




Cite this: *Analyst*, 2023, **148**, 2002

Probing the interaction of *ex situ* biofilms with plasmonic metal nanoparticles using surface-enhanced Raman spectroscopy†

Wafaa Aljuhani,^a Yingrui Zhang,^a Matthew P. Wylie,^b  Yikai Xu,^b  Colin P. McCoy^b and Steven E. J. Bell  ^{*a}

Biofilms are complex environments where matrix effects from components such as extracellular polymeric substances and proteins can strongly affect SERS performance. Here the interactions between SERS-enhancing Ag and Au particles were studied using *ex situ* biofilms (es-biofilms), which were more homogenous than *in situ* biofilm samples. This allowed systematic quantitative studies, where samples could be accurately diluted and analysed, to be carried out. Strong signals from intrinsic marker compounds were found for the *Pseudomonas aeruginosa* and *Staphylococcus aureus* extracted es-biofilms, which the standard addition method showed were due to 2×10^{-3} mol dm⁻³ pyocyanin or the equivalent of 1×10^{-4} mol dm⁻³ adenine, respectively. The es-biofilms hindered aggregation of Ag colloids more than Au but for both Au and Ag nanospheres the presence of es-biofilm reduced SERS signals through a combination of poorer aggregation and blocking of surface sites. For Ag, the effect of lower aggregation was to reduce the signals by a factor of ca. 2x, while site blocking gave a further 10x reduction for adenine. Similar results were found for Au nanospheres with adenine, although these particles gave low enhancement with pyocyanin. Nanostars were found to be unaffected by reduced aggregation and also showed lower site blocking effects, giving more reproducible signals than those from aggregated particles, which compensated for their lower enhancement factor. These results provide a rational basis for selecting enhancing substrates for use in *in situ* studies, where the further complexity means that it is important to begin with well-understood and controllable enhancing media.

Received 24th February 2023,

Accepted 4th April 2023

DOI: 10.1039/d3an00301a

rsc.li/analyst

Introduction

Bacterial biofilms are the predominant form of microbial life on Earth. They occur in many settings, most notably in water distribution systems and as pathological infections in humans and animals. Indeed 80% of all human bacterial infections are associated with biofilm formation.¹ These infections are particularly challenging to treat by conventional antibiotics because the microorganisms in the biofilm are protected by a strong sticky matrix formed by extracellular polymeric substances (EPS).¹ The EPS matrix has a complex structure, which primarily consists of polysaccharides, proteins, lipids and DNA. Bacteria in biofilms are particularly difficult to eradicate because the matrix prevents ingress of some antibiotics.²

It is important that methods which allow detailed information of the chemical composition and structure of the EPS

matrix, preferably *in situ*, are developed, since this will underpin strategies for administering, and monitoring the effectiveness of, biocides and for developing antifouling strategies. This is a challenging problem since biofilms are extremely complex, so studies normally concentrate on detecting and analysing just some of the components, for example by focusing on the bacteria within the films or the molecular structure of the EPS.^{3–5} These studies are made more difficult by the fact that the biofilms are very heterogenous so the structure and chemical properties will differ in different regions of the biofilm.³ For example, the bacteria tend to be located near the lower surface, *i.e.* close to the substrate on which the film has grown.³ We are interested in detecting small molecules present in the biofilms because this is relevant to understanding quorum sensing, response of bacteria in the films to external stimuli and the transport of antibiotics from the external surface to the interior where the bacteria are located. Current techniques do not provide the required combination of sensitivity and chemical specificity that is required to fully address these areas.³

The most widely used methods for quantification and identification of biofilm matrix use optical microscopy, which

^aSchool of Chemistry and Chemical Engineering, David Keir Building, Queen's University Belfast, BT9 5AG Belfast, UK. E-mail: s.bell@qub.ac.uk

^bSchool of Pharmacy, Queen's University Belfast, BT9 7BL Belfast, UK

†Electronic supplementary information (ESI) available: SERS spectra, standard addition plots. See DOI: <https://doi.org/10.1039/d3an00301a>

