CHEMISTRY

A European Journal

A Journal of

Accepted Article

Title: Complementary approaches to imaging sub-cellular lipid architectures in live bacteria using phosphorescent iridium complexes and Raman spectroscopy

Authors: Anna Maria Ranieri, Chiara Caporale, Valentina Fiorini, Alysia Hubbard, Paul Rigby, Stefano Stagni, Elizabeth Watkin, Mark I. Ogden, Mark J. Hackett, and Massimiliano Massi

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *Chem. Eur. J.* 10.1002/chem.201902023

Link to VoR: <http://dx.doi.org/10.1002/chem.201902023>

Supported by
ACES

WILEY-VCH

COMMUNICATION

Complementary approaches to imaging sub-cellular lipid architectures in live bacteria using phosphorescent iridium complexes and Raman spectroscopy

Anna Maria Ranieri^a, Chiara Caporale^a, Valentina Fiorini^b, Alysia Hubbard^c, Paul Rigby^c, Stefano Stagni^b, Elizabeth Watkin^d, Mark I. Ogden^a, Mark J. Hackett^a, Massimiliano Massi^{a*}

^a Curtin Institute for Functional Molecules and Interfaces, and School of Molecular and Life Sciences, Curtin University, Bentley 6102 WA, Australia.

^b Department of Industrial Chemistry "Toso Montanari", University of Bologna, viale del Risorgimento 4, 40136 Bologna, Italy.

^c Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, Perth 6009 WA, Australia

^d School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Kent Street, Bentley 6102, Australia.

*email: m.massi@curtin.edu.au

A family of three neutral iridium(III) tetrazolato complexes are investigated as bacterial imaging agents. The complexes offer a facile tuning of the emission colour from green (520 nm) to red (600 nm) in aqueous media, while keeping the excitation wavelength unchanged. The three complexes do not inhibit the bacterial growth of *Bacillus Cereus*, used as a model in this study, and exhibit extremely fast cellular uptake. After a minute incubation time, the non-toxic complexes show sub-cellular localisation in spherical structures identified as lipid vacuoles. Confocal Raman imaging has been exploited for the first time on live bacteria, to provide direct and label-free mapping of the lipid-enriched organelles within *B. cereus*, complementing the use of luminescent probes. Examination of the Raman spectra not only confirmed the presence of lipophilic inclusions in *B. cereus* but offered additional information about their chemical composition, suggesting that the lipid vacuoles may contain polyhydroxybutyrate (PHB).

Optical imaging is well suited to interrogate the chemical biology of living systems and has found extensive use to characterise cellular environments, with nanometre precision and high temporal resolution. The majority of the imaging techniques exploit the use of molecular probes, the design of which is an ongoing challenge, dictated by increasingly ambitious analyses requiring precise target specificity. At the same time, direct label-free analytical methods that will complement the use of molecular probes, is an emerging field (i.e. multimodal microscopy).^[1] Amongst other techniques, Raman spectroscopy is particularly attractive; because it provides sub-micron spatial resolution and does not suffer spectral interferences from H₂O. In this context, phosphorescent metal complexes of transition and lanthanoid elements offer useful tunability with respect to their

luminescence properties and target specificity, and have the potential to solve drawbacks associated with traditional organic fluorophores, such as self-quenching, photobleaching and discrimination from endogenous autofluorescence.^[2–4] Moreover, examples are emerging showing how these metal-based systems have the potential to be used as correlative probes with other imaging techniques such as electron microscopy.^[5] In the past few years, the potential of metal-based probes has started to be realised, with a wide range of new imaging tools developed from phosphorescent metal complexes which selectively target sub-cellular organelles (e.g., mitochondria, ribosomes, lipid droplets) in multicellular systems.^[4,6,7] In contrast, sub-cellular imaging tools for bacteria (single cell organisms) are less well developed. Compared to cellular and tissue imaging, bacterial imaging has several additional challenges. A typical bacterial cell has a size of less than 10 µm, that is close to, or smaller than, the spatial resolution of many conventional microscopes. Moreover, the small size of these organisms requires more excitation energy that often causes increased photobleaching^[8–11]. The main obstacle to imaging sub-cellular components in bacteria is represented by their robust cell envelope^[10]. Most of the current protocols to image bacteria rely on the use of a relatively limited number of commercial organic dyes^[12–15] or on the genetic manipulation of target proteins.^[10,11] The use of genetically encoded tags, however, can lead to artefacts and it is not applicable to label non-proteinaceous biomolecules such as glycans, nucleic acids and lipids.^[10,16] This restriction has been recognised in the past few years, with an increasing research effort focused on developing new markers to stain bacteria. While a wide range of organic fluorophores^[17–20] and bio-functionalised nanoparticles^[21,22] has been reported, most of these systems can only interact with the bacterial cell wall and are not internalised within the bacterial cells. This approach, therefore, limits the staining to the cell surface and hinders the visualisation of any sub-cellular environment or event.

