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Approaches to enhance the antimicrobial activity of carbapenems within bacterial biofilms

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Carbapenems are crucial antibiotics in the battle against bacterial infections, targeting both Gram-positive and Gram-negative bacteria with exceptional potency. These antibiotics are part of a group of vital 'last resort' antibiotics, reserved for severe infections caused by multi-drug resistant bacteria. However, their misuse poses a significant threat and the overuse of carbapenems accelerates the development of anti-biotic resistance, thereby jeopardizing the efficacy of these lifesaving drugs. Another contributing factor complicating this issue is the emergence of biofilms. These complex microbial communities are encased in a polymeric matrix and contribute to the onset of serious infections which are challenging to treat. This review explores the biofilm potency of different clinically approved carbapenems, delving into the latest strategies and delivery systems employed to augment their anti-biofilm activity. The goal is to provide valuable insights into the development of more potent carbapenems specifically tailored for combating biofilms.

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1. Introduction

1.1 History of carbapenems

Since the discovery of penicillin (Fig. 1, **1**) in 1928, the race to find new β-lactam antibiotics has been ongoing.[1] Although the history is unclear, the 'first' carbapenem discovered is accepted to be thienamycin (**2**), isolated in 1976 from a soil sample.[2] It was found to be an inhibitor of penicillin binding proteins (PBP) and was very effective against a variety of bacteria especially those displaying resistance to antibiotics at the time.[3,4] Carbapenems exhibit high potency across an exceptionally broad spectrum of pathogens, and lack the cross-resistance typically associated with other β-lactam antibiotics.[5] Gram-negative species such as *Pseudomonas aeruginosa* (*P. aeruginosa*), *Serratia*, and *Enterobacter* are susceptible to carbapenems. Additionally, carbapenems demonstrate activity against Gram-positive bacteria, including *Staphylococcus aureus* and *Staphylococcus epidermidis*, albeit with limited effectiveness against methicillin-resistant *Staphylococcus aureus* (MRSA).

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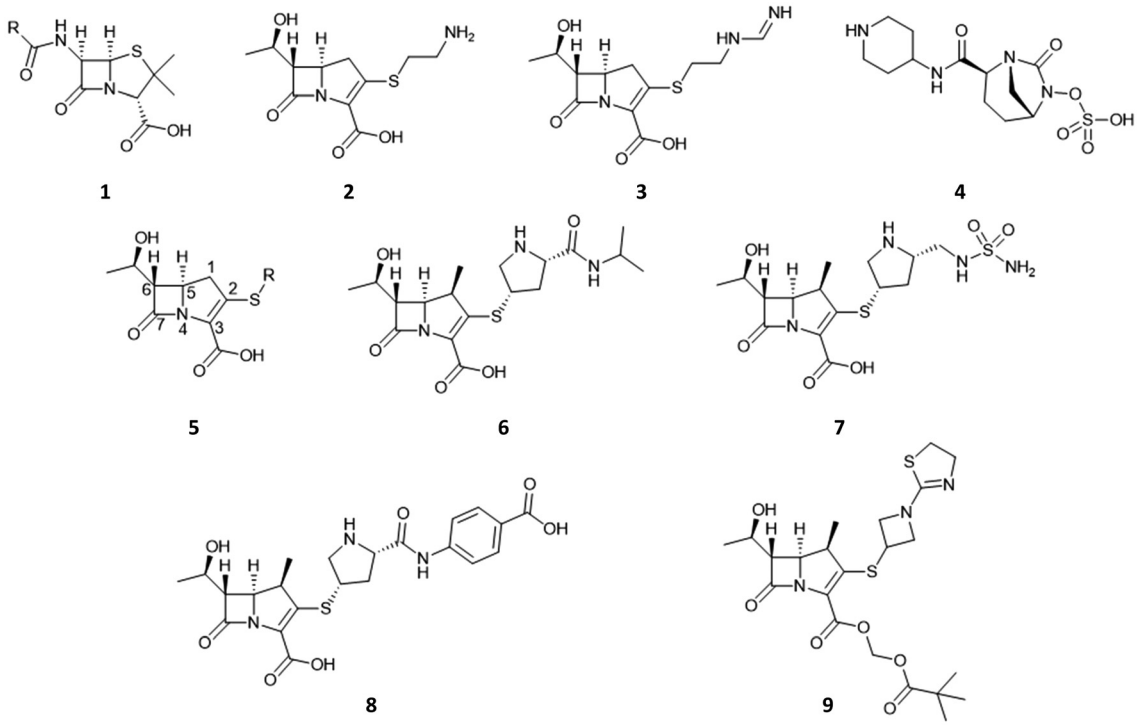


Fig. 1 Chemical structures of penicillin (**1**), thienamycin (**2**), imipenem (**3**), relebactam (**4**), meropenem (**6**), doripenem (**7**), ertapenem (**8**), tebipenem (**9**) and the general structure of a carbapenem (**5**).

The initial promising antimicrobial potency demonstrated by carbapenems generated significant interest and research activity to increase the potency of this new drug class such that it could be used clinically.[6] Thienamycin was used as a model to develop the range of carbapenems in use today. In 1985, the first carbapenem, imipenem (**3**),[7] was approved for clinical use. Despite its increased stability to base hydrolysis (compared to thienamycin), imipenem was still susceptible to degradation by the enzyme renal dehydropeptidase I (DHP-I).[8] It was found that less than 10% of the administered dose was recovered from urine due to DHP-I metabolism of imipenem at the renal brush border.[9] To improve recovery, imipenem was administered with the DHP-I inhibitor, cilastatin[10] which allowed this drug combination to be used safely and effectively in humans. In 2019, a drug called Recarbrio was approved by the FDA[11] to combat the hydrolysis of imipenem by β-lactamases. Recarbrio contained imipenem and cilastatin combined with relebactam (**4**), a β-lactamase inhibitor to treat complicated urinary tract infections.[12]

More recent carbapenem developments have involved the addition of a methyl group at the 1 position, shown in the general structure (**5**), which greatly improved stability against DHP-I. One of the most widely used carbapenems with this modification is meropenem (**6**). Studies on its pharmacokinetics show that almost 70% of meropenem was recovered from urine after 12 hours meaning DHP-I inhibitors were not needed.[13,14] Several other carbapenems have been developed such as doripenem (**7**) and ertapenem (**8**) with varying stabilities and activities. Tebipenem (**9**), approved in 2015, was the first orally bioavailable carbapenem which used a cleavable pivalate ester to deliver the prodrug.[15] There are also other carbapenems in development that target certain bacterial strains such as *Enterobacteriaceae* as well as methicillin-resistant *staphylococci* and *enterococci*[16] which are resistant to currently used carbapenems.[17] Further research is needed to develop new antibiotics to combat the increasing threat of antibiotic resistance.

Kathryn E. Fairfull-Smith

Prof. Kathryn E. Fairfull-Smith received her PhD degree in organic chemistry from Griffith Unviersity in 2004. She worked as a postdoctoral fellow at the University of Sheffield, England, and Queensland Unviersity of Technology (QUT) before beginning her independent academic career at QUT in 2009. She is currently a Director of the QUT Centre for Materials Science and the Associate Dean Research in the Faculty of Science. Her research focuses the use of organic synthesis to build molecules or functionalize surfaces and polymers for various applications, including the development of novel anti-biofilm agents and materials.

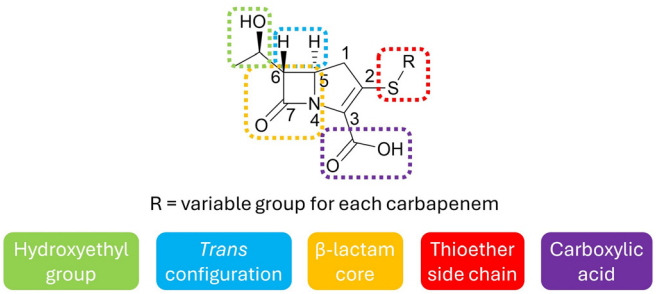


Fig. 2 General structure of a carbapenem highlighting the key functional groups.

1.2 Structure–activity relationships of carbapenem compounds

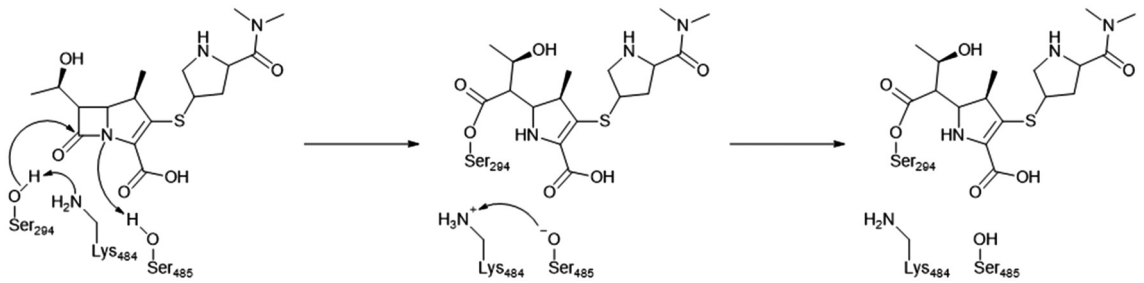
Carbapenems (Fig. 2) are identified by their fused 4- and 5-membered rings. However, in contrast to penicillin (**1**), carbapenems substitute the sulphur at C1 with a carbon whilst also adding an alkene between C2 and C3. The hydrogen atoms at C5 and C6 are in the *trans* configuration and there is also a hydroxyethyl group instead of an amide at C6 to further improve β-lactamase stability.[18] Carbapenems such as meropenem and ertapenem have a pyrrolidine side chain at C2 which has been shown to increase Gram-negative activity to produce a broader antibiotic spectrum.[19] The carboxylic acid group interacts with amino acid residues inside the protein's active site, allowing protein recognition and targeting. As mentioned, the methyl group at C1 in more recently developed carbapenems improves reabsorption of the drug by preventing its metabolism by DHP-I.