Several studies using SERS for *in situ* analysis of biofilms have been published.^{11–16} Most of these studies involved detection of pyocyanin, which is of interest for quorum sensing but also in the current context has been important in development of the technique since it gives very large SERS signals even at the low concentrations found in biofilms. The enhancing substrates used in previous studies were often colloidal silver

In the current study, initial SERS studies on intact biofilm highlighted the difficulties outlined above. For that reason, further experiments were performed using *ex situ* biofilms (*es*-biofilms) where biofilms were diluted and removed from their culture plates to produce a controllable homogenous liquid, thus removing complications associated with the structure and gel-like properties of the *in situ* biofilm. This allowed the biofilm-nanoparticle interactions that affect the SERS performance to be studied systematically. These results are interesting in their own right but more importantly they provide the understanding needed to underpin well-controlled studies on intact biofilms.

Ag colloid was prepared using the method reported by Lee and Meisel.¹⁹ Au colloid was prepared using the method reported by Frens with slight modification.²⁰ Briefly, 0.05 g of gold(III) chloride (HAuCl₄) was dissolved in 50 mL of deionized water and stirred and heated under reflux until boiling. Once boiling was reached, 5.6 ml of 1% (w/w) aqueous trisodium citrate was added to the precursor solution all at once. This resulted in a colour change of the solution from pale yellow to wine red. This colloid was then left to react for another 15 min before being cooled to room temperature. The colloid mixture was stored below 4 °C until required. Nanostar (NS) colloid was synthesized using a modified seedless protocol.²¹ Briefly,

For the standard addition method experiments, the control sample with no added analyte contained 150 μL NS colloid, 50 μL biofilm and 20 μL water. For pyocyanin measurements, four samples with final concentrations of added analyte of 1.0, 2.0, 3.0, 4.0×10^{-4} M were prepared. For adenine, the standard additions were recorded with final added concentrations of 1.0, 2.0, 3.0, 4.0×10^{-5} M.

Biofilm preparation

For *ex situ* biofilm preparation, a previously reported protocol was followed with some modifications.²²

An overnight cell culture of *P. aeruginosa* (PAO1) and *Staphylococcus aureus* (*S. aureus*, ATCC 29213) were diluted in Tryptic Soy Broth (TSB) to obtain 10^6 cfu ml⁻¹ inoculum. 150 μ L of this inoculum was inoculated into a 96-well microplate (sterilized, Thermo Scientific) and incubated at 37 $^{\circ}$ C and 100 rpm for 24 h. After 24 h, the broth was replaced with 150 μ L of fresh medium and incubated again for 24 h. At 48 h, the medium was removed, and each well was rinsed three times in water to remove any remaining medium and unattached cells. 5 μ L of sterile water was then added to the microplate and it was sonicated for 15 min to transfer the biofilm attached to the walls of the wells into the water. This process has previously been shown to have no effect on the viability of *S. aureus* samples.²³ Finally, the *ex situ* biofilm solutions in each well were aspirated into a container for further SERS analysis. For *in situ* experiments, quartz slides were added to each well of the 96-well microplate and cultured as described above. After biofilm growth was complete, the quartz slides were rinsed *in situ* with water and then removed for SERS analysis.

Results and discussion

In this work, three different enhancing substrates, citrate-reduced silver colloid, citrate-reduced gold colloid and Au/Ag nanostars (NS), were used to obtain SERS spectra of *P. aeruginosa* and *S. aureus* biofilms.

Initial experiments were carried out using simple quasi-spherical Au and Ag particles added to intact biofilms of *P. aeruginosa* and *S. aureus*, to establish the feasibility of this approach. In these experiments the particles were aggregated with salt and a droplet was placed on top of the pre-grown biofilms. It was found that the aggregated particles adsorbed to the biofilms and formed a visible layer on the surface which did not detach on rinsing. However, the particle distribution was very inhomogeneous at all length scales. Under low power

