Publishable Summary for 15HLT01 MetVBadBugs
Quantitative measurement and imaging of drug-uptake by bacteria with antimicrobial resistance

Overview

The innate resistance of Gram-negative bacteria to antibiotics is a consequence of the combinatorial effects of two permeability barriers: the outer and inner bacterial cell membranes, their ability to efflux antibiotics out of the cell and their capacity to form antibiotic tolerant biofilms that are up to 100 times more resistant than planktonic bacterial cells. The objectives of this project were to advance the measurement capability by providing the urgently needed essential metrology to quantitatively measure and image the localisation of antibiotics and to understand the penetration and efflux processes in bacteria and biofilms. The objectives were met and the project developed and demonstrated a range of technologies and methods to study microbial samples. These include chemical and optical imaging, but also a range of signal enhancement strategies for further advancing the measurements. Several techniques were adopted for the measurement of the samples in their close to native state, including measurements in liquid, near-ambient pressure or frozen-hydrated. Well controlled model systems and quantitative approaches were developed to aid the metrology of bacterial samples.

Need

It is universally acknowledged that the threat of antimicrobial resistance (AMR) to the health and prosperity of Europe and the world is real. The European Union has a major initiative to fight AMR. The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) is developing a strategic research agenda and is co-ordinating European research in Horizon 2020, in the Innovative Medicines Initiative (IMI) and in the EC's ERA-NET funding scheme. For example, the New Drugs for Bad Bugs (ND4BB) programme of the IMI involves nine large pharmaceutical companies in seven ND4BB projects, with a total committed budget of more than €600 million. They identified a metrology gap that EMPIR was uniquely placed to fill. The ND4BB project TRANSLOCATE stated that *"At present, there are no reliable and general methods for measuring these [drug] penetration processes in Gram-negative bacteria and this bottleneck substantially hinders the ability of scientists to optimise antimicrobial activity in intact bacterial cells"*. Furthermore, they identified the key need to quantify and image the penetration of drugs into bacteria and to measure the efflux processes. MetVBadBugs was directly focused at these metrology challenges. There was clearly no single technique that can deliver all the measurement answers needed by scientists studying AMR and developing new antibiotics. A robust metrology framework was needed, which built on fundamental studies of the techniques as well as being combined with cross measurement platform validation including pre-normative studies. The objectives of this project were clearly aimed at addressing these needs.

Objectives

This project addressed the following scientific and technical objectives:

1. To develop urgently needed new metrological capabilities for:
    - the label-free 3D imaging of antibacterial agents in bacteria. This required a new 3D chemical imaging instrument with 100 times better sensitivity and a high-spatial resolution (100 nm). The instrument would 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 would 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 would 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 would 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.

Progress beyond the state of the art

To address this project's needs, metrological innovation was necessary. This project was set-up to develop the technology and methods needed to enable an unprecedented 3D imaging of biocides in bacteria and biofilms.

This was undertaken using a novel 3D OrbiSIMS instrument with a mass resolution > 240,000, a mass accuracy < 1 ppm, and an image resolution for organics (< 1 micron for the argon cluster source, < 100 nm Bi nanoprobe). The high-performance MS/MS capability of this instrument aided molecular identification in complex biological matrices.

Advanced sample cryo-preparation and cryo-handling capabilities were developed, such as the high pressure freezing of the biofilm for analysis in high-vacuum methods. This enabled, for the first time, the high-resolution chemical imaging by SIMS of frozen-hydrated bacteria and biofilms without ultrastructural reorganisation within the studied samples.

Super resolution methods (STORM/PALM) were developed where the single molecule detection was combined with state-of-the-art genome editing techniques, which allowed us to directly localise and quantify expressed proteins on both isolated bacteria and complex biofilm environments with unprecedented accuracy.

Signal enhancement strategies and advanced sample preparation methods were developed; specially engineered and optimised tips for biological samples and corresponding signal enhancement were made for TERS experiments. Novel nanoengineered SERS substrates were employed to increase the sensitivity for antibiotic / biocide susceptibility measurements.