COMMUNICATION

Moreover, production of these dyes can be synthetically demanding due to their complex molecular architecture. This factor might limit mass production for widespread use and tunability to obtain a platform of bacterial markers with complementary properties. Intriguingly, complexes of Ru(II), Re(I), Ir(III) with luminescent properties have also been investigated in detail as potential antibacterial agents,^[23–27] but surprisingly they have not yet been studied as bacterial imaging agents. This growing effort to understand the mechanisms of prokaryotic cell biology is driven by the impact it has in clinical and industrial settings. Efficient treatment of bacterial infections, for example, requires continued, costly and resource consuming medical advances; but treatment can be accelerated, or made more efficient, through deeper understanding of the pathogen biology and mechanisms of infection.^[11,22,28] In an industrial context, biodegradation, bioremediation, and bio-fuel sectors are billion dollar enterprises that can benefit from innovative advances developed or leveraged from the unique chemical biology of bacteria.^[29,30] Unfortunately, despite the continuous advances in genome sequencing, many molecular mechanisms and biological functions in prokaryotic cells are yet to be delineated, and improved bacterial imaging has the potential to bridge the gap between genetic information and biological function.^[11,15,22] Given the lack of reports on luminescent metal complexes for bacterial imaging, we aim in this study to investigate the potential of a suite of phosphorescent metal complexes to act as molecular probes for bacteria. If this capability can be validated, then it lays the foundation for future studies to leverage the advantages of luminescent metal complexes for imaging applied to bacteria. Specifically, our methods centre on three phosphorescent Ir(III) tetrazolato complexes (Figure 1) that have been reported by our group.^[31,32] We have previously shown that a series of cationic iridium(III) complexes exhibited antimicrobial activity towards *Deinococcus radiodurans*, whereas the neutral analogues did not show any cytotoxic effect towards the same species.^[32] Based on these results, three neutral iridium complexes have been tested as molecular probes for the live imaging of the bacterial species *Bacillus cereus*. In addition, we have investigated the use of Raman microscopy analyses of live bacteria to complement the use of luminescent metal complex probes.

Figure 1. Chemical structure of the iridium(III) tetrazolato complexes and corresponding emission maxima ($\lambda_{\text{Ex}} = 405 \text{ nm}$).

The iridium complexes investigated as imaging agents were selected as they have strong potential to be developed into easily accessible probes for bacterial imaging. The probes can be readily synthesised by reacting the chloro-bridged dimer chloro-bridged dimer $[\text{Ir}(\text{C}^{\text{A}}\text{N})_2\text{Cl}]_2$ ($\text{C}^{\text{A}}\text{N} = 2\text{-phenylpyridine}$ or $2\text{-(2,4-difluorophenyl)pyridine}$) with an excess of the corresponding tetrazolato ligand at room temperature.^[31] Importantly, minor modifications in the chemical structure allow the tuning of the