that some particle aggregation could be observed for dried colloid/biofilm samples, it was not clear if the deposits were aggregates which had formed above the films and subsequently deposited or had formed within the top surface layer of the biofilm. This distinction is important because it is generally accepted that pre-aggregation creates aggregates in which many of the hot spots are inaccessible, since they lie within the particle assembly. Of course, there is an additional uncertainty, in that the concentration of the target molecules and their distribution within the biofilm is also not known in advance, so that the signal may vary across the sample due to heterogeneity in the film, as well as due to differences in the enhancing aggregates. In order to remove as many variables as possible but retain the important parts of the measurements, the focus of the studies was shifted from *in situ* measurements to *ex situ* measurements, where the biofilms were removed from the surface on which they were cultured and mixed, so that they could be treated as a homogenous controllable liquid. The preparation of the *ex situ* biofilm (*es*-biofilm) was carried out in a way which was designed to minimise disruption of the sample. The biofilms were grown in 96 well plates and extracted by adding the minimum amount of water (5 μL) to each well and could then be removed by pipette. Larger volumes were prepared by combining the extracts from numerous wells. In subsequent experiments, the addition of colloidal suspensions of nanoparticles and/or aqueous salt solutions resulted in *ex situ* samples which had a significantly higher water content than the *in situ* biofilms, which reduced their viscosity and made them easier to handle. In addition, this also removed complications associated with the structure and gel-like properties of the *in situ* biofilm. Of course this means that this approach cannot be used to monitor the structure which was present in the original biofilm but this is an inevitable consequence of the method. Initial *es*-biofilm experiments were carried out with *P. aeruginosa*. In this case, the *es*-biofilm was added to the colloid, to allow any interactions to occur, before the salt was added to promote aggregation. As shown in Fig. 2, the SERS spectra recorded with the Ag colloid in the 400–1800 cm^{-1} range are dominated by signals due to pyocyanin, a major virulence factor produced by *P. aeruginosa*. This assignment was confirmed by comparison of the *es*-biofilm signals with that of a 1×10^{-4} mol dm^{-3} solution of pyocyanin in water (also shown in Fig. 2), which is the concentration in the biofilm after dilution for SERS (see below). Detection of pyocyanin is as expected from the literature since pyocyanin has a very large scattering cross-section at this excitation wavelength.¹⁶ A significant advantage of using *es*-biofilm is that it can be used almost like a normal aqueous sample. For example, it is useful to establish the concentration of the pyocyanin in the biofilms. One way to measure this would be to use UV/Vis absorption spectroscopy, which would, in principle, be possible, since the pyocyanin is strongly coloured but in practice is difficult due to the low optical density of the relatively thin biofilms and the fact that the *in situ* biofilms are also heterogeneous. More generally, it is important to have a method available which could be used for other targets where

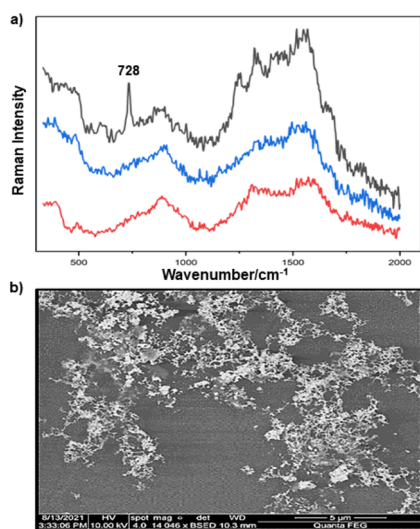
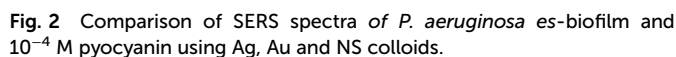
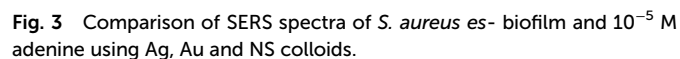


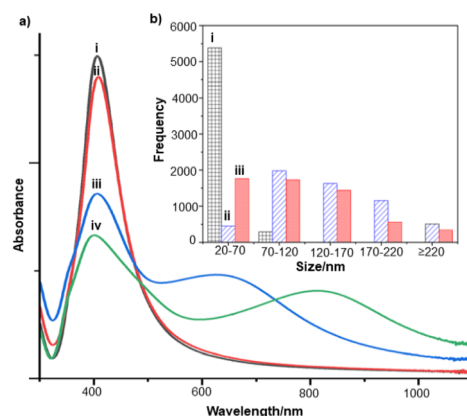
Fig. 1 (a) Au colloid-enhanced spectra recorded at three different positions of an *in situ* *S. aureus* biofilm. (b) SEM image of AuNPs adsorbed onto the surface of *S. aureus* biofilm. Scale bar = 5 μm .