X-ray based techniques were made applicable to bacterial samples in their natural environment. The use of an innovative NAP-XPS and synchrotron NAP-HAXPES instrument allowed the extended in-depth analysis of irregularly-surfaced bacterial and biofilm samples. Also, a novel liquid cell was used to enable quantitative chemical in-depth sample analysis (in their wet state). Quantitative NAP-XPS was made traceable to the SI by calibration using reference-free synchrotron (GI)-XRF.

The progress beyond-state-of-the-art of this project was demonstrated in a number of biological case studies, where the new capabilities were used to study complex bacterial samples.

Results

To develop urgently needed new metrological capabilities for: the label-free 3D imaging of antibacterial agents in bacteria. This required a new 3D chemical imaging instrument with 100 times better sensitivity and a high-spatial resolution (100 nm). The instrument would have a mass resolution of >100 000 and the ability to sample from sub-micron areas, simultaneously.

A method for the high-resolution and high-sensitivity imaging of biofilm samples was successfully developed. The beyond the state-of-the-art aspect lies in the analysis of the samples, by secondary ion mass spectrometry, in their frozen-hydrated state. Both the instrumentation as well as the methodology for sample

preparation and handling were developed. Here, for the first time, the microbial biofilm 3D architecture was successfully probed in the close-to-native sample state. The Orbitrap analyser, in addition to the conventional ToF analyser, allows for increased method sensitivity and for mass resolving power, enabling unambiguous identification of the compounds in samples. The method was tested on frozen-hydrated mature biofilms of *Pseudomonas aeruginosa.* Mapping of the 3D distribution of quorum sensing signals, nucleobases and bacterial membrane molecules, was achieved with high mass- and micrometre lateral resolution. A comparison with freeze-dried samples was also made, which showed significant differences between the frozen-hydrated and freeze-dried samples, highlighting the importance of the analysis of samples in their frozen-hydrated state.

Two methods to enhance SIMS sensitivity were investigated including matrix-enhanced SIMS (ME-SIMS) and laser post ionisation. Both methods gave promising results, however much longer studies, beyond the project, would be necessary to fully integrate either of the methods to the 3D OrbiSIMS technology. Therefore, they have not been implemented in the final method.

The potential of s-SNOM analysis to study bacteria-antibiotic interactions was also demonstrated. The technique is capable of chemical mapping with a resolution better than 50 nm. The analyses were performed on *Escherichia Coli* bacteria treated with Triclosan. The results obtained showed a clear interaction of the drug with the bacterial membrane, suggesting that the drug accumulates between the inner and the outer membranes of the bacteria. Yet, for the results to be conclusive, further study is needed. This work is being continued with other funding beyond the end of the project.

The traceable quantification of the vertical concentration profile of antibacterial agents in bacteria and biofilms. Measurements would be performed in liquid and at near ambient pressure.

This project extended already well-established measurement techniques: X-ray based techniques such as X-Ray Photoelectron Spectroscopy (XPS), and X-Ray Fluorescence analysis (XRF) to measure the uptake of antimicrobial agents or biocides in biofilms in their natural conditions and in liquid and at near ambient pressure.

Near-Ambient Pressure XPS (NAP-XPS) was evaluated for its potential use in biology-related fields. NAP-XPS makes it possible to characterise the bacterial surface with minimal sample preparation. Various sample preparation protocols were tested and optimised. Planktonic bacteria, bacterial biofilms and artificial biofilms have successfully been characterised by NAP-XPS under various conditions. As an example, the surface of *E. coli* was characterised by NAP-XPS from a humid to a dry state. It was possible to identify components originating from polysaccharides, lipids, and proteins/peptides, however the analysis of samples held in vacuum causes substantial changes to the bacterial surface, pointing to the importance of the analysis of biological samples in their close-to native states.