emission colour, while using the same excitation wavelength. Indeed, upon excitation at 405 nm, the emission maxima range from 520 nm to 600 nm in aqueous media (see Figure 1, and SI). As the intention of this work was to develop probes for live-imaging of bacteria, an important initial step was to determine their impact on the bacterial growth. The toxicity of the complexes towards *B. cereus* was determined by monitoring the kinetics of growth in the presence of the complexes at the concentration used for the imaging experiments (20 μM in nutrient broth, 0.1% DMSO). Notably, the complexes do not significantly affect maximum cell yield (see SI). With the fundamental requirements of synthetic accessibility and non-toxicity confirmed, the potential of F_2IrPTz , F_2IrCN and IrQn as molecular probes for bacteria was assessed by confocal microscopy (Figure 2). The complexes were incubated within live *B. cereus* directly in the growth medium (nutrient broth, 0.1% DMSO). The emission of the complexes was detected after only one minute of dosing at room temperature (Supporting Video 1). This extremely fast cellular uptake is remarkable, if one considers that previously reported protocols involve from 30 minutes up to two hours incubation time.^[14,26,27] All the complexes were excited with a 405 nm laser and the emission collected in the 500–700 nm region (see SI), consistent with the emission properties of the complexes in aqueous media (Table S1, SI). Acquisition of *z-stacks* images confirmed that the complexes were effectively internalised within the live bacteria and were not interacting with the outer side of the membrane (See Supporting Video 2). Importantly, time-lapse experiments showed that no evident photobleaching from the complexes was observed after imaging over one hour period, acquiring a *z-stack* every minute (Supporting video 3).

Figure 2. Confocal fluorescence microscopy demonstrates the uptake and compartmentalisation of iridium complexes in *B. cereus*. Scalebars 5 μm .

The resolution of the images showed that all of the complexes were highly localised to the same well defined, droplet-like micro domains within *B. cereus*. The spherical shape of the inclusions within the bacterial cytoplasm resemble lipid vacuoles. In order to prove our

COMMUNICATION

conclusion of lipid staining, a further staining experiment was trialled with BODIPY (SI), showing a very similar localisation pattern and therefore suggesting that the iridium complexes show specificity for lipid bodies in the bacteria (a direct comparison of the confocal images for bacteria stained with the iridium complexes and BODIPY can be found in the SI). Importantly, the three complexes exhibit the same sub-cellular localisation. The ability to have probes with a wide range of emission profiles but consistent sub-cellular localisation is beneficial when considering experiments with multiple probes, so that the specific emission of each marker can be easily discriminated, as they can be excited and/or detected independently. To the best of our knowledge, our protocol is the first to image lipid distributions within prokaryotes using phosphorescent metal complexes. Indeed, imaging of lipid inclusions in bacteria has to date only been achieved with traditional organic dyes.^[14,15] Significantly, an increasing research effort has focused on the study of the lipid metabolism in bacterial cells.^[15,30,33] In fact, the role of neutral lipids both in the pathogenesis or in the antibiotic biosynthesis of different microorganisms has been investigated.^[34,35] Moreover, further studies on bacterial lipid accumulation have been prompted by the potential use of prokaryotes as a source of single cell oil or biopolymers (e. g. exopolysaccharides, EPS or polyhydroxyalkanoates, PHAs) for a wide range of derived industrial products.^[29,30] To date, however, many dynamics involving lipid production, trafficking and storage in bacteria are poorly understood, and the capability of imaging lipid distribution in live bacteria is of key importance. Direct lipid imaging in living cells is also possible with vibrational spectroscopies such as Raman spectroscopy. The sub-micron spot sizes achieved with confocal Raman microscopy are well suited to studying sub-cellular lipid distributions, hence this technique has previously been applied in situ within ex vivo tissue sections, and living cell culture,^[1,36] but is yet to be applied to image sub-cellular lipid distributions in bacterial cells. Classical bulk analyses of *Bacillus* species has been used to study lipophilic inclusions or granules that are enriched in lipids and other lipophilic compounds, such as polyhydroxybutyrate (PHB).^[37] Raman spectroscopic mapping was used in this study to provide direct biochemical imaging of lipid-enriched inclusions within live *B. cereus*. The ester carbonyl band which is an established spectroscopic marker for lipophilic environments in bacteria,^[38,39] was detected with confocal Raman mapping at 1725 cm⁻¹, (shoulder at 1735 cm⁻¹), revealing distinct lipid enriched regions within live *B. cereus* (Figure 3). The lipid enriched domains display similar morphology to those observed in the imaging experiments with the iridium complexes. Further examination of the Raman spectra suggest that the lipid enriched regions may contain PHBs, based on the pronounced characteristic peak observed at 843 cm⁻¹.^[39] To the best of our knowledge, this is the first time that confocal Raman microscopy has been used to image lipids at sub-cellular spatial resolution in living bacteria. Many previous studies using Raman spectroscopy to analyse biological samples report strong spectral interference from autofluorescence of endogenous fluorophores.^[40] To overcome endogenous autofluorescence longer excitation wavelengths are typically used, but this worsens spatial resolution and decreases Raman scattering power. Alternatively, sophisticated coherent anti-Stokes Raman scattering has been developed to overcome endogenous autofluorescence in biological samples,^[41] but such methods do not reveal the complete Raman spectrum, and the instrumentation is highly specialised. The collection of Raman spectra with a 532 nm excitation wavelength in this study is possible with routinely available benchtop Raman equipment, thus allowing accessible opportunities to image lipids at sub-cellular spatial