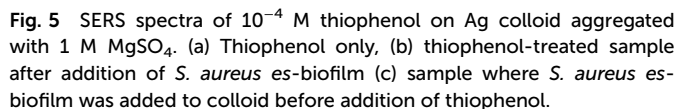


es-biofilm samples. One final observation was that these data also show a small band at 730 cm^{-1} , which does not match with the pyocyanin spectra (expanded spectra comparing both are shown in the Fig. S3†). This peak is in the position expected for the strongest band in adenine or adenine-containing molecules, including ATP, NAD, RNA and DNA.²⁴ Further studies on *S. aureus* (see below) show this band much more clearly. Surprisingly, with Au colloid, the spectra of the *es*-biofilm showed features that were identical to those observed for the blank sample, with no obvious pyocyanin bands (Fig. 2). This lack of signal might be due to the lower affinity of pyocyanin for the surface of the Au particles used here than for the Ag particles. To test if this was the case, the SERS spectrum of a $1 \times 10^{-4}\text{ mol dm}^{-3}$ aqueous sample of pyocyanin (the same concentration as used in the experiments with Ag colloid) was recorded. The spectrum, shown in Fig. 2, is very similar to that of the Au colloid alone and there are only very weak pyocyanin bands, demonstrating that pyocyanin has low affinity for the Au colloids used in these studies. The complete lack of pyocyanin bands in the biofilm is presumably due to the combination of this low affinity and the effect of the EPS matrix interfering with the binding or aggregation of the Au colloid. While *P. aeruginosa* is a good test organism because many *P. aeruginosa* strains produce pyocyanin, which is very easy to detect, it is clearly different from most bacteria because they do not produce this strong marker compound.²² It was therefore important to move to a more representative biofilm to be able to get a better understanding of SERS in biofilms. Here *S. aureus* was chosen because it is clinically relevant and readily forms biofilms. Although the *es*-biofilms of *S. aureus* did not contain any pyocyanin, it was still possible to observe strong bands in the SERS spectra obtained using both Ag and Au colloids (Fig. 3). In this case the signals closely matched those of adenine, whose spectra were also recorded using the

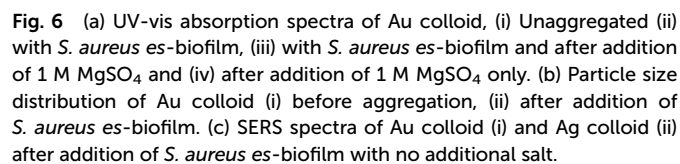


at longer wavelengths, indicating the formation of aggregates, as expected. However, in the presence of *es*-biofilm, addition of the salt to the silver colloid gave a new band which was less shifted to the red than was the case for the samples without biofilm present (Fig. 4a). It is well known that the location of the second band strongly depends on the size of the aggregates, so the smaller shift of the second peak indicates formation of smaller aggregates in the presence of biofilm. This is a clear indication that the *es*-biofilm can indeed hinder aggregation for Ag colloid. These observations were further confirmed with Dynamic Light Scattering (DLS) which showed that while salt addition did significantly increase the average size of the particles/aggregates in both cases, the extent of aggregation was lower in the sample containing *es*-biofilm than that in a simple aqueous solution. This is particularly clear at lower end of the size range, where even after salt addition the biofilm-containing samples still retained a large number of particles in the 20–70 nm size range (Fig. 4b). The data showing that the *es*-biofilm reduces aggregation helps to explain why the biofilm addition did not create aggregates in the absence of added salt, even though aggregation might be expected since the biofilm was grown in a medium which contained NaCl. Presumably here the effect of the salt which was added as part of the *es*-biofilm was countered by the ability of the biofilm to also reduce aggregation through protein adsorption. While these results show that the biofilm affects aggregation, they do not allow the effect of aggregation and of blocking of the surface to be separated, since the Ag colloid does still aggregate to a significant extent, even in the presence of *es*-biofilm and so might be SERS active, although less enhancing than simple aqueous samples. In order to resolve this problem, experiments were carried out with a very strongly binding analyte, thiophenol, which we can be confident will adsorb to the surface and not then be displaced by other biofilm components.²⁷ Fig. 5 compares the spectra of 10^{-4} mol M^{-3} thiophenol with simple Ag colloid and Ag colloid with