The key structural feature of carbapenems, which underpins their antibiotic activity, lies in the β-lactam ring and this moiety is conserved in all β-lactam antibiotics. These antibiotics can inhibit penicillin binding proteins (PBPs) which are enzymes found in bacterial cell walls that catalyse the synthesis of peptidoglycan,[20] a key component of the cell walls. β-Lactam antibiotics kill bacteria by permanently acylating some key amino acid residues in the PBPs active site.[21] Inhibiting PBPs halts cell wall synthesis and leads to death of the bacteria. A proposed mechanism for the irreversible acylation of serine 294 in *P. aeruginosa* PBP3 by meropenem is shown in Scheme 1.[22] Lysine 484 and serine 485 act as proton carriers, shuttling protons around and increasing the rate of reaction. Serine 294 is left acylated by meropenem blocking the enzyme's function. It has been suggested that meropenem is removed by hydrolysis leaving the serine with just an acyl group, however the mechanism for this last step is unclear.[23] Carbapenems can bind to many types of PBPs in a wide range of bacterial species which makes them broad spectrum antibiotics.[24]

β-Lactams such as the cephalosporins and penicillins utilise an acylamino group at C6 whereas carbapenems have a hydroxyethyl group at this position. This is the most important modification as the hydroxyethyl group forms a zwitterion with the carboxylic acid, allowing the drug to pass through bacterial porin channels giving free access to the cell wall.[25] However, the primary effect of this change gives carbapenems greatly enhanced stability to β-lactamases compared to other β-lactams. An array of publications utilising X-ray crystallography and molecular dynamics simulations have revealed the mechanisms behind this stabilising effect.[26] Other types of β-lactams also acylate β-lactamases but the enzyme-drug hybrid is hydrolysed rapidly so that the enzyme can react with another drug molecule. Carbapenems also react initially in the same way, however the hydrolysis step is incredibly slow at a 10 000-fold reduction in reaction rate[27] due to the hydroxyethyl group. This is caused by hydrogen bond formation between the hydroxyl moiety and asparagine 132. This forces the methyl group of the hydroxyethyl chain to sit inside a pocket reserved for a water molecule.[28] This single water is vital for the final hydrolysis step and without it the β-lactamase cannot regenerate to hydrolyse other carbapenem molecules. Therefore, carbapenems also act as suicidal inhibitors of β-lactamases allowing the remaining drug molecules to access their initial target.[29]

1.3 Carbapenems in biofilms

Carbapenems, like most antibiotics, are primarily designed and used to treat infections caused by planktonic bacteria. However, a significant proportion of bacterial infections (some studies estimate up to 80%) are caused by biofilms.[30] As most antibiotics are significantly less effective at combating biofilms, many of these biofilm-associated infections are difficult



Scheme 1 A proposed mechanism for the irreversible acylation of PaPBP3 by meropenem. Reproduced from ref. 22 with permission from ASPET, copyright 2020.

to treat with current antibiotics.[31] This is especially true for carbapenems, as a small number of studies comparing their effectiveness against planktonic bacteria to those in a biofilm show a significant increase in antibiotic concentration is required to inhibit the growth of cells in a biofilm. For example, the minimum inhibitory concentration (MIC) for meropenem against planktonic *Klebsiella pneumoniae* is 16 μg mL^{-1} (Table 1), however, in a biofilm, the minimum biofilm inhibitory concentration (MBIC) of meropenem is 128 μg mL^{-1}.[32] Similarly, the MIC for imipenem in *Burkholderia pseudomallei* is 0.125 μg mL^{-1} and the corresponding MBIC is 256 μg mL^{-1} (Table 1).[33] Thus, the ability of bacterial biofilms to tolerate antibiotics is a serious global health concern. Furthermore, the ineffectiveness of antibiotics in biofilms is exacerbated by the tendency of bacteria in a biofilm to have a higher rate of mutation enabling the development of resistance mechanisms,[34] rendering these antibiotics ineffective even against planktonic cells. Therefore, new solutions are needed to treat biofilm-associated infections.

2. Bacterial biofilms

2.1 What is a biofilm?

Many types of bacteria can form structured communities of cells called biofilms. They consist of multiple layers of aggregated cells coated in a protective layer known as an extracellular polymeric substance (EPS). The EPS is a collection of various biomolecules secreted from the developing bacterial colony. It mainly consists of proteins, lipids, and glycoproteins[35] that form the structural matrix that 'houses' and protects the colony. In most colonies, the bacterial cells account for less than 10% of the dry mass, whereas the biofilm is responsible for the other 90%.[36] The biofilm matrix is a 3-dimensional structure that adheres the colony to a surface and holds the cells in place allowing for more complex operations in the colony. The biofilm can hold secreted enzymes near to the surface creating an external digestion system where nearby nutrients or dead bacterial cells can be recycled for use by the colony.[37] The main function of the EPS is to provide protection to the bacterial colony, from various environmental factors such as water loss, pH or temperature changes, loss of nutrients and the host's immune system.[38] Biofilms also provide significant protection against antibiotics both *via* physically blocking the drug and slowing diffusion, as well as facilitating the exchange of genetic material or allowing the development of persister cells.[39]

"The five-step biofilm model" shown in Fig. 3,[40] was developed from research on *P. aeruginosa*. It is a simplified model of a very complex process that varies greatly in real world applications and different species. The model describes a cyclic pathway that bacteria follow when forming a biofilm. It begins when free individual planktonic cells attach to a surface.[41] This process is reversible, so the planktonic bacteria can be removed or detach from the surface easily. The next stage begins when the bacterial cells start producing the EPS. This sticky matrix firmly adheres the cells to the surface in an irreversible manner. The biofilm then enters the first maturation stage where the bacterial cells begin to divide forming clusters that are several layers thick. The increased production of EPS and cell division allows the microcolonies to grow into an established colony where different cells have specific roles to upkeep the biofilm known as the second maturation stage. The final stage of the biofilm life cycle is called dispersion. This is where bacterial cells are released from the biofilm as individual cells and are free to attach to other surfaces and start the cycle again.[42] It is important to remember that this is a simplified model and biofilms in a non-laboratory setting may behave quite differently. However, the model is a useful starting point to understand biofilm formation.

Since many of the current antibiotics are less effective against biofilms there is a huge need for new treatments that attack biofilms directly. There are two main strategies for treating biofilms. The first is biofilm inhibition, where the biofilm is prevented from forming. The second is biofilm eradication, where established biofilms are broken down and destroyed. Preventing the formation of biofilms is extremely difficult in the human body as generally the signs of infection are only apparent once the biofilm has established itself and has begun to spread virulence factors.[43] Once the patient requires treatment, alternative strategies are needed to combat the mature biofilm.

2.2 Biofilm testing methods

There are a variety of quantitative and qualitative methods used for evaluating biofilm growth and assessing the effectiveness of various treatments. It is important to understand each method and its inherent advantages and disadvantages before conclusions can be drawn regarding the efficacy of carbapenems in biofilms.[44]

Viable cell enumeration is the direct counting of live cells or colony forming units (CFU) to determine the number of viable cells. In this method the biofilm is homogenised and suspended in a medium where aliquots are then taken. Each aliquot undergoes serial dilution which is then plated onto an agar plate. Each plate is incubated (between 24–72 hours) to grow the colony which is then counted by eye. Using the volume of each aliquot and the dilution factor, the mean number of cells in the biofilm can be calculated. This method can be used to create a calibration curve by comparing the optical density (OD) of the original biofilm medium with the experimental cell count. This allows the cell number of the biofilm to be calculated by measuring the OD of the sample without having to plate and count the colonies. This technique does not require specialised equipment and can be done in most laboratories with good aseptic technique. However, this experiment is very laborious, and time consuming to obtain reproducible results and can be affected by counting errors and biases depending on the researcher especially with high colony densities on each plate. This method isn't ideal for testing antibiotics on biofilms as homogenisation removes the

Table 1 Planktonic and biofilm assay data of carbapenems, alone or combined with other treatments

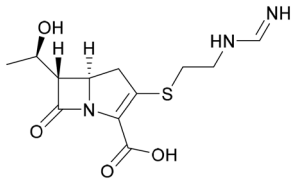
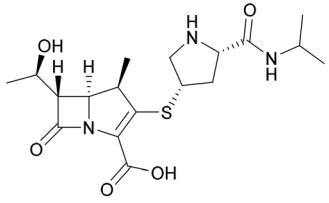
Compound	Strain	MIC (μg ml^{-1})	MBIC (μg ml^{-1})	MBEC (μg ml^{-1})	Other data types	Assay method: planktonic (biofilm)[a]	Ref.
Imipenem  **3**	*P. aeruginosa* ATCC	3.9		7.81		Visible cell enumeration (crystal violet)	104
	PAO1	15.6		31.25			
	T129	15.6		15.62			
	T46	15.6		15.62			
	O7	7.8		15.62			
	O46	7.8		15.62			
	T2	15.6		15.62			
	O135	7.8		15.62			
	O129	15.6		32.25			
	K. pneumoniae BAMC 07-18	0.39			12.5 μm ml^{-1} = 5 log reduction CFU	Broth microdilution (visible cell enumeration)	105
	B. pseudomallei Bp096	0.5	>256	>256		Broth microdilution (resazurin)	33
	Bp041	1	>256	>256			
	Bp011	0.125	256	>256			
	Bp045	1	>256	>256			
	B. pseudomallei 377	2			% Cell viability – planktonic-25.3 biofilm-82.8	Broth microdilution (visible cell enumeration)	67
	M6 (defective biofilm formation)	2			% Cell viability – planktonic-18.3 biofilm-46.2		
	P. aeruginosa strains[b]	4				E test strip	106
	P. aeruginosa 214	2		>2560	MBC planktonic 128	Visible cell enumeration	107
	P. aeruginosa PAO1 10^5	1		512	MBC planktonic 8	E test strip (visible cell enumeration)	77
	PAO1 10^6	1		512	MBC planktonic 16		
	PAO1 10^7	1		>2048	MBC planktonic 32		
	PAΔDDh2Dh3 10^5	0.5		256	MBC planktonic 8		
	PAΔDDh2Dh3 10^6	0.5		1024	MBC planktonic 16		
	PAΔDDh2Dh3 10^7	0.5		1024	MBC planktonic 32		
Meropenem  **4**	*P. aeruginosa* strains[b]	16				E test strip	106
	P. aeruginosa PAOΔmutS	2	16			Crystal violet	108
	CW44	4	64				
	P. aeruginosa PAO1	0.38				E test strip	109
	PAOΔmutS	0.5					
	CW8	1.5					
	CW35	0.5					
	CW44	0.5					
	E. coli KBN12P05816	16				Broth microdilution	110
	KBN12P06081	16					
	K. pneumoniae C2	32		512		Visible cell enumeration	32
	C4	16		128			
	B. cenocepacia LMG 18828	8	Similar to MIC			Resazurin staining	111
	B. cepacia LMG 18821	16	Similar to MIC				
	B. contaminans LMG 16227	4	Similar to MIC				
	P. aeruginosa ATCC 27853	0.5	Similar to MIC				

Table 1 *(Contd.)*

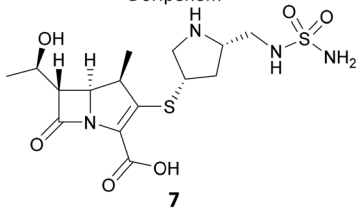
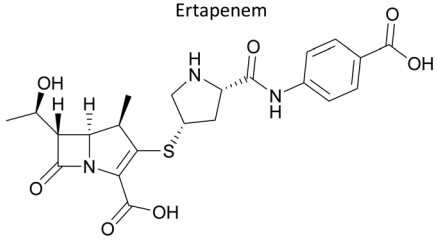
Compound	Strain	MIC (μg ml^{-1})	MBIC (μg ml^{-1})	MBEC (μg ml^{-1})	Other data types	Assay method: planktonic (biofilm)[a]	Ref.
	E. coli ATCC 25922	0.016	Similar to MIC				
	A. baumannii AB1	32				Broth microdilution	112
	AB2	64					
	P. aeruginosa PA01	4				Broth microdilution	113
	P. aeruginosa PA01	1	2	>256		Broth microdilution (optical density)	85
	HUB3	16	16	>256			
	Acinetobacter baumannii Ab15151	0.5		400	MBC planktonic 4	Broth microdilution (visible cell enumeration)	91
	Ab1770	2		>3200	MBC planktonic 4		
	Ab2075	2		>3200	MBC planktonic 4		
	Ab15151 (pOXA-82-2)	32		3200	MBC planktonic 32		
	Ab1987	64		>3200	MBC planktonic 64		
	Ab2147	64		>3200	MBC planktonic 64		
	P. aeruginosa PA01	2	32			Broth microdilution (optical density)	114
	K. pneumoniae BAMC 07-18	0.156	25			Broth microdilution (crystal violet)	115
	P. aeruginosa MB699	64				Crystal violet	116
	Proteus mirablis UFPEDA 767	0.78		3.12		Crystal violet	117
	ICP4	2000		500			
	CP523S	0.78		3.12			
	K. pneumoniae BAMC 07-18	1				Broth microdilution (visible cell enumeration)	105
Doripenem  **7**	*P. aeruginosa* strains[b]	2				E test strip	106
	P. aeruginosa PAO1	1			Cell count reduction Δ log10 2.5 CFU cm^{-2}	Broth microdilution	94
	HUB1	>128			Cell count reduction – no effect		
	HUB2	16			Cell count reduction – no effect		
Ertapenem  **8**	*S. aureus* MSSA 3	0.25		2048	MBC planktonic 0.25	Optical density (crystal violet)	103
	MSSA 4	0.25		2048	MBC planktonic 0.25		
	MSSA 12	0.25		2048	MBC planktonic 2		
	MSSA 13	0.25		2048	MBC planktonic 1		
	MSSA 15	0.25		2048	MBC planktonic 2		
Meropenem + ZnO-NPs	*P. aeruginosa* PU15	4				Broth microdilution	118
Meropenem alone	PU15	512					
YXL-13	*P. aeruginosa* PAO1	>1112	>1112			Optical density	119
Meropenem + YXL-13	PAO1		1				