resolution in a relatively quick time frame (~1 hour per bacterium). Although Raman microscopy has a number of limitations compared to fluorescence microscopy, (e.g. longer data acquisition times, poorer detection limits), the direct imaging capability makes the technique ideal to validate and gain further insight about the chemical specificity of luminescent complexes. Moreover, it allows a facile, label-free complementary route to monitor the morphology of live bacteria over time, to assess any effect of the dyes on the lipid metabolism.

Figure 3: Direct detection of lipid inclusions in living bacteria using confocal Raman microscopy. (A-C) Confocal Raman microscopy images showing outline of bacterial cell (A, intensity of $\nu(\text{C-H})$), lipid esters (B, intensity of ester $\nu(\text{C=O})$), and overlay (C). Representative Raman spectra of bacterial cell body and lipid enriched inclusion (D, spectra offset for clarity). Spectra within the lipid-enriched region show characteristic ester $\nu(\text{C=O})$ and skeletal $\nu(\text{C-C})$, consistent with presence of lipids or lipophilic substances such as PHBs. Scale bar in A-C = 1 μm .

The results strongly suggest that the three iridium tetrazolato complexes not only successfully act as stains for living bacteria, they also show a lipid-specific affinity and sub-cellular localisation. The lipid nature of the inclusions has been confirmed by means of Raman spectroscopy, used for the first time to image lipids in live bacteria, complementing the use of luminescent probes. Chemical modifications of the complexes allow a facile colour tuning of the emission by using the same excitation wavelength, without affecting the sub-cellular localisation. Critically, the three complexes investigated do not inhibit the cell growth for the strain object of this study. These results highlight the potential to use this family of complexes as non-toxic labels for in-vivo imaging of bacteria, offering widely varying emission colour and consistent sub-cellular specificity. Work is underway to extend this library of metal-based markers to develop structure-activity relationships, as well as exploring their behaviour in other bacterial strains.

Acknowledgements

A.M.R. and C.C. thank Curtin University for the CIPRS Scholarship.

Keywords: Iridium • Imaging Agents • Raman Spectroscopy • Bacterial Imaging • Luminescence