Analyst, 2023, **148**, 2002–2011 | **2007**

2008 | *Analyst*, 2023, **148**, 2002–2011

It is useful to note that all the experiments described above were carried out using *es*-biofilm which was initially diluted by



Analyst, 2023, **148**, 2002-2011 | **2009**

A significant advantage of the NS particles is that they sit as numerous randomly distributed individual particles within the *es*-biofilm and this homogenous particle distribution leads to a more uniform signal enhancement. In contrast, the Ag and Au colloids need to be aggregated with salt, which means that the samples are intrinsically more inhomogeneous, with local high concentration regions and areas where the number of particles is very low. This leads to much more variation in the absolute SERS intensity recorded at different points within the *es*-biofilm compared to the NS samples (Fig. 8 and S7†).

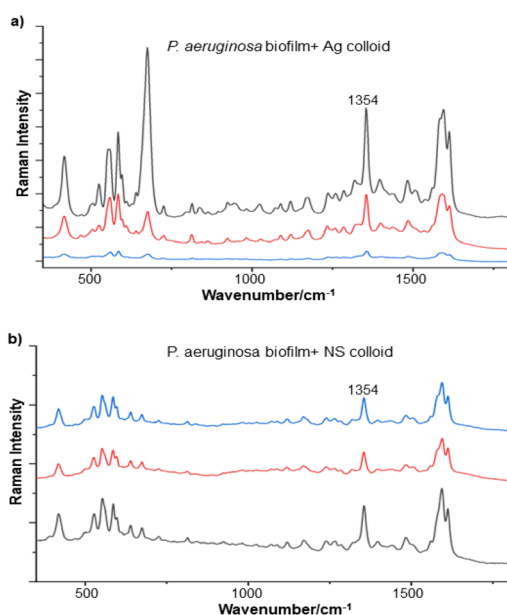


Fig. 8 Surface enhanced Raman spectra of *P. aeruginosa* es-biofilm obtained with (a) Ag colloid and (b) NS colloid. Three technical replicates recorded on the same day using the same es-biofilm sample are shown for each colloid.

Conclusion

The work presented above provides a new approach for using SERS to study biofilms. This *ex situ* method allowed for quantitative measurements of the intrinsic marker compounds present in biofilms in a way which is very difficult or impossible to carry out in very heterogeneous *in situ* biofilm samples. In particular, it allowed standard addition methods to be used to quantify the signals from the intrinsic biomarkers and therefore the various factors which control the overall signals which are detected in the complex biofilm matrix to be untangled. Here, the fact that strong signals could be detected from intrinsic marker compounds in two different species and that these could be quantified even in the presence of strong matrix interference demonstrates the power of this approach. Similarly, the observation and quantification of matrix effects on different enhancing media is central to further studies in this area. It is clear that both hindered aggregation and blocking of surface sites are important in determining overall performance. In the current study, the nanostars tested were found to be superior to standard aggregated Ag and Au colloidal nanospheres and this will be important for our future studies of *in situ* biofilm samples. In addition, this work also provides a general approach which can be adopted more widely as a basis for developing and validating SERS enhancing media for biofilm studies in a systematic way. This will allow them to be optimised before moving on to much more complex *in situ* measurements where their performance relative to other materials would otherwise be very difficult to determine.

Author contributions

W. A. contributed to conceptualization, methodology, investigation, data analysis, writing – original draft, review and editing; M. W. contributed to methodology, review and editing; Y. X. contributed to methodology; Y. Z. contributed to investigation; C. P. M contributed to conceptualization, review and editing, and S. E. J. B. contributed to conceptualisation, methodology, data analysis, resources, supervision, writing – original draft, review and editing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

W. A. acknowledges the Ministry of Education in Saudi Arabia for funding support. Y. Z. acknowledges the Chinese Scholarship Council (202008370188) for funding support. Y. X. acknowledges the Leverhulme Trust Early Career Fellowship (grant ECF2020703).