For the X-ray fluorescence technique, a vacuum compatible liquid cell was adapted so that it could fit a biofilm for XRF measurements. First in situ-measurements of biomaterial containing liquids (haemoglobin and methaemoglobin) in the newly developed liquid cell were successfully carried out by means of reference-free X-ray fluorescence spectrometry. However, due to the technical failure of facilities at BAMline, where the measurements were supposed to be carried out, it was not possible to finalise and apply the measurement to biofilm samples within the lifetime of the project. Nonetheless, the results obtained with haemoglobin suggest that the iodine penetration through the biofilm can potentially be measured and quantified in this liquid cell, which will be tested as soon as the BAMline is back in operation.

XRF was also used for the successful quantification of three different reference samples: i) ionic liquids and ii) a model membrane with added known concentrations of iodo-benzoic-acid, PVP-iodine, Triclosan and Linezolid, and iii) iodine-implanted low Z crystal matrices. All the samples showed that they are suitable model systems for the calibration of XPS, FTIR and 3D OrbiSIMS techniques for biofilm characterisation.

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 would be developed to support measurements in complex biological environments.

This objective was achieved. Two different optical strategies to study the interaction and localisation of drugs in bacteria and biofilms were developed during the project. In particular, a super-resolution microscopy platform for single-molecule localisation in bacteria and a microfluidic Raman-DEP (dielectrophoretic) device to follow dynamic interactions of the bacteria with antibiotics were developed. The super-resolution microscopy platform was validated by performing super-resolution imaging of fluorescent antibiotics on the surface of the bacteria and bacterial proteins (efflux pumps), which are involved in antimicrobial resistance, both in planktonic cells

and in biofilms. Alongside the microscopy platform, an engineered strain of the bacterium *E. coli* was developed in which the efflux pump component AcrB was labelled with a fluorescent protein that was suitable for Photoactivated Localisation Microscopy (PALM), which enabled us to obtain quantitative data on the expression and distribution of this well-studied protein with an unprecedented level of detail.

Moreover, a microfluidic device based on the combination on Raman-DEP techniques was developed to detect dynamic changes in a Raman bacterial profile during drug susceptibility testing. This procedure allowed us to obtain direct, real-time measurements of a suspension of planktonic bacteria without labelling or other time-consuming sample preparation processes. The present methodology was validated by testing *E. coli* susceptibility towards ciprofloxacin by monitoring spectral changes in the chemical fingerprint of the bacteria over a 3 hour span. Raman data processed with supervised multivariate data analysis were able to detect subtle spectral differences at a molecular level between treated or untreated bacterial cells after only 1 hour of treatment.

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 would be investigated.

This objective was achieved as biofilm models were developed that could be successfully imaged by OrbiSIMS, Nano-SIMS and also by Raman spectroscopy. Molecules of interest, including antimicrobials, were detected and quantified. For example, Triclosan and Ciprofloxacin were detected within bacteria by Raman Microscopy. As a measure of efficacy, fluorescent microscopy (with or without confocal analysis) monitored the rate of bacterial death within biofilms, which correlated with the concentration of antimicrobial. This analysis was undertaken using the model bacterium *Pseudomonas aeruginosa*, and the antimicrobials ciprofloxacin, tobramycin and benzalkonium chloride. In addition, analysis of *Staphylococcus aureus* provided supporting evidence with triclosan alone or in combination with a range of antibiotics including ciprofloxacin and vancomycin. The optimised protocols were used to evaluate antimicrobial resistant strains of the bacterium *Pseudomonas aeruginosa* (deficient in Mex efflux pumps) to see if they accumulated antimicrobials, and more microcolonies of a similar structure were seen to form within the biofilms.

The construction of an artificial biofilm model consisting of a thick layer of alginate doped with iodine was also worked on. The aim was to use the model sample for the interlaboratory – inter-technique measurement. Due to planar inhomogeneity of the iodine distribution within a sample, the constructed artificial biofilms were not suitable for the round-robin studies among the partner laboratories within the MetVBadBugs project. However, the model samples created could be used as a reference material for the VAMAS interlaboratory study on SIMS depth profiling through frozen hydrated organic materials.

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.