COMMUNICATION

- [1] M. D. de Jonge, S. Vogt, R. Siegele, P. A. Lay, J. A. McQuillan, D. D. Cohen, N. H. Hunt, D. Paterson, G. E. Grau, H. J. Ball, M. J. Hackett, F. El-Assaad, J. B. Aitken, M. J. Tobin, E. A. Carter, *Sci. Adv.* **2015**, *1*, e1500911.
- [2] M. P. Coogan, V. Fernández-Moreira, *Chem. Commun.* **2014**, *50*, 384.
- [3] K. K. W. Lo, *Acc. Chem. Res.* **2015**, *48*, 2985.
- [4] C. Caporale, M. Massi, *Coord. Chem. Rev.* **2018**, *363*, 71.
- [5] R. E. Daniels, L. K. McKenzie, J. R. Shewring, J. A. Weinstein, V. N. Kozhevnikov, H. E. Bryant, *RSC Adv.* **2018**, *8*, 9670.
- [6] F. L. Thorp-Greenwood, M. P. Coogan, L. Mishra, N. Kumari, G. Rai, S. Saripella, *New J. Chem.* **2012**, *36*, 64.
- [7] D. Septiadi, A. Aliprandi, M. Mauro, L. De Cola, *RSC Adv.* **2014**, *4*, 25709.
- [8] J. P. Schneider, M. Basler, *Philos. Trans. R. Soc. B Biol. Sci.* **2016**, *371*: 20150499.
- [9] C. Coltharp, J. Xiao, *Cell. Microbiol.* **2012**, *14*, 1808.
- [10] O. Kocaoglu, E. E. Carlson, *Nat. Chem. Biol.* **2016**, *12*, 472.
- [11] Z. Yao, R. Carballido-López, *Annu. Rev. Microbiol.* **2014**, *68*, 459.
- [12] P. Stiefel, S. Schmidt-Emrich, K. Maniura-Weber, Q. Ren, *BMC Microbiol.* **2015**, *15*, 1.
- [13] S. Schlafer, R. L. Meyer, *J. Microbiol. Methods* **2017**, *138*, 50.
- [14] C. Zhang, L. Yang, Y. Ding, Y. Wang, L. Lan, Q. Ma, X. Chi, P. Wei, Y. Zhao, A. Steinbüchel, H. Zhang, P. Liu, *Nat. Commun.* **2017**, *8*:15979.
- [15] M. Wältermann, A. Hinz, H. Robenek, D. Troyer, R. Reichelt, U. Malkus, H. J. Galla, R. Kalscheuer, T. Stöveken, P. Von Landenberg, A. Steinbüchel, *Mol. Microbiol.* **2005**, *55*, 750.
- [16] P. V. Chang, C. R. Bertozzi, *Chem. Commun.* **2012**, *48*, 8864.
- [17] P. Shieh, M. S. Siegrist, A. J. Cullen, C. R. Bertozzi, *Proc. Natl. Acad. Sci.* **2014**, *111*, 5456.
- [18] A. G. White, N. Fu, W. Leevy, J.-J. Lee, M. Blasco, B. Smith, *Bioconjug. Chem.* **2010**, *21*, 1297.
- [19] S. Saurabh, A. M. Perez, C. J. Comerici, L. Shapiro, W. E. Moerner, *J. Am. Chem. Soc.* **2016**, *138*, 10398.
- [20] X. Ning, S. Lee, Z. Wang, D. Kim, B. Stubblefield, E. Gilbert, N. Murthy, *Nat. Mater.* **2011**, *10*, 602.
- [21] C. A. Strasser, M. Otter, R. Q. Albuquerque, A. Hone, Y. Vida, B. Maier, L. De Cola, *Angew. Chem. Int. Ed.* **2009**, *48*, 7928.
- [22] V. Hoerr, L. Tuchscher, J. Hüve, N. Nippe, K. Loser, N. Glyvuk, Y. Tsytsyura, M. Holtkamp, C. Sunderkötter, U. Karst, J. Klingauf, G. Peters, B. Löffler, C. Faber, *BMC Biol.* **2013**, *11*:63.
- [23] L. Lu, L. J. Liu, W. C. Chao, H. J. Zhong, M. Wang, X. P. Chen, J. J. Lu, R. N. Li, D. L. Ma, C. H. Leung, *Sci. Rep.* **2015**, *5*, 1.
- [24] S. V. Kumar, W. K. C. Lo, H. J. L. Brooks, L. R. Hanton, J. D. Crowley, *Aust. J. Chem.* **2016**, *69*, 489.
- [25] S. V. Kumar, S. O. Scottwell, E. Waugh, C. J. McAdam, L. R. Hanton, H. J. L. Brooks, J. D. Crowley, *Inorg. Chem.* **2016**, *55*, 9767.
