

Title: Quantitative measurement and imaging of drug-uptake by bacteria with antimicrobial resistance

Abstract

The innate resistance of Gram-negative bacteria to antibiotics and other antimicrobials is a consequence of (a) the permeability barrier of the outer membrane, (b) their ability to efflux antibiotics out of the cell and (c) their capacity to form antibiotic tolerant biofilms that are up to 100 times more resistant than planktonic (suspended) cells. There is an urgent need for metrology to quantitatively measure and image the localisation of antibacterial agents in bacteria and biofilms and to understand penetration and efflux processes. This should be achieved by developing new metrological capabilities for the label-free 3D imaging of antibacterial agents in bacteria, the traceable quantification of the vertical concentration profile of antibacterial agents in bacteria and biofilms, and for the real-time quantitative measurement of drug-uptake in bacteria and biofilms. Well-controlled model systems should also be developed to allow the cross-platform measurement of penetration, accumulation and efflux of antibacterial agents, as should signal enhancement strategies and advanced sample preparation methods for studying antibacterial agents.

Keywords

Antimicrobial resistance, medical devices, Gram-negative bacteria, biofilms, high-resolution molecular imaging, antibiotics, pharmaceuticals, drugs

Background to the Metrological Challenges

The threat of antimicrobial resistance (AMR) to the health and prosperity of Europe and the world is real. For example, Jim O'Neill's review states that "AMR infections currently claim at least 50 000 lives each year across Europe and the US alone" and "300 million people are expected to die prematurely because of drug resistance over the next 35 years". This could lead to a drop in the world's GDP of 2 % to 3.5 % with economic losses of \$60 to \$100 trillion. For modern health systems, with treatments that rely heavily on antibiotics, there could be a return to the dark age of medicine where surgery would be too risky to undertake, immuno-suppressed patients would be susceptible to infections, and there would be a significant increase in maternal deaths.

Europe is taking a leading role in combatting AMR through the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) [1] and the Innovative Medicines Initiative (IMI) New Drugs for Bad Bugs programme (ND4BB) [2] which is addressing the need for antibacterial drug discovery and development (e.g. only two new classes of antibiotics have been discovered in the last 30 years). The ND4BB - TRANSLOCATION project aims to address drug penetration into Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii*, which have a double membrane and protein pumps that can rapidly efflux antibiotic drugs. They also found that "there are no general reliable methods for measuring these penetration and efflux processes in Gram-negative bacteria, a bottleneck that substantially hinders the ability of scientists to optimise antimicrobial activity in intact bacterial cells". When communities of bacteria become enmeshed in a 'biofilm' (an extracellular matrix of polymeric substances which "can sequester and degrade antimicrobial agents"), for example at the surface of a medical device or in a wound, they become 100 times more drug resistant than planktonic (suspended) cells. Novel, direct methods need to be developed for their chemical measurement.

Measurement and metrology capabilities need to be advanced. For cross-measurement platform studies, new assays are needed to better understand the mechanisms by which Gram-negative bacteria resist the actions of conventional antibiotics. This includes investigating the efficacy of novel antibacterial agents and efflux pump inhibitors. Well-controlled model systems are required to allow the cross-platform measurement of penetration, accumulation and efflux of antibacterial agents in single cells, suspended cellular aggregates and biofilms, including binding to biofilm matrix components. Assays based on genetically amenable

laboratory strains and clinical isolates of Gram-negative bacteria, which include isogenic strains with mutations in key envelope, efflux pump and biofilm-matrix associated genes are needed.

Currently, there is no method to image the uptake of a drug by a single bacterium. Although, secondary ion mass spectrometry (SIMS) combines chemical analysis capabilities with 3D analysis, the current spatial resolution of 5-pixels is insufficient. A resolution of 100 nm is needed, which requires signal enhancement of 100 times. Also, the current mass resolution of ~ 10 000 is insufficient to identify drugs from the biological background. Therefore, a new generation instrument is needed with > 100 000 mass resolution.

To improve drug and biocide design, it is important to measure uptake quantitatively, however, this can only be done indirectly at present. One possibility is to measure concentration profiles of drugs with sub 100 ppm concentration using grazing incidence X-ray fluorescence with reference-free metrology. However, novel multi-purpose wet cells will be needed to measure solid-liquid interfaces. X-ray photoelectron spectroscopy (XPS) is a non-destructive chemical depth profiling method for organic samples. However, it cannot yet be used on biofilms as XPS currently only measures dehydrated samples on flat surfaces. Full dehydration of biofilms needs to be avoided and standards need to be developed. 2D Confocal Raman Spectroscopy is a non-invasive, label-free imaging technique, which can be used to qualitatively measure drug penetration and localisation inside cells with μm resolution. However, quantitative correlations of Raman intensity to the amount of substance, and standards with known concentration of drugs are urgently needed.

High-resolution imaging techniques are needed for imaging the location of membrane proteins, such as porins, to enable drug efflux mechanisms to be studied. Fluorescence imaging is one such tool and recently, super-resolution optical microscopy techniques have broken the diffraction barrier of around 250 nm. Also, microscopy techniques based on the localisation of stochastically photoactivable and photoswitchable dyes have been reported to achieve 20 nm planar spatial resolution. However, advances in both axial resolution and volume sectioning capabilities are necessary for 3D imaging and to visualise and quantify the location of molecules of interest at bacterial surface membranes. Scanning near-field optical microscopy (s-SNOM) provides topographic data and information about the optical properties of the sample, with a spatial resolution < 50 nm. However, the sensitivity is too low and e.g. high-brightness broadband IR radiation sources are needed. Advances in numerical methods and data interpretation and processing are required for biological materials.