Protocols were developed for the freezing of planktonic bacteria and biofilms by high pressure freezing to enable 'liquid,' albeit vitrified, water into the vacuums required for high performance metrology instruments. The immobilisation of water in these samples reduces the ultrastructural reorganisation and the loss or translocation of exo/endo -genous molecules, which occurs with dehydration. Complete vitrification of the water within a biological specimen was essential as the formation of ice crystals within a cell can cause disastrous effects on ultrastructure. High pressure freezing was chosen for biofilm samples as it allows vitrification of the samples up to 500 µm thick. Considerable effort went in to assessing a chemical imaging cryoprotectant that was compatible with SIMS. 150 mM solution of ammonium formate was finally chosen as the most compatible with further chemical imaging. Ammonium formate does not interfere with SIMS analysis and it is commonly used to wash biological samples from salts, which otherwise have a very strong signal in the mass spectrum and can suppress other ions. Its efficiency as a cryoprotectant, was evaluated by electron microscopy imaging of frozen-hydrated bacterial samples. The SOP for the high pressure freezing of bacteria and biofilms has been created and published on the project website.

Procedures were developed for the fabrication of efficient tip-enhanced Raman spectroscopy (TERS) probes and surface-enhanced Raman spectroscopy (SERS) substrates for highly sensitive Raman measurements on bacteria and biofilms. A procedure for the measurement of the TERS enhancement factor independently from

the SPM apparatus, operator arbitrariness and most external factors was developed, in the form of an isolated tip covered in a SAM of organic molecules. This new standard procedure for the precise quantification of the EF allowed the optimisation of the AFM-TERS tips production process, which is necessary for measurements on bacteria and biofilms. A novel substrate for further amplification of TERS signals was also engineered, consisting of a 2D array of pyramidal structures, showing promising results and high enhancements with respect to common techniques. The standardisation of TERS spatial measurement capabilities was also pursued, by means of a traceable spatial standard for TERS chemical imaging, which was conceived, constructed and tested with different organic compounds as target molecules. It was proven that this could be employed as a traceable universal standard for TERS spatial calibration. For the production of SERS-active surfaces, a homogenous array of flexible, gold-coated SiNWs was obtained combining nanosphere lithography and metal assisted chemical etching. The influence of the long-range ordering of the SiNWs distribution was studied as well, and the substrate was consequently optimised. The measurements showed a linear increase of the SERS signal for increasing values of correlation lengths, coherently with a more ordered and dense distribution of hotspots on the surface.

Impact

This project has provided better, faster and more reliable tools for the scientific and industrial communities in sectors such as medical, pharmaceutical, medical devices and biomedical discovery.

A total of 11 papers have been published in peer-reviewed journals and are open access, and a further 3 papers have been submitted or are in preparation. Additionally, 42 conference presentations and posters were presented over the lifetime of the project. There were also a number of conferences, training sessions and workshops, where the results were shared with a variety of different stakeholders to aid the uptake of the work completed in this project. The dissemination included an interview on BBC World service ‘The Inquiry’ about antimicrobial resistance, seminars, communication with the public through “Massacre the microbes activity” at the University of Nottingham and a Schools study Day at Nottingham Castle.

Dissemination of the results will continue beyond the end of the project. There are several publications in preparation and also a number of talks and posters to be presented during upcoming meetings and conferences. In addition, there will be a stand showcasing bacteria and biofilm research, as a public engagement, at The Royal Society summer exhibition in London in July 2019. Also, a special session, dedicated to MetVBadBugs will take place during the annual 18th ECASIA conference held in September 2019.

Impact on industrial and other user communities

This project advanced the measurement capability and provided the essential metrology needed for measuring drugs in bacteria and biofilms. This will have an impact across healthcare and industry sectors, including large pharma, clinicians, wound and infection centres and instrument vendors.

The implementation of the 3D OrbiSIMS instrument and the development of cryo-SIMS methodology will have an impact on research in all areas of drug discovery. It will enable researchers to perform high-resolution and high-sensitivity label-free 3D imaging of antibacterial agents in bacteria at the sub-cellular scale in their close to native state. This unprecedented measurement capability can be further translated to studies of animal and human samples and have impact on other life science sectors.