Table 1 *(Contd.)*

Compound	Strain	MIC (μg ml^{-1})	MBIC (μg ml^{-1})	MBEC (μg ml^{-1})	Other data types	Assay method: planktonic (biofilm)[a]	Ref.
Meropenem alone	PAO1	0.25	8				
IDR 1018 (DJK-5) [DJK-6][c]	*K. pneumoniae* Kp1450421	32 (8) [16]	32 (8) [16]			Broth microdilution	120
	Kp1789769	32 (16) [8]	32 (8) [4]				
	Kp1825971	>64 (>64) [64]	64 (>64) [32]				
	Kp2144392	32 (32) [8]	4 (4) [2]				
	Kp2210477	32 (16) [>64]	32 (8) [>64]				
Meropenem alone (Imipenem alone)	*K. pneumoniae* Kp1450421	>8 (>8)				Broth microdilution	120
	Kp1789769	>8 (>8)					
	Kp1825971	>64 (>64)					
	Kp2144392	>8 (>8)					
	Kp2210477	>8 (>8)					
Meropenem + DJK-6	*K. pneumoniae* Kp1825971		4			Broth microdilution	120
Imipenem + DJK-6	Kp1825971		8				
Imipenem + berberine (Imipenem alone)	*P. aeruginosa* ATCC 27853	1 (1)				Broth microdilution	121
	PA012	32 (256)					
Imipenem + PF-5081090 (Imipenem alone)	*A. baumannii* ATCC 19606	0.25 (1)				Broth microdilution	122
	Ab-84	64 (128)					
	Ab-108	64 (256)					
	Ab-167	64 (256)					
	Ab-176	64 (256)					
	Ab-7	16 (4)					
	Ab-31	32 (4)					
Meropenem + MSNs (Meropenem alone)	*P. aeruginosa* A isolate 1	4 (8)	16 (32)			Broth microdilution (optical density)	123
	PA isolate 2	8 (16)	32 (128)				
	PA isolate 3	8 (16)	16 (64)				
	PA isolate 4	8 (32)	16 (64)				
	PA isolate 5	2 (8)	32 (64)				
	PA isolate 6	2 (8)	32 (256)				
	PA isolate 7	8 (64)	32 (64)				
	PA isolate 8	16 (64)	64 (256)				
	PA isolate 9	2 (8)	16 (64)				
	PA isolate 10	8 (32)	32 (128)				
Imipenem + PCLNs (Imipenem alone)	*K. pneumoniae* ATTCC 4352	0.6 (0.6)				Broth microdilution (crystal violet)	124
	KMU5.5	5 (312.5)			Biofilm adhesion reduction – 74% (<30%)		
	KMU4.5	2.5 (625)					
	KMU2.3	2.5 (80)					

[a] Rows without a method in brackets (), means both planktonic and biofilm assays were tested the same way. [b] The mean MIC of the specified antibiotic against 435 clinical isolates of *P. aeruginosa*. [c] Compounds in brackets () or [ ] correspond to the MIC, MBIC or MBEC with the same brackets.

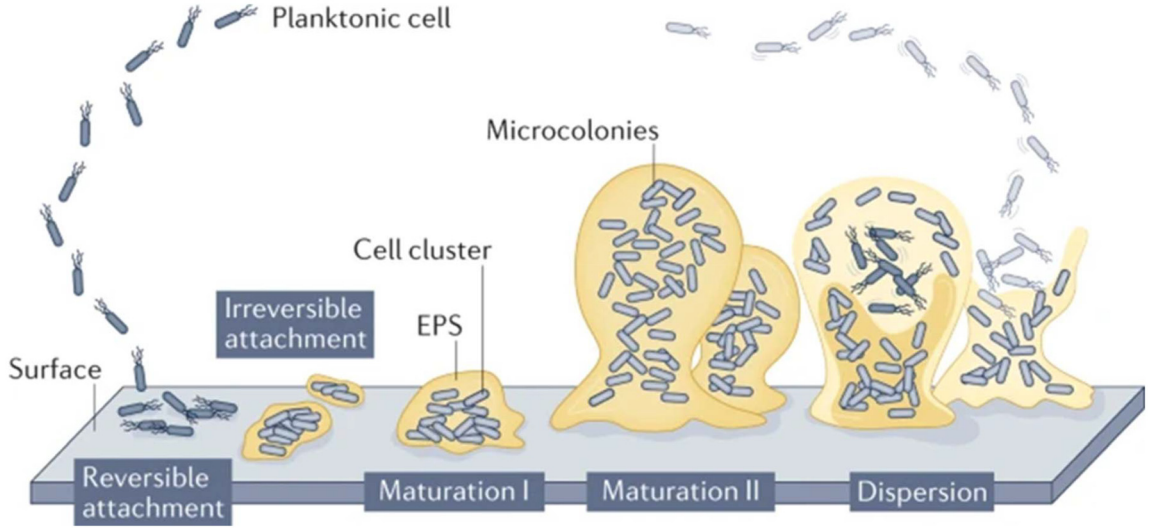


Fig. 3 The five stages of biofilm formation. Reproduced from ref. 42 with permission from Springer Nature, copyright 2022.

protective biofilm from the cells allowing previously resistant bacteria to be killed by the antibiotics in solution.

To improve the automation and eliminate human biases of biofilm measurements, flow cell counting was developed. For this method, a medium containing the homogenised biofilm flows through a very narrow opening where cells are counted as they pass through. The simpler method uses a Coulter counter which is a device that flows charged particles in an electrolyte solution through an opening. When a particle enters the gap, it completes an electrical circuit which is registered as a change in voltage. This voltage change is specific for cells or polymers in the EPS matrix allowing the counting of individual cells and other biofilms components. This technique is very user friendly and much more sensitive than other biofilm measurements however it is limited to dilute samples as the aperture can get clogged[45] and it cannot differentiate between live and dead cells.

A similar method called flow cytometry can be used however this is much more expensive with each unit costing between $80 000–150 000.[46] A medium of cells pass through a narrow opening where a laser is used to detect each cell and it's properties. The cytometer can detect scattering and absorbance of the laser as well as fluorescence from the cells. This allows not only highly accurate cell counts, but also information regarding cell dimensions, surface makeup, and metabolic state. Further information can be gathered using cell staining or fluorescent tags such as green fluorescent protein (GFP).[47]

A variety of microscopy methods are available for cell counting and three-dimensional biofilm observation. As bacterial cells are so small, typically 1–10 μm in length,[48] this is at the edge of what standard compound light microscopes can observe. Fluorescent microscopes can be used to increase the information gained from a biofilm by using a variety of fluorescent stains that allow observation of specific cellular structures and can be specific to live or dead cells. However, these microscopes, as well as the specific light filters and dyes needed, are very expensive and is time intensive to observe and count cells. This method also becomes more difficult as the biofilm matures, not only due to increased cell density but the colony also forms a third dimension. A Petroff-Hausser counting chamber has a highly accurate sample volume well, with an etched grid, this can be used to determine more mature biofilm cell densities.[49]

Confocal laser scanning microscopy (CLSM) is a highly specialized type of microscopy that can produce high resolution and high contrast images of biofilms which can then be reconstructed to produce 3D images. CLSM utilises a pinhole aperture that only allows light from the specific focal area while blocking all other light. This produces a highly focussed image with no background noise. A very sensitive detector such as a photomultiplier tube is needed due to the tiny number of photons passing through the aperture. This method takes a photo of a single 2D point of the biofilm with a diameter of 500 nm (the wavelength of light). This focal area is scanned across the whole sample where every single point image is collated to produce the final 3D image.[50] Additional lasers of varying wavelengths can be used to excite fluorescent markers or stains inside the biofilm to further increase the information available from the sample.[51] These instruments have very high costs associated and require highly trained users to get accurate and focussed images.

There are a variety of indirect methods developed to measure biofilm development. They use specific indicators that correlate to a desired biofilm characteristic such as number of cells, biomass, or metabolic activity. Dry mass is a measure of the mass of a biofilm after all the water has been removed by an oven. It allows for very quick and cheap determination of the size of the biofilm. This method cannot differentiate between cells in the biofilm and the extracellular

matrix so total cell counts can't be estimated. Also, the cells are killed during the process so further analysis by other methods is not possible.

Crystal violet (CV) staining is the most commonly used assay for observing cells in a biofilm. It is used to differentiate between Gram-positive and Gram-negative cells due to Gram-positive bacteria remaining stained after decolourisation with ethanol whereas Gram negative cells lose their colour.[52] The triarylmethane dye can permeate the membranes of both gram type cells allowing it to be used for all biofilm assays. However, CV cannot be used to differentiate between live or dead cells.

CV is a dye commonly used alongside a microtiter plate assay which allows immature biofilms to be studied at low cost (Fig. 4). Microtiter plate assays are also used to test the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) of various antibiotics and other antibiofilm agents. The method for this assay is simple to do and doesn't require advanced equipment and produces reproducible results. A sterile growth medium is loaded into a multi-well microtiter plate (usually 96). The bacteria to be studied is inoculated into each well and the plate is incubated for 24–48 hours. The growth medium and planktonic cells are washed from the plate with deionised (DI) water leaving the biofilm behind adhered to the plate. Depending on the analysis required the biofilm can be scraped off to measure biomass or use one of the previously discussed methods for total cell counts. Dyes can be added such as CV or more specialised ones that can differentiate between live and dead cells.[53] Microtiter assays allow for high-throughput screening of lots of compounds simultaneously and can be repeated quickly. The main disadvantage of using this assay for antibiofilm testing is that the drug is usually added during inoculation before the biofilm has started to form.[54] This can lead to false positives where the planktonic bacteria are inhibited before forming a biofilm, making the drug seem more effective against biofilms.[55]

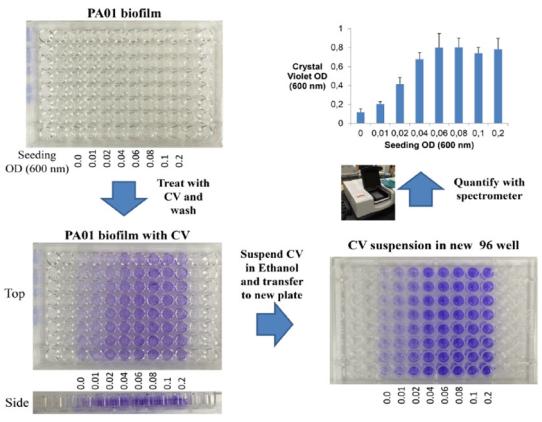


Fig. 4 Schematic of a crystal violet assay of *P. aeruginosa* biofilms on a microtiter plate. Reproduced from ref. 44 with permission from Research & Reviews, copyright 2017.