The successful use of the aforementioned techniques will depend on the development of advanced sample preparation methods that enable 'liquid,' albeit vitrified, water in the vacuum of high performance metrology instruments. Current methods only vitrify the water in samples up to a depth of 20 μm , which is sufficient for studying individual bacteria, but not a biofilm. Advances are needed to enable biofilms and bacteria to be reliably prepared for chemical metrology. Chemical analysis standards also need to be developed. Recent advances in nano-object manipulation and nanofabrication by electron and ion beams have great potential for the study of bacteria e.g. probing the localisation and arrangement of microbial clusters with high-resolution SIMS. Novel approaches are required to "sculpt" the bacteria to expose their interior structure in a controlled way. Furthermore, nanofabrication of sample surfaces could enhance the sensitivity of techniques.

Objectives

Proposers should address the objectives stated below, which are based on the PRT submissions. Proposers may identify amendments to the objectives or choose to address a subset of them in order to maximise the overall impact, or address budgetary or scientific / technical constraints, but the reasons for this should be clearly stated in the JRP-Protocol.

The JRP shall focus on the development of measurement capability and metrology to support the development of new drugs for bacteria with antimicrobial resistance in the context of EU funded drug-discovery research. The overall goal is to measure and image the penetration of antibacterial agents into Gram-negative bacteria and biofilms.

The specific objectives are

1. To develop new metrological capabilities for:
 - the label-free 3D imaging of antibacterial agents in bacteria. This requires a new 3D chemical imaging instrument with 100 times better sensitivity and a high-spatial resolution (100 nm). The instrument should have a mass resolution of >100 000 and the ability to sample from sub-micron areas, simultaneously.
 - the traceable quantification of the vertical concentration profile of antibacterial agents in bacteria and biofilms. Measurements should be performed in liquid and at near ambient pressure.

- imaging surface macromolecules, such as porins or metal-transport proteins, to study the efflux mechanisms in Gram-negative bacteria and to give real-time quantitative measurements of drug-uptake in bacteria and biofilms. Numerical modelling and algorithms should be developed to support measurements in complex biological environments.
2. To develop well-controlled model systems to allow cross-platform measurement of penetration, accumulation and efflux of antibacterial agents in single cells, in suspended cellular aggregates, as well as in biofilm communities including binding to biofilm matrix components. The efficacy of novel antibacterial agents and efflux pump inhibitors should be investigated.
 3. To develop signal enhancement strategies and advanced sample preparation methods for studying antibacterial agents in bacteria and biofilms including:
 - advanced cryo preparation methods to enable 'liquid' (vitrified) water to be present in the vacuum of high-performance metrology instruments without ultrastructural reorganisation and translocation of exo/endo-genous molecules.
 - novel methods to nano-sculpt bacteria for chemical imaging at 50 nm resolution.
 - nano structured substrates for enhanced sensitivity.
 4. To facilitate the take up of the technology and measurement infrastructure developed by the project by healthcare professionals (hospitals and health centres) and industry (pharmaceutical companies), in order to fight the threat from antimicrobial resistance to the health and prosperity of Europe.

These objectives will require large-scale approaches that are beyond the capabilities of single National Metrology Institutes and Designated Institutes, and it is expected that multidisciplinary teams will be required. To enhance the impact of the research, the involvement of the appropriate user community such as medical practitioners, medical (academic) hospitals and industry is strongly recommended, both prior to and during methodology development.

Proposers should establish the current state of the art, and explain how their proposed project goes beyond this.

EURAMET expects the average EU Contribution for the selected JRPs in this TP to be 1.8 M€, and has defined an upper limit of 2.1 M€ for this project.

EURAMET also expects the EU Contribution to the external funded partners to not exceed 35 % of the total EU Contribution to the project. Any deviation from this must be justified.

Any industrial partners that will receive significant benefit from the results of the proposed project are expected to be unfunded partners.

Potential Impact

Proposals must demonstrate adequate and appropriate participation/links to the "end user" community, describing how the project partners will engage with relevant communities during the project to facilitate knowledge transfer and accelerate the uptake of project outputs. Evidence of support from the "end user" community (e.g. letters of support) is also encouraged.

You should detail how your JRP results are going to:

- Address the SRT objectives and deliver solutions to the documented needs,
- Feed into the development of urgent documentary standards through appropriate standards bodies,
- Transfer knowledge to the medical sector.

You should detail other impacts of your proposed JRP as specified in the document "Guide 4: Writing Joint Research Projects (JRPs)".

You should also detail how your approach to realising the objectives will further the aim of EMPIR to develop a coherent approach at the European level in the field of metrology and include the best available contributions from across the metrology community. Specifically the opportunities for:

- improvement of the efficiency of use of available resources to better meet metrological needs and to assure the traceability of national standards
- the metrology capacity of EURAMET Member States whose metrology programmes are at an early stage of development to be increased

- organisations other than NMIs and DIs to be involved in the work

Time-scale

The project should be of up to 3 years duration.

Additional information

The references were provided by PRT submitters; proposers should therefore establish the relevance of any references.

- [1] Strategic Research Agenda; Joint Programming Initiative on Antimicrobial Resistance; 2013
- [2] Innovative Medicines Initiative (IMI) New Drugs for Bad Bugs project, www.nd4bb.eu