NPL has already started offering the 3D OrbiSIMS instrument as a measurement service. For example, a large pharmaceutical company has been using the methodology developed at NPL and expertise to study biofilms. The number of 3D OrbiSIMS instruments worldwide is increasing and this project contributed to the uptake of this technique and pioneered the essential metrology for the studies of bacterial samples.

The adaptation of the liquid cell has been assessed for the analysis of biological molecules and enabled the traceable quantification of the vertical concentration profiles of antibiotics in bacteria and biofilms. This will have an impact on a wide range of biological and biomedical research as it will enable studies of biological samples by XRF methods, which has not been possible thus far due to the high-vacuum requirements of these techniques.

The developed protocols for well-controlled bacterial model systems not only give insights into the mechanisms of antimicrobial resistance but also permit the same processes to be studied using different techniques, thus

providing complementary information. Likewise, analysis instrument vendors will benefit from the availability of quantifiable reference samples for the benchmarking of methods and, in turn, advancing measurement capability.

The sensitivity of Raman techniques for complex biological samples have been vastly enhanced through the nanofabrication of nano-structured substrates (for SERS) and specialised tips (for TERS). These methods have been designed for studying bacterial samples but end users of the techniques are also expected to benefit as the developments could be translated and tailored to study other complex biological samples.

Impact on the metrology and scientific communities

The development of novel, beyond the state-of-the-art capabilities: cryo-3D OrbiSIMS, super-resolution 3D imaging, signal enhancement strategies for TERS and SERS measurements, quantitative XRF using a liquid cell and metrology for ambient-pressure and SI-traceable NAP-XPS have an important impact on the scientific communities. Good practice guides for well-controlled bacterial model systems and for cryogenic sample preparation have been produced and published, which are expected to have a significant impact to the wider community.

The consortium organised a successful focus meeting 'Cool tools for microbial imaging' at the Microbiology Society Annual Meeting 2018. The meeting highlighted that in recent years there have been extraordinary advances in the technologies available to study microbial biology. The development of new techniques to probe individual cells and molecules is a major driver of scientific advance. The focus meeting covered and showcased new advanced techniques that have been successfully applied to microbiology, including mass spectrometry imaging, light and electron microscopy and Raman spectroscopy. These have diverse applications which attracted a broad audience across all the Microbiology Society divisions.

Sample preparation is one of the most critical steps for reproducible high-resolution imaging. To ensure uptake of the results of this project and to promote the topic within the scientific community, NPL and NIBSC organised the 89th IUVSTA workshop "Biological and soft matter sample preparation for high-resolution imaging by high-vacuum techniques". The workshop attracted 58 people across a mixed audience (industry, academia etc.) for an extended scientific debate.

The project had a strong role in training, including e-Learning and webinars and teaching. Training courses for PhD students on the formation of biofilms were organised and there were series of two-day SPM workshops to improve the scientific community's knowledge of Graphics card-based computing for an inverse problem solution. Training was also given to the consortium in bacterial culturing and in the preparation of biofilms for imaging.

Impact on relevant standards

A technical report entitled "Surface chemical analysis of bacteria and biofilms" has been submitted to ISO TC 201 through DIN as a new work item proposal. The report summarises the current status of the characterisation of bacteria and biofilm by XPS, XRF, FTIR, IR-s-SNOM, SIMS, Raman-spectroscopy and super-resolution microscopy. This provides a good overview of the analytical techniques, requirements for sample preparation and what information each method provides in the context of biofilm analysis.

The adaptation of the liquid cell for a potential application to analyse biofilms using quantitative reference-free XRF was given as an outlook in the talk "Characterisation of bio-molecular nano-layers by means of reference-free X-ray Spectrometry".

A presentation was given at the annual workshop on AP-XPS in December 2017 about the development of instrumentation for measuring biofilms with NAP-XPS, and two application notes related to the subject were published.