A way to overcome the limitations of this assay is to use a Calgary biofilm device. This innovative design uses a specialised lid that fits onto standard 96 well microtiter plates.[56] The lid has polystyrene pegs suspended from it that are submerged in the bacterial medium. This allows the biofilm to grow around the pegs instead of the walls of the plate. After incubation, the lid and pegs are transferred to another plate containing sterile growth medium and the drug to be tested. After further incubation, the biofilm is removed from the peg by sonication where it can be analysed by various dyes or viable cell enumeration. Each peg can also be removed and individually analysed used CLSM to study the 3D structure of the biofilm.

Instead of measuring biofilms with dyes or by colony counting, their metabolic activity can be observed using tetrazolium salts. A variety of salts have been developed which allow for quantification of biofilm metabolism using fluorescence spectroscopy.[57] The salt is diluted in an appropriate growth medium, and the biofilm is left to incubate in the medium for 1–3 hours. The enzymes from the biofilm cells reduce the colourless salt into a formazan molecule which is detected by fluorescence spectroscopy. The accumulation of formazan in the growth medium allows for real time analysis of biofilm metabolism.[58,59]

All these biofilm analysis techniques have inherent advantages and disadvantages, with many opportunities for errors to affect the results. Comparing biofilm activity data across the reported literature is difficult, particularly when different analysis methods are used. This issue is further complicated in the literature with the definition of MBEC. It is often defined as the lowest concentration that prevents visible growth,[60] however some publications define it as the concentration that eradicates 99.9% of the biofilm compared to growth controls.[61] In order to draw conclusions from the literature each result requires careful consideration of assay type and conditions.

3. Carbapenem biofilm activity

3.1 Imipenem

Imipenem is a type of carbapenem used clinically around the world. It was the first carbapenem approved for clinical use in 1985[7] and despite its age, is still used for a variety of diseases such as bacterial septicaemia, urinary tract infection (UTI) and endocarditis.[62] As a carbapenem it has a wide spectrum of antibiotic activity including potency against many strains that are resistant to other antibiotics. It is not effectively absorbed through the gastrointestinal tract and is normally administered intramuscularly with a bioavailability of 89%.[63] As discussed in section 1.1, imipenem is quickly metabolised in the kidneys, and requires a double formulation of imipenem and cilastatin to work effectively.[8,10] Imipenem also suffers from severely reduced antibiofilm efficacy compared to its planktonic activity.

Melioidosis is an endemic disease found in Southeast Asia and Brazil. It is caused by the bacteria *B. pseudomallei* and has

quite high lethality rates. Melioidosis is the third most frequent cause of death from infectious diseases in Thailand (after HIV and tuberculosis).[64] It is usually treated by a variety of antibiotics[65] but recently imipenem resistant isolates have been reported.[66] It has been suggested in the literature that this rise of resistance is related to *B. pseudomallei* forming biofilms as this increases the rate that the bacteria develop resistance.[67,68] Bandeira and colleagues[33] investigated the difference in activities of imipenem on *B. pseudomallei* in planktonic and biofilm cells. They firstly categorised the 9 tested strains as weak, moderate, and strong biofilm producers by staining with crystal violet and measuring the optical density at 570 nm. They then measured the planktonic MIC, MBIC, and MBEC values of each strain using Resazurin cell viability assays. Resazurin is a redox indicator that is used to monitor the number of viable cells.[69] Like tetrazolium salts, it is metabolised by active cells where the colour change from non-fluorescent blue to fluorescent pink can be detected and quantified.[70] Every strain had an MIC of at most 1 μg mL^{-1} like Bp038.

A few strains like Bp066 had an MIC of 0.5 μg mL^{-1}. Even Bp011 had an MIC of 0.125 μg mL^{-1}. These results show that imipenem is incredibly active against a variety of *B. pseudomallei* strains. Intriguingly, the biofilm data showed that imipenem was completely inactive in all 9 strains at the maximum tested concentration at just inhibitory levels with MBICs of >256 μg mL^{-1}. It goes without saying that there was no eradication below 256 μg mL^{-1}. There was no correlation with the biofilm production of each strain compared to its resistance to antibiotic treatment. It has been shown that the secretion of β-lactamases is one of the primary carbapenem resistance mechanisms in *B. pseudomallei* cells.[71] Isolates of *P. aeruginosa* biofilms have been shown to increase their production and excretion of β-lactamases into the EPS matrix by exposure to β-lactam antibiotics.[72] *B. pseudomallei* may also use this mechanism to develop increased resistance to imipenem explaining its inability to kill biofilms.

Cystic fibrosis is a relatively uncommon but severe disease that affects roughly 100 000 people worldwide.[73] It is caused by a mutation on the cystic fibrosis transmembrane conductance regulator (CFTR) gene leading to a dysfunctional CFTR protein.[74] Th is disrupts the cells' ability to regulate salt transportation leading to extremely sticky mucous especially in the bronchi and bronchioles of the lungs. The mucous normally traps pathogens but it is too thick to be removed and turns into a breeding ground for bacteria leading to serious infections and organ damage.[75] *P. aeruginosa* is one of the major pathogens in cystic fibrosis infections and readily forms biofilms on the epithelial lining of the lungs.[76] This leads to chronic lung infections that are very difficult to treat. To improve our understanding of how *P. aeruginosa* forms biofilms in cystic fibrosis, Hengzhuang and colleagues[77] investigated how β-lactamase levels affected imipenem and ceftazidime (Fig. 5, **10**) (a cephalosporin) in biofilm activity. The group used two strains of *P. aeruginosa*, PAO1 and PAΔDDh2Dh3, a β-lactamase overproducing mutant. They

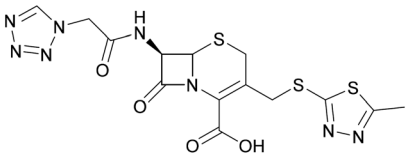


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Fig. 5 Chemical structure of ceftazidime (**10**).

measured the minimum bactericidal concentration (MBC) of planktonic cells using microtiter plate assays and the MBEC was calculated from time kill curves of each strain when exposed to imipenem. The group used three concentrations of cells for each strain at 10^5, 10^6 and 10^7 CFU mL^{-1}. In planktonic PAO1, MBCs of 32, 32 and 128 μg mL^{-1} were recorded for ceftazidime respectively. Imipenem gave MBCs of 8, 16 and 32 μg mL^{-1} in the same strains. The difference between the antibiotics is displayed during the PAΔDDh2Dh3 strain MBC tests. Ceftazidime gave MBCs in the mutant of 32, 128 and >512 μg mL^{-1} for each cell concentration. Whereas imipenem was completely unaffected by the increased β-lactamase production with MBCs of 8, 16 and 32 μg mL^{-1}. This shows how important carbapenems are and why their inherent resistance to β-lactamases is so vital for these antibiotics. However, when treating the biofilms of each *P. aeruginosa* strain with imipenem, the efficacy drops dramatically. Each biofilm was cultured on alginate beads at the same concentrations as the planktonic cells, 10^5, 10^6 and 10^7 CFU mL^{-1}. In PAO1 imipenem gave MBECs of 512, 512 and >2048 μg mL^{-1} respectively. In PAΔDDh2Dh3, imipenem gave MBECs of 256, 1024 and 1024 μg mL^{-1} respectively. This huge decrease in antibiotic activity in biofilm compared to planktonic cells shows how important new treatments are for combating biofilm infections. In addition, imipenem in the biofilm had similar activities between the mutant and refence strains, showing how little effect these specific β-lactamases have on imipenem.

3.2 Meropenem

Meropenem is a broad-spectrum, carbapenem antibiotic frequently prescribed for the treatment of severe bacterial infections. First patented in 1983, meropenem is on the World Health Organization's list of essential medicines[78] and is recommended to only be used as a last resort when other routes have failed. Meropenem is listed as a "critically important antimicrobial for human medicine"[79] and if enough strains of bacteria develop resistance, millions of patients will die from infections. Meropenem is administered for a variety of infections such a meningitis, pneumonia, and sepsis[80] and permanently inhibits PBPs in bacterial cell walls, stopping its activity and killing the cell. This mechanism of action is well understood in planktonic cells, but there is limited research on how meropenem acts in a biofilm. However, there are some examples that can showcase the difference between its activity in planktonic and biofilm states.

Biofilms are prevalent in many types of industries, but they become extremely damaging to human health in the medical field.[81,82] Orthopaedic implants such a titanium screws and plates, as well as prosthetics are particularly susceptible to biofilm formation. Recently, there has been significant increases in Gram-negative bacilli (GNB) infections caused by biofilms forming on implants[83] which are limiting therapeutic options.[84] To find a potential solution, Benavent and colleagues[85] investigated how meropenem affects biofilms of *P. aeruginosa* in both meropenem susceptible (PAO1) and meropenem resistant strains (HUB3). They also investigated how the addition of colistin, an antibiotic specifically for Gram-negative infections,[86] may improve anti-biofilm activity. They measured the MIC, MBIC, and MBEC of both strains with respect to meropenem. As expected, the MIC for PAO1 and HUB3 were 1 and 16 μg mL^{-1} respectively. The MBIC results showed a slight increase for PAO1 at 2 μg m^{-1} with HUB3 remaining the same at 16 μg mL^{-1}. However, the MBEC results show how biofilms improve resistance to antibiotics with meropenem failing to eradicate both strains at 256 μg mL^{-1}. However, the biofilms in this study were evaluated after 48 hours, so meropenem may have a greater effect with a less mature biofilm. The addition of colistin to the meropenem infusion produced a more active treatment of −4.98 $\log_{10}$CFU mL^{-1} after 54 hours compared to meropenem monotherapy with a value of −3.8 $\log_{10}$CFU mL^{-1}. This over 10-fold increase in activity shows there is room for optimisation of meropenem therapy, especially for infections caused by dangerous microbes.