- [26] F. Li, M. Feterl, Y. Mulyana, J. M. Warner, J. G. Collins, F. R. Keene, *J. Antimicrob. Chemother.* **2012**, *67*, 2686.
- [27] A. K. Gorle, M. Feterl, J. M. Warner, S. Primrose, C. C. Constantinoiu, F. R. Keene, J. G. Collins, *Chem. Eur. J.* **2015**, *21*, 10472.
- [28] M. van Oosten, M. Hahn, L. M. A. Crane, R. G. Pleijhuis, K. P. Francis, J. M. van Dijk, G. M. van Dam, *FEMS Microbiol. Rev.* **2015**, *39*, 892.
- [29] R. Subramaniam, S. Dufreche, M. Zappi, R. Bajpai, *J. Ind. Microbiol. Biotechnol.* **2010**, *37*, 1271.
- [30] Hector M. Alvarez, *Biochimie* **2016**, *120*, 28.
- [31] C. Caporale, C. A. Bader, A. Sorvina, K. D. M. MaGee, B. W. Skelton, T. A. Gillam, P. J. Wright, P. Raiteri, S. Stagni, J. L. Morrison, S. E. Plush, D. A. Brooks, M. Massi, *Chem. Eur. J.* **2017**, *23*, 15666.
- [32] V. Fiorini, I. Zanoni, S. Zacchini, A. L. Costa, A. Hochkoeppler, V. Zanotti, A. M. Ranieri, M. Massi, A. Stefan, S. Stagni, *Dalton Trans.* **2017**, *46*, 12328.
- [33] D. J. Murphy, *Protoplasma* **2012**, *249*, 541.
- [34] J. L. Cocchiaro, Y. Kumar, E. R. Fischer, T. Hackstadt, R. H. Valdivia, *Proc. Natl. Acad. Sci.* **2008**, *105*, 9379.
- [35] A. B. F. Barletta, L. R. Alves, M. C. L. Nascimento Silva, S. Sim, G. Dimopoulos, S. Liechowski, C. M. Maya-Monteiro, M. H. F. Sorgine, *Sci. Rep.* **2016**, *6*, 1.
- [36] H.-J. van Manen, Y. M. Kraan, D. Roos, C. Otto, *Proc. Natl. Acad. Sci.* **2005**, *102*, 10159.
- [37] **2016**, 198.
- [38] V. Ciobotă, E. M. Burkhardt, W. Schumacher, P. Rösch, K. Küsel, J. Popp, *Anal. Bioanal. Chem.* **2010**, *397*, 2929.
- [39] J. De Gelder, D. Willemse-Erix, M. J. Scholtes, J. I. Sanchez, K. Maquelin, P. Vandenabeele, P. De Boever, G. J. Puppels, L. Moens, P. De Vos, *Anal. Chem.* **2008**, *80*, 2155.
- [40] K. L. Summers, N. Fimognari, A. Hollings, M. Kiernan, V. Lam, R. J. Tidy, D. Paterson, M. J. Tobin, R. Takechi, G. N. George, I. J. Pickering, J. C. Mamo, H. H. Harris, M. J. Hackett, *Biochemistry* **2017**, *56*, 4107.
- [41] J. P. Pezacki, J. A. Blake, D. C. Danielson, D. C. Kennedy, R. K. Lyn, R. Singaravelu, *Nat. Chem. Biol.* **2011**, *7*, 137.

COMMUNICATION

COMMUNICATION

Iridium(III) tetrazolato complexes are investigated as bacterial imaging agents, representing the first example of metal-based probe staining lipids in bacteria.

Complementing the use of these luminescent probes, confocal Raman imaging has been exploited for the first time on live bacteria, providing direct mapping and chemical characterisation of these lipid vacuoles.

Anna Maria Ranieri, Chiara Caporale, Valentina Fiorini, Alysia Hubbard, Paul Rigby, Stefano Stagni, Elizabeth Watkin, Mark I. Ogden, Mark J. Hackett, Massimiliano Massi

Page No. – Page No.

Complementary approaches to imaging sub-cellular lipid architectures in live bacteria using phosphorescent iridium complexes and Raman spectroscopy