The project's progress and engagement have been reported at all ISO TC 201, VAMAS and CCQM-SAWG and CCQM-CAWG meetings. The project enabled partners to provide input to the development of the existing and new standards. For example, contribution to a draft standard for the measurement of sputtering yield for organic materials and participation in a CCQM pilot study on 'the amount of substances at a buried interface'. Partners have been regularly involved in networking activities at user meetings and participated in standard- and technical committees especially: ISO TC 201, NA 062-08-16 AA and BIPM/CCQM SAWG.

Longer-term economic, social and environmental impacts

Economic benefits:

The O'Neill review on antimicrobial resistance (https://amr-review.org/) commissioned two independent studies by RAND Europe and KPMG, who modelled two scenarios for the situation in 2050. Using the available data, which under-reports AMR effects, they conservatively estimate that "300 million people are expected to die prematurely because of drug resistance over the next 35 years". The drop in the world's GDP will be between 2 % to 3.5 % with a corresponding loss of economic output between 60 and 100 trillion USD.

As an example, the Adult Intensive Care Unit (ICU) at the Queen's Medical Centre cares for >3500 critically ill patients every year. A single patient day in an intensive care unit costs the NHS more than £2 k, and mortality rates average approximately 20 %. The annual budget for Critical Care within just one hospital is more than £15 M. The potential benefit of interventions that reduce the length of stay and or clinical risk within this environment is huge.

The antibiotic resistance of bacteria, in the biofilm mode of growth, contributes to the chronicity of infections such as those associated with medical devices. Biofilms have become a common cause of medical, difficult-to-treat infections. The impact of the project could be seen from its potential benefits in treating chronic infections caused by biofilms. Chronic infections such as wounds cost the healthcare industry billions of dollars each year.

The results of the project are also expected to aid the pharmaceutical industry overall. With its annual output of € 220 B, its approximately 800,000 employees and as the world's major trader in medicinal products, the EU pharmaceutical industry is of strategic importance to the European economy. It is a major asset with regard to its contributions to economic growth, the labour market and the European science and technology base (estimated € 30,000 million in R&D in Europe in 2012). The world market for medical products is expected to reach nearly $1.17 trillion by 2017. It is therefore essential for the EU to maintain its competitive edge.

Social benefits**:**

The O'Neill review states that "Antimicrobial-resistant infections currently claim at least 50,000 lives each year across Europe and the US alone. As a result of AMR, modern health systems and treatments that rely heavily on antibiotics could be severely undermined". Surgery would become far more dangerous and too risky to undertake. Modern chemotherapy drugs give increasingly successful patient outcomes but suppress the patient's immune system, making them susceptible to infections. In the last century, the world has witnessed a 50-fold decrease in maternal deaths and it is, more-or-less, taken for granted that childbirth is safe. Without action on AMR this modern-day concept will change.

Bacterial infection and biofilms are a major source of complications and even death upon severe surgical treatment and chemotherapy. Biofilms on implants are especially difficult to detect and treatment often fails due to the complex structure of the biofilm. This often requires the costly exchange of the implant and an impairment of life quality for years.

Environmental benefits:

The effects of antibiotics on the environment are still not entirely understood. However, one major concern is the rise of antibiotic-resistant strains of bacteria due to increased antibiotic use in humans and animals. This can critically disturb natural bacterial ecosystems and lead to a serious threat to human health. Biocidal Product Regulation (EU 528/2012) now requires evidence that a biocidal product will not give rise to microbial resistance. Increased research into new antibiotics and antibiotic alternatives is urgently needed to prevent the resistance from forming.

The developed technologies and methodologies are transferable to other biological systems or soft matter samples such as cells, tissues or food, and this will support uptake of the developed metrology by other academic and industry sectors, e.g. veterinary sciences, food production, marine biology, etc.