Like *P. aeruginosa*, *Acinetobacter baumannii* is listed as 'critical' on the World Health Organisation's list of priority pathogens for new antibiotics,[87] specifically for carbapenem resistant strains.[88] This is a serious bacterial pathogen that is primarily acquired through hospital infections. In the last decade it has been largely associated with soldiers returning from combat zones such as Iraq and Afghanistan.[89] *A. baumannii* forms biofilms that make it much more difficult to treat and can increase the rate of antibiotic resistance.[90] Wang and colleagues[91] investigated the anti-biofilm activity of a variety of antibiotics including meropenem, on six *A. baumannii* strains (Ab15151, Ab1770, Ab2075, Ab15151 (pOXA-82-2), Ab1987, Ab2147). They measured MBCs which they defined as the antibiotic concentration that reduced the number of viable cells by ≥99.9%. They used visible cell enumeration by incubating both planktonic and biofilm cells with serially diluted antibiotics and counting the number of cells that survived the treatment. Ab15151 was used as a reference strain, and along with Ab1770, Ab2075, are all, carbapenem susceptible *A. baumannii* (CSAb). Ab15151 (pOXA-82-2) is a strain with the bla_{OXA-82} gene mutation inserted into the plasmid as found in emerging strains of carbapenem resistant *A. baumannii* (CRAb) in Taiwan.[92] Ab1987 and Ab2147 are also CRAb strains. Each strain was exposed to meropenem and all three of the CSAb strains that were tested showed MBCs for meropenem in planktonic cells of 4 μg mL^{-1}. The planktonic MBCs for meropenem in the other three strains were considerably higher with 32 μg mL^{-1} in Ab15151 (pOXA-82-2) and 64 μg mL^{-1} in Ab1987 and Ab2147 which is expected due to their inherent resistance. Concerningly, the MBCs for meropenem against all strains except Ab15151 (400 μg mL^{-1}) were >3200 μg mL^{-1}, implying meropenem had virtually no effect on the biofilms of any of these strains, even the carbapenem susceptible ones. Wang's group also tested the anti-biofilm activity of meropenem in combination with other antimicrobials, sulbactam, colistin, and tigecycline over 24 and 48 hours. The CSAb reference strain Ab15151 after 24 hours of meropenem exposure at 2 μg mL^{-1} reduced the bacterial load by 1.33 $\log_{10}$CFU mL^{-1} compared to no antibiotics. Exposure to meropenem for 48 hours reduced the bacterial load by 5.33 $\log_{10}$CFU mL^{-1} which is classed as bactericidal (>log 3 reduction). This shows how these antibiotics require extended treatment times to be effective against biofilms. The combination therapies of meropenem + sulbactam, colistin and tigecycline after 24 hours reduced bacterial loads by 2.05, 1.92, and 2.5 $\log_{10}$CFU mL^{-1} respectively. After 48 hours, the bacterial loads decreased by 5.58, 4.57 and 5.15 $\log_{10}$CFU mL^{-1} respectively. All combination therapies were better at biofilm reduction over 24 hours compared to meropenem alone. However, over 48 hours, colistin and tigecycline performed worse in combination with meropenem, with sulbactam only being slightly better. This suggests that there may be some interference mechanism at play between the different antibiotics. As meropenem requires porins (membrane protein channels) to cross the cell membrane,[93] these transport proteins may be blocked by the other antibiotics, reducing meropenem's diffusion rate and therefore its ability to kill the bacteria. However, this study only used one concentration for each antibiotic, with sulbactam at 4 μg mL^{-1} and the rest at 2 μg mL^{-1}. There may be more optimal concentrations for each antibiotic which would allow them to act synergistically. The CRAb strain Ab15151 (pOXA-82-2) showed significantly higher biofilm resistance with just meropenem exposure showing a decrease of 1.25 $\log_{10}$CFU mL^{-1} after 24 hours and only 1.41 $\log_{10}$CFU mL^{-1} after 48 hours. This shows how this strain is extremely resistant to meropenem. Interestingly, despite the lack of meropenem activity, the combination of meropenem with sulbactam and tigecycline almost bring the bacterial load reduction in line with the carbapenem susceptible strain. But meropenem and colistin exposure showed very low antibiofilm activity (1.00 and 1.42 $\log_{10}$CFU mL^{-1} after 24 and 48 hours respectively) against the CRAb strain Ab15151. Comparable tests against two clinical strains from each resistance category show wildly different results to the previously tested reference strains. For example, the CRAb strain Ab1987, when exposed to meropenem and tigecycline, had a bacterial load reduction of 0.21 $\log_{10}$CFU mL^{-1} despite the previously mentioned strain having a reduction of 4.98 $\log_{10}$CFU mL^{-1}. These results highlight the differences between reference strains and clinically isolated strains and how important it is to select the most appropriate strain for testing. Comparing different strains even with similar characteristics is very difficult and care must be taken when drawing conclusions from results like these.

3.3 Biofilm activity of other carbapenems

As imipenem and meropenem are the most commonly prescribed carbapenems, there is a significant focus on them in the literature. There are fewer reports on the potency of other carbapenems and even less on their anti-biofilm activities. This is a problem as eventually bacteria will develop resistance to the commonly used carbapenems, and there will only be a limited understanding of other carbapenems and their optimal antibiotic activities. Tamayo and colleagues[94] have expanded this area of research by investigating how doripenem (7) (a carbapenem first approved for UTIs in 2007)[95] activity is affected by multidrug-resistant *P. aeruginosa* biofilms in combination with colistin. The group used three strains, carbapenem susceptible reference PAO1, and two clinical isolates, HUB1 which is extensively drug resistant as well as HUB2 which is multi-drug resistant. Both clinical strains caused outbreaks in the Hospital Universitario de Bellvitge in Barcelona, Spain.[96,97] The MICs of doripenem in each strain was measured by broth microdilution. In PAO1, doripenem had an MIC of 1 μg mL^{-1}. In HUB1 it was >128 μg mL^{-1} and HUB2 was 16 μg mL^{-1}. Unfortunately, the group did not calculate MBIC and MBEC values for doripenem in biofilms, but they did several experiments regarding the bacterial killing of each drug over time. They found that doripenem monotherapy at 25 μg mL^{-1} was effective at reducing the biofilm of PAO1 after 72 hours by 2.5 $\log_{10}$CFU cm^{-2} however this wasn't bactericidal. As expected, doripenem was completely ineffective against the carbapenem resistant strains HUB1 and HUB2. Combination treatment of doripenem 25 μg mL^{-1} and colistin 1.25 μg mL^{-1} displayed some additive effects against all three strains, reducing the biofilm initially at 32 hours by 4.0 $\log_{10}$CFU cm^{-2} in PAO1. However, the biofilm regrew after 72 hours to a cell count that was higher than doripenem monotherapy after 72 hours. This shows how important eradication values are when it comes to treating biofilm infections as even a small number of surviving cells can regrow and form a new biofilm that may have acquired antibiotic resistance.

Ertapenem (**8**) is another relatively new carbapenem first authorised for use in 2001[98] with limited published data on its antibiofilm activity. It is shown to be effective against a range of Gram-positive and Gram-negative bacteria and is used for complicated intra-abdominal and surgical site infections.[99] *S. aureus* is one of the main causes of bacterial endocarditis, a life-threatening inflammation of heart chambers and valves.[100] Biofilm formation on the epithelium of the heart makes endocarditis extremely difficult to treat leading to a very high mortality.[101,102] Gilbertie and colleagues[103] suggested that ertapenem in combination with cefazolin (Fig. 6, **11**) may synergistically eradicate biofilms of methicillin susceptible *S. aureus* (MSSA) as a possible treatment for endocarditis. The group used ten strains but five of them were clinical bloodstream isolates of MSSA from patients with endocarditis, so the focus will be on the isolates. Firstly, the group used broth microdilution to determine the planktonic MBCs of each strain when exposed to ertapenem or cefazolin. The five MSSA strains were 3, 4, 12, 13, 15. The MBCs of ertapenem against each strain at 10^5 CFU mL^{-1} was 0.25, 0.25, 2, 1 and 2 μg mL^{-1} respectively. The MBCs of cefazolin for each strain was 0.25, 0.125, 2, 1 and 2 μg mL^{-1} respectively. These results are very similar for both antibiotics in planktonic cells. Comparing these results to the biofilms of each strain shows how resistant MSSA biofilms are to these antibiotics. They calculated the MBECs using a Calgary biofilm device and used proteinase-K to detach biofilms from the pegs for further incubation. For both antibiotics, the MBEC values for all 5 isolates were 2048 μg mL^{-1} however it is possibly higher than this as this was the maximum concentration of each antibiotic tested and no greater than (<) symbol is apparent in the data. Excitingly, the treatment of these biofilms with a combination of ertapenem and cefazolin showed promising results with very strong synergy being shown across all 5 strains. In isolate 3, the combined concentration of both antibiotics to produce biofilm eradication was 128 μg mL^{-1} for ertapenem and 64 μg mL^{-1} for cefazolin. This is a 16-fold reduction in MBEC compared to ertapenem alone with a fractional inhibitory concentration (FIC) of 0.09. This trend continued with the other 4 clinical isolates. Despite being significantly higher than the planktonic data, these combinations are 8 to 32 times more potent than ertapenem's monotherapy MBEC and is a promising start to improving therapies for these horrible diseases.

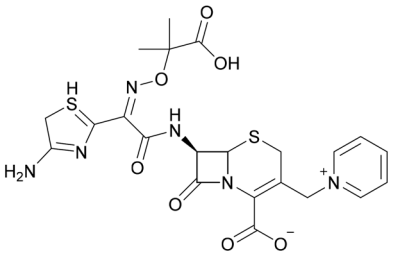

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Fig. 6 Chemical structure of cefazolin (**11**).

4. Strategies for improving carbapenems in biofilms

With the prevalence of multi-drug resistant bacteria increasing at an exponential rate especially in intensive care units, the overreliance on antibiotics will ultimately lead to a significant number of deaths.[125,126] Combined with the difficulty of developing new antibiotics, bacterial resistance is a huge problem. One possible solution is to combine known antibiotics with compounds that can disrupt biofilms as new treatment options. This will prolong the useful lifetime of existing antibiotics and provide a longer lead for the development of new antimicrobial strategies.

4.1 Quorum sensing inhibitors

One alternative antimicrobial approach is to interfere with chemical signalling pathways in bacteria. Bacterial communication has been studied for over 50 years with the term "quorum sensing" (QS) introduced by Dr Steven Winans in 1994 to define bacteria's coordinated response within colonies and carry out colony-wide functions to improve survival in adverse environmental conditions.[127,128] Over the last decade, interest in this area has grown, especially with the quest for new antibacterial medicines.

Bacteria use signalling molecules called autoinducers to coordinate responses to environmental stimuli to aid with cell survival and proliferation. (QS) is the regulation of gene expression in response to changing cell populations[129] which describes the production, release, and detection of these autoinducers.[130] The autoinducers generally work by a minimum threshold concentration mechanism. At low cell densities, the concentration of autoinducers is low. As the cell density increases, the autoinducer concentration also increases until it reaches a threshold level that allows the bacteria to regulate their gene expression in response to the population density. There are many processes that are controlled *via* quorum sensing, for example, bioluminescence, virulence factor production and biofilm formation.[130] Most of these processes are difficult for a few bacteria to accomplish as they are resource intensive but can be used effectively with a large group of cells working towards a concerted goal.

