H. E., Van Beilen, J. W., Ali, S., Svistunenko, D., Mclean, S., Hellingwerf, K. J., Sanguinetti, G. & Poole, R. K. 2016. Carbon Monoxide Gas Is Not Inert, but Global, in Its Consequences for Bacterial Gene Expression, Iron Acquisition, and Antibiotic Resistance. *Antioxidants & redox signaling*.

Wareham, L. K., Mclean, S., Begg, R., Rana, N., Ali, S., Kendall, J. J., Sanguinetti, G., Mann, B. E. & Poole, R. K. 2018. The Broad-Spectrum Antimicrobial Potential of [Mn(CO)4(S2CNMe(CH2CO2H))], a Water-Soluble CO-Releasing Molecule (CORM-401): Intracellular Accumulation, Transcriptomic and Statistical

Analyses, and Membrane Polarization. *Antioxidants & redox signaling,* 28**,** 1286-1308.

Waters, V. & Ratjen, F. 2015. Standard versus biofilm antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis. *Cochrane database of systematic reviews,* 3**,** CD009528.

Wegiel, B., Larsen, R., Gallo, D., Chin, B. Y., Harris, C., Mannam, P., Kaczmarek, E., Lee, P. J., Zuckerbraun, B. S., Flavell, R., Soares, M. P. & Otterbein, L. E. 2014. Macrophages sense and kill bacteria through carbon monoxide-dependent inflammasome activation. *The Journal of clinical investigation,* 124**,** 4926-40.

Weinstock, B. & Niki, H. 1972. Carbon monoxide balance in nature. *Science,* 176**,** 290-2.

Wilson, J. L., Jesse, H. E., Hughes, B., Lund, V., Naylor, K., Davidge, K. S., Cook, G. M., Mann, B. E. & Poole, R. K. 2013. $Ru(CO)_3Cl$(Glycinate) (CORM-3): A carbon monoxide-releasing molecule with broad-spectrum antimicrobial and photosensitive activities against respiration and cation transport in *Escherichia coli*. *Antioxidants & redox signaling*.

Wolfgang, M. C., Jyot, J., Goodman, A. L., Ramphal, R. & Lory, S. 2004. Pseudomonas aeruginosa regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 6664-8.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., Birrer, P., Bellon, G., Berger, J., Weiss, T., Botzenhart, K., Yankaskas, J. R., Randell, S., Boucher, R. C. & Doring, G. 2002. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *The Journal of clinical investigation,* 109**,** 317-25.

Wright, G. D. 2010. Antibiotic resistance in the environment: a link to the clinic? *Curr Opin Microbiol,* 13**,** 589-94.

Wu, L. & Wang, R. 2005. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacological Reviews,* 57**,** 585-630.

Xue, J. & Habtezion, A. 2014. Carbon monoxide-based therapy ameliorates acute pancreatitis via TLR4 inhibition. *The Journal of clinical investigation,* 124**,** 437-47.