List of publications

1. T. Vignolini, L. Gardini, V. Curcio, M. Capitanio and F. S. Pavone, *Optimization of Highly Inclined Optical Sheet Illumination for Super-Resolution Microscopy*, Biophysical Journal 114 (3), 14a https://doi.org/10.1016/j.bpj.2017.11.121

2. Hornemann, D. Sinning, S. Cortes, L. Campino, P. Emmer, K. Kuhls, G. Ulm, M. Frohme, B. Beckhoff, *A pilot study on fingerprinting Leishmania species from the Old World using Fouriertransform infrared spectroscopy*, Analytical & Bioanalytical Chemistry 409 (29), 6907 – 6923 https://doi.org/10.1007/s00216-017-0655-5
3. P Sanjuan-Alberte, MR Alexander, RJM Hague, FJ Rawson, *Electrochemically stimulating developments in bioelectronic medicine,* Bioelectronic Medicine 4, 1 https://doi.org/10.1186/s42234-018-0001-z
4. SA Khaled, MR Alexander, DJ Irvine, RD Wildman, MJ Wallace, S Sharpe, *Extrusion 3D printing of unique geometries of paracetamol single formulation with tunable release profiles,* AAPS PharmSciTech 19 (8), 3403 – 3413 https://doi.org/10.1208/s12249-018-1107-z
5. J Meurs, MR Alexander, PA Levkin, S Widmaier, J Bunch, DA Barrett, *Improved extraction repeatability and spectral reproducibility for liquid extraction surface analysis–mass spectrometry using superhydrophobic–superhydrophilic patterning,* Analytical Chemistry 90 (10), 6001-6005 https://doi.org/10.1021/acs.analchem.8b00973
6. A Hüsler, S Haas, L Parry, M Romero, T Nisisako, P Williams, *Effect of surfactant on Pseudomonas aeruginosa colonization of polymer microparticles and flat films,* RSC Advances 8 (28), 15352-15357 https://doi.org/10.1039/C8RA01491D
7. M. Kjærvik, K. Schwibbert, P. Dietrich, A. Thissen, W.E.S. Unger*; Surface characterisation of Escherichia coli under various conditions by near-ambient pressure XPS*, Surface and Interface Analysis 50 (11), 996-1000 https://doi.org/10.1002/sia.6480
8. Eleonora Cara, Luisa Mandrile, Federico Ferrarese Lupi, Andrea Mario Giovannozzi, Masoud Dialameh, Chiara Portesi, Katia Sparnacci, Natascia De Leo, Andrea Mario Rossi & Luca Boarino*, Influence of the long-range ordering of gold-coated Si nanowires on SERS,* Scientific Reports 8, 11305 https://doi.org/10.1038/s41598-018-29641-x
9. Alessio Sacco, Dario Imbraguglio, Andrea M. Giovannozzi, Chiara Portesi and Andrea M. Rossia, *Development of a candidate reference sample for the characterization of tip-enhanced Raman spectroscopy spatial resolution*, RSC Advances 8 (49), 27863-27869 https://doi.org/10.1039/C8RA03762K
10. Hermann, P., Kästner, B., Hoehl, A., Kashcheyevs, V., Patoka, P., Ulrich, G., Feikes, J., Ries, M., Tydecks, T., Beckhoff, B., Rühl, E., and Ulm, G *Enhancing the sensitivity of nano-FTIR spectroscopy.* Opt. Express 25, 16574–16588. https://doi.org/10.1364/OE.25.016574
11. T Vignolini, L Gardini, M Capitanio, FS Pavone, *Quantitative imaging of efflux pumps in planktonic and biofilm-associated bacteria through single-molecule localization microscopy*, Proceedings of SPIE 11140 https://doi.org/10.1117/12.2535451

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Internal Funded Partners: 1 NPL, United Kingdom 2 BAM, Germany 3 CMI, Czech Republic 4 DoH, United Kingdom 5 INRIM, Italy 6 PTB, Germany	External Funded Partners: 7 LENS, Italy 8 UNOTT, United Kingdom	Unfunded Partners: 9 FCI, United Kingdom 10 GSK, United Kingdom 11 ION-TOF, Germany 12 SNM, United Kingdom 13 SPECS, Germany
RMG: -		