Quorum sensing inhibitors (QSIs) are molecules that block the signalling pathways in biofilms, disrupting gene regulation and leading to the breakdown of the biofilm. This is referred to as "quorum quenching".[131] There are 3 main strategies used for quorum quenching: (1) enzymatic degradation of the autoinducer; (2) blocking the autoinducer signal receptors and (3) inhibiting autoinducer synthesis.[132] There are a range of compounds that have been shown to inhibit QS, many of which are derived from natural products.[133] By studying the inhibitory effects of various naturally synthesised molecules, researchers can modify their structures to improve their activities. The main benefit of these QSIs is that they don't kill bacteria and only interfere with growth and virulence factors, removing the selection pressure for developing resistance.[134]

QSIs are found in a variety of forms both natural and artificial but they all have the potential to be used as antibacterial therapeutics. Zinc oxide nanoparticles (ZnO-NPs) are a type of QSI that have been shown to inhibit the *P. aeruginosa* signalling molecule pyocyanin. It is a vital virulence factor that is used for biofilm formation and is extremely toxic to human cells.[135,136] Although the mechanism for inhibition is still unclear, ZnO-NPs can decrease the production of pyocyanin up to 80%.[137] El-Telbany and colleagues[118] demonstrated how combining meropenem with zinc oxide nanoparticles showed synergistic antibiofilm activity compared to meropenem monotherapy. They measured MICs of both meropenem and the nanoparticles in planktonic *P. aeruginosa* strain PU15. This strain was chosen as it is highly carbapenem resistant and produced the thickest biofilm making it more difficult to treat, improving the validity of the results. The MIC of meropenem alone was 512 μg mL^{-1} which is incredibly high for planktonic cells showing just how resistant PU15 is to meropenem. The MIC for ZnO-NPs was 64 μg mL^{-1}. However, when meropenem and ZnO-NPs were administered together the MIC dropped to 4 μg mL^{-1}. This is a considerable decrease, which is highlighted by the FIC index. The group calculated an FIC of 0.007 which is highly synergistic (a value of less than 0.5 shows synergy). Unfortunately, the group did not calculate MBIC or MBEC values for these compounds in biofilms, however they did observe the effects using scanning electron microscopy (SEM). The microscope images in Fig. 7 showed that a combination of ZnO-NPs and meropenem. At 8 and 4 μg mL^{-1}, respectively, completely eradicated the preformed *P. aeruginosa* PU15 biofilm. This highly effective treatment is a very promising way to increase the efficacy of carbapenems in biofilms.

Another class of natural QSIs are the *N*-acyl homoserine lactone (AHL) inhibitors. The general structure of an AHL **12** is shown in Fig. 8. AHLs follow a complex cascade for gene regulation and QS but there are structurally similar molecules that can inhibit these signalling pathways causing disruption of bacterial processes, such as biofilm formation. Jiang and colleagues[119] demonstrated how an analogue of an AHL molecule, called YXL-13 (Fig. 8, **13**), was able to synergistically improve the activity of meropenem in biofilms of *P. aeruginosa*. The group first showed how meropenem is

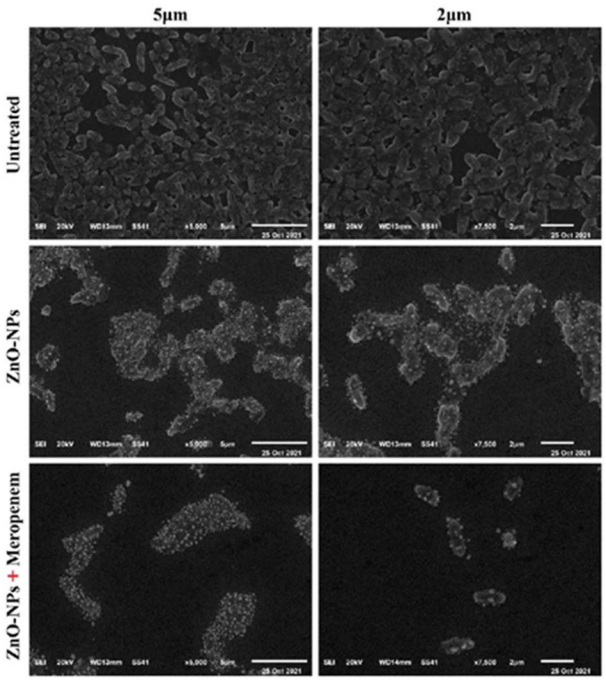

Fig. 7 SEM images of biofilms formed by *P. aeruginosa* PU15 (scale bars 5 μm and 2 μm). Untreated biofilm (top), ZnO-NPs treated biofilm (middle), and meropenem–ZnO-NPs combination-treated biofilm (bottom). Reproduced from ref. 118 with permission from MDPI, copyright 2022.

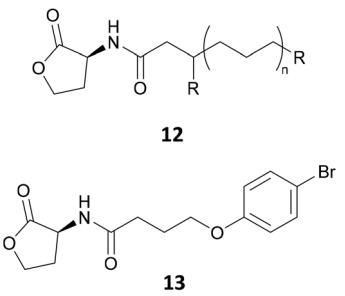

Fig. 8 Chemical structures of a general AHL **12** and the AHL analogue YXL-13 (**13**).

inhibited by biofilms of PAO1 with an MIC of 0.25 μg mL^{-1} compared to the MBIC of 8 μg mL^{-1}, which was a 32-fold increase. The MBIC for meropenem in PAO1 biofilms was still relatively low compared to other MBIC results (Table 1). However, PAO1 is a carbapenem susceptible strain so this value would increase significantly if tested with the carbapenem resistant *P. aeruginosa* strains found (with increasing frequency)[138] clinically. The group then used a checkerboard assay to measure how meropenem performed in combination with YXL-13 in biofilms of PAO1. A checkerboard assay is used to measure the effects of drug combination therapies. Varying concentrations of each compound is dispensed along the rows and columns to get a wide array of combinatorial information.[139,140] Meropenem alone had an MBIC of 8 μg mL^{-1} and a combination of meropenem and YXL-13 had an MBIC of 1 μg mL^{-1}. This 8-fold increase in activity shows how YXL-13 increased the susceptibility of the biofilm to meropenem. The measured FIC index of 0.128 demonstrated that each compound acted synergistically to inhibit bacterial growth. This is an exciting area of drug development as there is a large range of QSIs that could be utilised to improve activities of existing antibiotics, or even "resurrect" old antibiotics that have lost their activity due to resistance.

4.2 Antibiofilm peptides

Whilst a large proportion of new anti-biofilm agent research is focused on synthetic small molecules, an alternate strategy utilising cationic amphipathic antimicrobial peptides (AMPs) has emerged as a promising way of disrupting mature biofilms.[141] When used in combination with antibiotics, the peptide can inhibit or disrupt the biofilm and EPS layer allowing the antibiotics to kill the unprotected bacteria. Many of these peptides are derived from host defence peptides (HDPs) which are naturally occurring in all organisms that are vital to the innate immune system for killing pathogens.[142] By taking these peptides and modifying them, researchers have been able to reduce biofilm mass up to 80% in *P. aeruginosa* biofilms using the human derived peptide LL-37.[143] Ribeiro and colleagues[120] have used synthetic HDPs to investigate the effects they have on biofilms of *K. pneumoniae* in combination with carbapenems. Initially they measured how each peptide performed in biofilms of five clinical isolates of *K. pneumoniae*. Each isolate was multidrug resistant, and they all tested positive for β-lactamase production as well as harbouring bla_{KPC} genes. These genes code for a β-lactamase enzyme known as *Klebsiella pneumoniae* carbapenemase (KPC) and are easily transferable by horizontal transfer of plasmids[144] which is a major cause of concern for hospitals around the world.[145] The three peptides IDR 1018, DJK-5 and DJK-6 were tested by broth microdilution to find the MICs and MBICs of each peptide in the five isolates. Each peptide showed similar biofilm inhibition properties with MBICs of 4–32 μg mL^{-1} in all five isolates. Flow cell assays examining the effect of each peptide on mature two-day old biofilms showed that DJK-6 had the highest activity in *K. pneumonia* biofilms showing almost complete dispersal at a concentration of 2 μg mL^{-1}. DJK-6 was then used for synergy studies with a variety of antibiotics: meropenem, imipenem, cefotaxime, colistin, and polymyxin against biofilms of the isolate KpC1825971. Only the two carbapenems, meropenem and imipenem, showed synergy with the AMPs, the other three were statistically indifferent. Due to the production of β-lactamase enzymes in KpC1825971, unsurprisingly meropenem and imipenem had MBICs of >64 μg mL^{-1}. Compared to other literature data these MBICs are more likely to be around 256 μg mL^{-1}.[32] Combining these carbapenems with DJK-6 showed very exciting anti-biofilm results. The MBIC for meropenem and imipenem combined with DJK-6 was 4 and 8 μg mL^{-1} respectively and both gave an FIC of 0.4 showing synergy. This result is even more impressive given the definitions used by Ribeiro's team. Many researchers define MBIC as only partial inhibition of bacterial growth,[33] some literature has defined MBIC as >90% of biofilm inhibition compared to a control.[146] Ribeiro's group defined MBIC as inhibition of 100% of the biofilm. This is very difficult to achieve especially with such low concentrations of antimicrobials, making their results better than other data reported in the literature. However, this variation in definitions continues to make comparisons between biofilm literature extremely difficult. This huge increase in anti-biofilm activity of both carbapenems, especially in a highly resistant strain, shows just how effective AMPs can be as future drug candidates when combined with antibiotics.

5. Carbapenem delivery systems targeting biofilms

In recent years, the persistence of bacterial biofilms has emerged as a formidable challenge in the field of healthcare, posing significant obstacles to effective treatment strategies. As antibiotic resistance continues to escalate, novel approaches to combat biofilm-associated infections are imperative. This section explores novel, carbapenem biofilm delivery systems using innovative technologies designed to specifically target and dismantle bacterial biofilms. Given that most biofilm infections are associated with medical devices, chronic wounds, and prosthetic implants,[39,147] the ability to target these areas more effectively would greatly improve the progno-

sis of millions of affected patients. Due to the limited exploration of carbapenems in biofilms, some of the following treatments are only tested in planktonic bacteria. Nevertheless, it is valuable to incorporate these treatments in this review, as they have the potential to be effectively employed in antibiofilm therapies.

5.1 Nanoparticle delivery systems

As biofilms form physical barriers that prevent antibiotics from accessing the bacterial cells inside, a significant challenge in antibiofilm treatments is achieving a sufficiently high dose of antibiotics within the cells to effectively eliminate them. One solution to this problem involves encapsulating antibiotics inside nanoparticles, providing protection against degrading enzymes and enabling more targeted therapies. The ability to control the release of an antibiotic from a nanoparticle carrier holds the potential to develop highly effective therapies for biofilms.

Sepsis is a relatively common but life-threatening condition, with fatality rates for severe sepsis at 50% and 80% for septic shock.[148] With a cost of $38 billion annually in the USA alone[149] new treatment options are desperately needed to prevent the spread of infections that lead to sepsis. Even with current methods, antibiotic resistance makes treatments increasingly difficult. Qing and colleagues[150] have developed an innovative thermally activated nanoparticle that can transport carbapenems to the site of the biofilm. They formulated a thermo-responsive nanostructure (TRN) from two natural fatty acids with a tuneable melting point. Once the carbapenem is encapsulated by the TRN, the hydrophobic particle is wrapped in a phospholipid called DSPEPEG2000 for increased biocompatibility and solubility. During capsule formulation they found that using lauric acid and stearic acid in a ratio of 3.5 : 1 produced a TRN with a melting point of 43 °C which is ideal for use in human tissue. The TRN was heated using an IR laser (808 nm, 0.5 W cm^{-2}) with low intensity to minimise surrounding tissue damage. A diagram explaining how the drug therapy works is shown in Fig. 9. IR-780 iodide (IR780) is a NIR fluorescence dye that has been utilised in photothermal therapy before[151] and is the component used to absorb IR energy and heat the TRN. The drug loaded nanoparticles were initially tested for their compatibility with mouse embryo cells (3T3 cells) and human umbilical vein endothelial cells (HUVECs). TRN showed no clear cytotoxic effects on cells with 90% viability in both cell lines. The group conducted *in vitro* testing of the loaded nanoparticle on clinical isolates of multidrug resistant *Escherichia coli* (MDREC) and methicillin resistant *Staphylococcus aureus* (MRSA). The naming conventions for each test condition are shown in the Fig. 9 caption. In both bacterial species exposure to IMP@TRN and IMP/IR780@TRN showed no decrease in CFU counts. This shows that the nanoparticle has no leakage of drug when not exposed to IR which is ideal for a targeted therapy. Upon exposure to IR radiation, IMP/IR780@TRN + NIR drastically reduced the planktonic cell counts, with both species showing a decrease of 4.5 $\log_{10}$CFU mL^{-1}. Imipenem exposure at three times the concentration that was inside the TRN only caused a cell count reduction of 2.5 $\log_{10}$CFU mL^{-1} in MDREC and 1 $\log_{10}$CFU mL^{-1} in MRSA. Showing that the combined therapy was up to 1000 times more effective at killing the bacteria than imipenem alone. This impressive combination of antibiotic and nanoparticle demonstrates how effective targeting of the encapsulated antibiotic can show much greater results compared to free antibiotics. This treatment strategy would work extremely well for patients with biofilms on medical implants such as a knee replacement. The laser would be placed on the skin above the biofilm which would melt the nanostructure and open the capsule releasing the drug inside the biofilm. This would likely be much more effective than injecting free carbapenem into the bloodstream.

Biofilms of carbapenem resistant *P. aeruginosa* are a major problem particularly for treatment of chronic wounds including burns. *P. aeruginosa* is an opportunistic pathogen that is notorious for colonising burn wounds leading to severe infections.[152] The ability of this bacteria to readily form a biofilm at the burn site leads to severe complications with treatment. Biofilms are known to cause chronic wounds in burn victims due to constant inflammation being triggered by the immune system unable to clear the biofilm.[153] This perpetual cycle of inflammation and wound reopening leaves the patient in immense pain and at risk of bacteraemia and sepsis.[154] A study found that almost 80% of chronic wounds are associated with biofilms,[155] and that *P. aeruginosa* is responsible for 77% of burn wound mortalities.[156] With the majority of these deaths likely caused by antibiotic resistant strains of bacteria it is very important that new ways of treating chronic burn wounds caused by biofilms are developed.

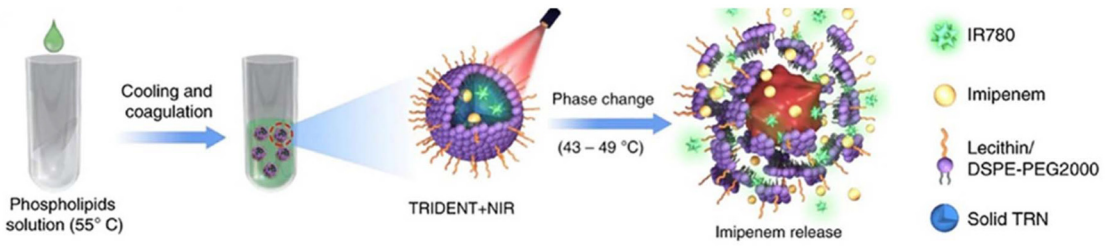

Fig. 9 Schematic illustration of the synthesis and infrared triggered antibiotic release of imipenem by the nanoparticle TRIDENT. IMP@TRN is imipenem in the nanoparticle with no IR. IMP/IR780@TRN is imipenem + the IR780 dye in the nanoparticle with no IR. IMP/IR780@TRN + NIR is imipenem + the IR780 dye in the nanoparticle with IR exposure. Reproduced from ref. 150 with permission from Nature, copyright 2019.

The utilisation of nanotechnology to selectively target biofilms should increase the effectiveness of carbapenems. Memar and colleagues[123] have utilised mesoporous silica nanoparticles (MSNs) to effectively transport meropenem directly to the cells of *P. aeruginosa* biofilms. The MSNs were synthesised using a previously described procedure,[157] and meropenem was loaded onto the nanoparticles by stirring for 24 hours in solution. MSNs are more stable than similar nanoparticles such as liposomes due to the strong Si–O bond and resistant chemical and biological degradation. Depending on the synthetic route and other additions, they can be fully functionalised for pore size (loading concentration), or surface components (able to target specific cells).[158] The researchers used a formulation that was designed to mimic the lipid-bilayers of bacterial cell membranes allowing the nanoparticle to pass through into the cell.[159] Memar used 10 carbapenem resistant clinical isolates of *P. aeruginosa* for testing. The mechanisms for resistance were overexpression of efflux pump (4 strains), decreased expression of porins (4 strains), and carbapenemase production (2 strains). Firstly, the planktonic activity of meropenem alone and meropenem loaded MSNs were calculated by broth microdilution to find the MICs. In all isolates meropenem exhibited MICs from 8 μg mL^{-1} up to 64 μg mL^{-1}. Meropenem loaded MSNs showed a good increase in activity with an average 8-fold decrease in MICs for all isolates compared to meropenem alone. Even the most resistant isolates had impressive MIC reductions at 8 and 16 μg mL^{-1} respectively compared to meropenem monotherapy MICs of 64 μg mL^{-1}. This trend continued with the antibiofilm assays, where the group used the OD_{650} of crystal violet stained biofilms to calculate the MBICs for each isolate. The lowest MBIC for any isolate treated with meropenem alone was 32 μg mL^{-1}. However, most isolates were between 64 and 256 μg mL^{-1}. These results align the rest of the published literature that biofilms cause a significant decrease in antibiotic activity compared to planktonic cells. When compared to meropenem loaded MSNs the data shows that the nanoparticles can promote the activity of meropenem and more efficiently deliver the antibiotic to the bacteria. Across all isolates, the MBICs decreased by 2 to 8 times during combined therapies. The MBICs for isolate one and nine had the best activity of meropenem loaded MSNs at 16 μg mL^{-1}. The cell viability assays also showed that meropenem loaded MSNs were not cytotoxic up to 512 μg mL^{-1}. This report demonstrates that silica nanoparticles can effectively increase the efficacy or carbapenems against *P. aeruginosa* biofilms however the researchers did not discuss nanoparticle mode of action providing improved anti-biofilm activity. More research is needed into the physiochemical properties of the nanoparticles and how, mechanistically, they interact with cell membranes. Although promising, the biofilm inhibition of meropenem loaded MSPs is not active enough for a therapeutic treatment. It is possible that the outer surface of the nanoparticle could be refined to interact less with the polymers found in the EPS of biofilms. As most of the EPS is negatively charged due to the dominance of carboxyl and hydroxyl groups,[160,161] specialised anionic substrates could be added to the outside of the MSPs to stop unwanted interactions with the EPS and allow quicker diffusion, improving anti-biofilm activity.

Carbapenem hydrolysing enzymes are currently the main factor for serious antibiotic resistance to carbapenems. Acquired mutations such as reduced porins and more efflux pumps are generally overshadowed by the effect of carbapenemases.[162] A more recent example of the usefulness of nanoparticles on anti-biofilm activity was demonstrated by Shaaban and colleagues.[124] They used poly ε-caprolactone (PCL) polymers to encapsulate imipenem and cilastatin in nanoparticles with mean diameters of 100 nm. In the following discussion, "imipenem" refers to the physical mixture of equal weight of imipenem and cilastatin. The group measured the MICs of imipenem alone and imipenem encapsulated in PCL nanoparticles (PCLNs) in various strains of *Klebsiella pneumoniae*. ATTCC4352 (*K. pneumonia*) was used as an imipenem susceptible reference strain. The other three strains used KMU5.5, KMU4.5 and KMU2.3 were all clinical isolates collected from patients at Mansoura University Hospital, Egypt, that had displayed enhanced carbapenemase production. Imipenem alone had MICs of 0.6, 312.5, 625 and 80 μg mL^{-1} for *K. pneumonia* strains respectively. The MICs for imipenem loaded PCLNs were 0.6, 5, 2.5 and 2.5 μg mL^{-1} respectively. Unsurprisingly, the reference strain wasn't affected by the nanoparticles but the three isolates, especially KMU4.5, show a substantial increase in antibiotic activity (up to 250 times) when imipenem was protected by the PCLNs from carbapenemases. The group also measured the anti-adhesion activity of each exposure type in biofilms of *K. pneumonia*. The simple but effective assay showed that at sub-inhibitory concentrations (1/4 MIC), imipenem encapsulated in PCLNs significantly reduced the adhesion of KMU5.5 biofilms by 74% compared to imipenem alone which reduced adhesion by less than 30%.

With the increase in antibiotic resistance being driven by biofilm related infections,[39] we need to develop new methods for treating biofilms. This could be finding new antibiotics that are more effective against biofilms or repurposing current antibiotics with new methods that improve their efficacy.[163] Milani and collagues[104] have utilised nanoliposomes to encapsulate imipenem to try and improve its antibiofilm activity. The synthesis of β-lactamases and mutations of porin tertiary structure[93] are the main routes bacteria use to acquire resistance to carbapenems, and biofilms help to speed up this process. By encasing the antibiotic in a nanoliposome it is protected from degrading enzymes, allowing for targeted drug delivery and can bypass a bacterial strains defence that are more resistant to antibiotics.[164] The group measured MIC values using microtiter assays with serial dilution of imipenem. They tested nine clinical strains of *P. aeruginosa* with imipenem loaded liposomes and the free drug form. To avoid unnecessary data, the strains ATCC, PAO1, T129, and O129 have been chosen for analysis with the remaining strains shown in Table 1. The planktonic MICs of imipenem as a free drug were 3.9, 15.6, 15.6 and 15.6 μg mL^{-1} respectively. Imipenem encapsulated in the liposomes had MICs of 1.9, 3.9,

3.9 and 7.8 μg mL^{-1}. Liposomes increase the efficacy of imipenem in all the planktonic strains. They then tested imipenem on the biofilms of each strain to calculate the MBEC of free imipenem and inside a liposome. They used 96 well plates to inoculate and grow the biofilms. Crystal violet was used to stain the biofilm after washing and an ELISA plate reader measured absorbance at 570 nm. This was used to determine if a biofilm has formed and how strong of a producer each strain was. Following the order of ATCC, PAO1, T129, and O129, the free imipenem had MBEC values of 7.81, 31.25, 15.62 and 31.25 μg mL^{-1}. The nanoliposome data is promising with MBECs of 3.9, 15.62, 15.62, 15.62 μg mL^{-1} respectively. With almost all the strains showing a reduction of half the eradication concentrations of imipenem in liposomal form compared to its free drug state, this could potentially be a promising method to increase the efficacy of imipenem especially against carbapenem resistant strains. It is important to note that the MBEC values for free imipenem are extremely low compared to other literature data. Even compared to other data described earlier, they are 1 to 2 orders of magnitude lower than expected. This could potentially be due to errors using crystal violet staining for biofilms. As crystal violet stains all cells, alive or dead, this can greatly affect the results of biofilm assays. Therefore, it is important to take care when choosing the methods for biofilm assay experiments and choose stains that are more specific to get more accurate results.

5.2 Bone cements

Bone cements are materials used in orthopaedic surgery to anchor prosthetic implants to bones, particularly in joint replacement procedures. These cements are commonly employed in total hip and knee arthroplasty (replacement) surgeries. It is mainly used to fix the implant in place, providing a stable interface between the implant and the bone. The most common bone cement is polymethylmethacrylate (PMMA), a type of acrylic resin. PMMA is ideal due to its high biocompatibility, and quick setting time. The surgeon can mix the components (a liquid MMA monomer and a powered MMA-styrene co-polymer) and apply the cement, holding the protistic in place without the mixture running.[165] Bone cement is especially useful as antibiotics can be added to the powder to create a formulation that delivers the drugs directly to the site where they are needed. During the curing process, the polymerisation of MMA is exothermic (enthalpy of polymerization of MMA to PMMA is 57.8 kJ mol^{-1}),[166] so the main requirements for antibiotics inside bone cements is that they are thermally resistant, and they do not adversely affect the cements mechanical properties.[167] With some studies reporting up to 20% of joint replacement surgeries being complicated by biofilm formation,[168] it is vital that new solutions are found.

Despite carbapenems being the gold standard antibiotic for multi-drug resistant bacteria, there is limited research on their incorporation into bone cement. There is a lot of literature that use other antibiotics in bone cements such as cefotaxime and vancomycin,[169] but interest in carbapenems is much less. It is possible that carbapenems are less thermally stable[170] and do not survive the curing process or they do not last long enough due to their highly strained β-lactam core,[171] making them useless in surgical cements. However, with the increase of multi resistant Gram-negative bacteria, such as *P. aeruginosa* or *Enterobacteriaceae*, carbapenems are desperately needed in bone cements to fight implant biofilm infections. Schmid and colleagues[172] have identified this problem and investigated how the stability of meropenem and imipenem in bone cement formulations. They initially tested both carbapenems in solution with heating to temperatures of 37–90 °C for 30–120 minutes. Meropenem had a degradation of 3% at 37 °C and 75% at 90 °C after 120 minutes. Imipenem (known to be less stable) degraded by 4% at 37 °C and 95% at 90 °C. This was quite surprising, and is extremely useful for medicinal chemists, as even at 37 °C for 30 minutes, meropenem and imipenem degraded by 0.4% and 1% respectively. Fortunately for bone cements, powdered forms of each carbapenem are used which are much more thermally stable, with meropenem and imipenem showing 5% and 13% degradation at 90 °C for 120 minutes. Once the group established that each carbapenem would mostly survive the polymerisation stage they tested each carbapenem in PMMA bone cements. Each carbapenem was mixed with three bone cements Palacos R, Copal G + V, and Copal spacem (250 mg in 15 g). After polymerisation, the cement was dissolved in ethyl acetate and the antibiotics were extracted with water. Meropenem and imipenem remaining mostly intact after the high temperature treatment. Meropenem showed a degradation of 29% with Palacos R, 23% with Copal G + V, and 8% with Copal spacem. Imipenem degraded by 31, 27, and 22% in each cement respectively. Schmid also measured the temperature change of the cement during polymerisation. The cements reached a maximum temperature of 110 °C which decreased to 30 °C after 30 minutes. This means there is only a limited time that each carbapenem must resist the high temperatures, and given the results, meropenem and imipenem are stable enough to be used as an antibiotic in biofilm targeting bone cements. The results of this research inspired further exploration into using meropenem in bone cements.

Recently, Wei and colleagues[173] conducted a full evaluation of various antibiotics loaded onto bone cement and what effect they have on, compressive strength, rate of release, antibiofilm activity and effect on a rat model. The physical tests are not relevant for this review, but it is worth noting that the antibiotics did not compromise the strength of the cement, and most of each antibiotic was released after 1 day, with very slow release over the next 7–28 days. Even after 28 days, enough meropenem was released from the cement to kill all bacteria inoculated into the wells in all 4 tested bacterial species, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Proteus vulgaris* as demonstrated in Fig. 10. The group grew biofilms on PDM Diagnostic Disks, submerged in inoculated wells containing each bacterial species. The disks were then exposed to elution samples from the cement at time points 1–28 days. The biofilm diameter was measured with callipers to determine the antibiofilm activity of the antibiotics released from the

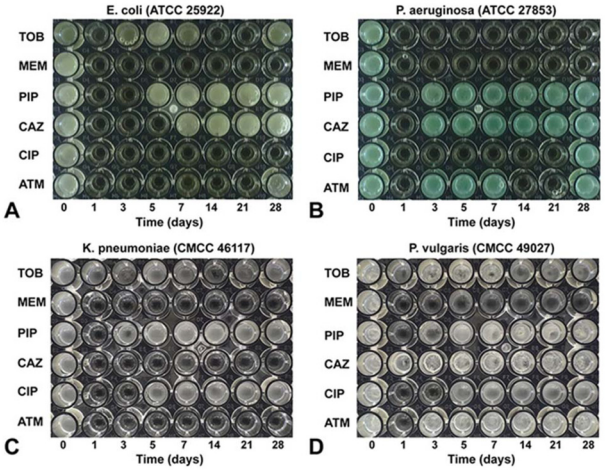

Fig. 10 Antibacterial activities against Gram-negative bacteria in elution samples from the antibiotic-loaded acrylic cements over a 28 day elution period. The wells that appear cloudy indicate bacterial growth. (A) *E. coli*, (B) *P. aeruginosa*, (C) *K. pneumoniae*, and (D) *P. vulgaris*. Reproduced from ref. 173 with permission from Springer Science, copyright 2022.

cement. During the 28-day study period, meropenem cement showed the best antimicrobial activity against the four tested bacteria. In *P. aeruginosa* the biofilm diameter was decreased 74% by meropenem, and the second-best antibiotic ceftazidime showed a decrease of 60%. Even after 28 days meropenem decreased the biofilm by 40% with the other antibiotics at mostly 0% reduction in biofilm size. This shows just how effective meropenem eluting from bone cement was at killing bacterial biofilms, not just after short amounts of time, but continued to show the best eradication throughout all 28 days. The bacterial killing of eluted meropenem over the whole experiment is shown in Fig. 10. This constant bacterial inhibition is very important to stop biofilms reforming on the surface of an implant. The researchers could have measured the concentrations of meropenem in each tested sample to get a better understanding of how close each antibiotic was to its MBIC. The group used rats to test meropenem bone cement *in vivo*. Rats had cement injected into their knee joints under anaesthesia, then a 1.5×10^6 CFU mL^{-1} suspension of *P. aeruginosa* (ATCC 27853) was injected into the articular cavity to start an infection. After 14 days the rats were euthanised and the tibia and surrounding tissue was removed for examination. The bacterial cell counts of the bone, soft tissue, and cement was analysed compared to rats that had no meropenem cement injection but were infected with *P. aeruginosa*. The tissues were sonicated to remove the biofilms from the bone and tissues. In the control, the bone, soft tissue, and cement had cell counts of 5×10^5 CFU mL^{-1}. In treated rats, the bone had a bacterial load of 1×10^1 CFU mL^{-1}, and the soft tissue was also 1×10^1 CFU mL^{-1}. Unsurprisingly the cement had no bacteria growing on it at 0 CFU mL^{-1}. This 50 000-fold decrease shows just how effective targeted meropenem therapies are on biofilms. The locally increased concentration of meropenem has very high efficacy compared to a normal intravenous injection. More research is needed to see how this targeted biofilm strategy performs in human joint replacements, but these results show that meropenem loaded bone cement could relieve many patients who suffer from perpetual biofilm growth on implants.

6. Summary and perspective

One significant challenge identified while examining the existing literature on the antimicrobial activity of carbapenems in biofilms is the absence of sufficient published data on the subject. Despite being one of the leading antibiotics in use against multi-drug resistant bacteria and with most human infections being caused by biofilms,[174] there is a clear lack of research in this area. Biofilms must be considered when evaluating the effectiveness of new antimicrobial treatments, especially with the hazards they pose to human health.

The array of literature reviewed in this work all displayed a similar trend when carbapenems were tested in planktonic and biofilm cell states. They show that carbapenems are severely inhibited in their antibacterial activity by biofilms; even carbapenem susceptible strains showed greatly increased resistance.[91] The risk of mutated resistant strains forming due to the inherent nature of biofilms (presence of persister cells and high mutation rate) combined with the reduced carbapenem activity results in therapeutics that are effectively useless. This will be devastating to the entire medical field who relies on these vital antibiotics to conduct regular surgeries and provide treatments to millions of patients every year.

Another difficulty encountered when reviewing the literature is the variety is testing methods and definitions used. There is no standard definition for each of the measurements used, including MIC, MBIC, MBEC, and MBC. For example, with MBIC, some research groups only use partial biofilm inhibition as the standard, some use 90% and others look for complete inhibition. This can wildly vary the concentrations values reported for each experiment making comparisons between the literature extremely difficult. In addition, the range of assays used by different research groups and disciplines makes this issue worse. Comparing broth microdilution, visible cell enumeration, and optical density measurements creates its own challenge as each assay method has its own inherent flaws such as, interpretation influenced by the observer, sampling errors due to low cell densities or inability to distinguish dead or alive cells. This requires better standardisation regarding biofilm testing and definitions. There have been some attempts to produce some standardised testing conditions for biofilms,[175,176] but these have not been readily adopted by the scientific community more broadly, so more work is needed to agree on specific definitions and testing methods. As mentioned previously in section 3.2, the maturity level of a biofilm can have a big impact on how it is affected by various treatments. Almost all biofilms tested in this review were 24–48 hours old, and some reports didn't even disclose

biofilm age. As a biofilm matures, its structure becomes more organized and denser, creating a thicker barrier that restricts the penetration of antibiotics into the biofilm.[177] Mature biofilms may also exhibit enhanced resistance mechanisms, such as the overexpression of efflux pumps, the production of carbapenemases and the development of persister cells.[178] In a clinical setting, it may take more than a week before a biofilm presents itself by making the patient sick, which gives the biofilm a lot of time to mature. So how relevant are the biofilms currently tested compared to hospitalised patients with biofilm infections?

Carbapenems already possess a very wide spectrum of antimicrobial activity and they are some of most potent antibiotics available, but the inhibition by biofilms makes them less useful. More research is needed on delivery methods to sites of biofilm infections. As shown in this review nanoparticles and bone cements that encapsulate carbapenems into their structure show incredible anti-biofilm activity compared to the antibiotic alone. This area of research requires more investigation to improve the solutions established previously and develop new ways to target carbapenems to biofilms. Discovering new antibiotics is crucial; however, repurposing existing antibiotics can significantly reduce both research expenses and time. Quorum sensing inhibitors, discussed in section 4.1, show very promising biofilm inhibition and dispersal characteristics. The examples reviewed had great synergy with meropenem and further investigation into these biofilm inhibitors could provide exciting results. Verderosa and colleagues[179] observed that combining the antibiotic ciprofloxacin with nitroxides, a biofilm dispersal agent, as a hybrid molecule resulted in increased antibiofilm activity compared to administering each compound separately. Exploring the application of this approach to carbapenems and other quorum sensing inhibitors[180] (QSIs) could offer intriguing possibilities for developing enhanced anti-biofilm therapies.

In conclusion, this review highlights the critical challenges with limited research on the activity of carbapenems in biofilms. Despite their importance in combating multi-drug resistant bacteria, biofilms significantly inhibit carbapenems, raising concerns about their efficacy in clinical infections compared to *in vitro* results. In addition, standardisation issues in testing methods and definitions, coupled with overlooking the maturity of biofilms, further complicate the evaluation of antibiofilm therapies. Future research should focus on enhancing delivery methods to improve the effectiveness of carbapenems against biofilms. Addressing these challenges is crucial for advancing biofilm research and optimising treatments with carbapenems.

Author contributions

The authors confirm contribution to the paper as follows: review conception: S. W. and K. E. F-S; draft manuscript preparation: M. A. L; manuscript reviewing and editing: M. A. L, S. W and K. E. F-S.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Conflicts of interest

There are no conflicts to declare.

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