References

- 1 L. K. Vestby, T. Grønseth, R. Simm and L. L. Nesse, *Antibiotics*, 2020, **9**, 1–29.
- 2 H. C. Flemming, T. R. Neu and D. J. Wozniak, *J. Bacteriol.*, 2007, **189**, 7945–7947.
- 3 M. Magana, C. Sereti, A. Ioannidis, C. A. Mitchell, A. R. Ball, E. Magiorkinis, S. Chatzipanagiotou, M. R. Hamblin, M. Hadjifrangiskou and G. P. Tegos, *Clin. Microbiol. Rev.*, 2018, **31**, 1–49.
- 4 M. Okshevsky and R. L. Meyer, *J. Microbiol. Methods*, 2014, **105**, 102–104.
- 5 M. Alhede, K. Qvortrup, R. Liebrechts, N. Høiby, M. Givskov and T. Bjarnsholt, *FEMS Immunol. Med. Microbiol.*, 2012, **65**, 335–342.
- 6 A. M. Donald, *Nat. Mater.*, 2003, **2**, 511–516.
- 7 N. P. Ivleva, P. Kubryk and R. Niessner, *Anal. Bioanal. Chem.*, 2017, **409**, 4353–4375.
- 8 B. Kideog, W. Zheng, Y. Ma and Z. Huang, *Theranostics*, 2019, **9**, 1348–1357.
- 9 C. Zong, M. Xu, L. J. Xu, T. Wei, X. Ma, X. S. Zheng, R. Hu and B. Ren, *Chem. Rev.*, 2018, **118**, 4946–4980.
- 10 S. Keleştemur, E. Avcı and M. Çulha, *Chemosensors*, 2018, **6**, 1–15.
- 11 N. P. Ivleva, M. Wagner, H. Horn, R. Niessner and C. Haisch, *Anal. Chem.*, 2008, **80**, 8538–8544.
- 12 S. Keleştemur and M. Çulha, *Appl. Spectrosc.*, 2017, **71**, 1180–1188.
- 13 E. Efeoglu and M. Culha, *Appl. Spectrosc.*, 2013, **67**, 498–505.
- 14 S. De Marchi, D. García-Lojo, G. Bodelon, J. Pérez-Juste and I. Pastoriza-Santos, *ACS Appl. Mater. Interfaces*, 2022, **13**, 61587–61597.
- 15 C. Q. Nguyen, W. J. Thrift, A. Bhattacharjee, S. Ranjbar, T. Gallagher, M. Darvishzadeh-Varcheie, R. N. Sanderson, F. Capolino, K. Whiteson, P. Baldi, A. I. Hochbaum and R. Ragan, *ACS Appl. Mater. Interfaces*, 2018, **10**, 12364–12373.
- 16 G. Bodelón, V. Montes-García, V. López-Puente, E. H. Hill, C. Hamon, M. N. Sanz-Ortiz, S. Rodal-Cedeira, C. Costas, S. Celiksoy, I. Pérez-Juste, L. Scarabelli, A. La Porta, J. Pérez-Juste, I. Pastoriza-Santos and L. M. Liz-Marzán, *Nat. Mater.*, 2016, **15**, 1203–1211.
- 17 S. Dominguez-medina, J. Blankenburg, J. Olson, C. F. Landes and S. Link, *ACS Sustainable Chem. Eng.*, 2013, **1**, 833–842.
- 18 T. O. Peulen and K. J. Wilkinson, *Environ. Sci. Technol.*, 2011, **45**, 3367–3373.
- 19 P. C. Lee and D. Meisel, *J. Phys. Chem.*, 1982, **86**, 3391–3395.
- 20 G. Frens, *Nat., Phys. Sci.*, 1973, **241**, 20–22.
- 21 S. He, Y. M. E. Kyaw, E. K. M. Tan, L. Bekale, M. W. C. Kang, S. S. Y. Kim, I. Tan, K. P. Lam and J. C. Y. Kah, *Anal. Chem.*, 2018, **90**, 6071–6080.
- 22 G. Bodelón, V. Montes-garcía, V. López-puente and E. H. Hill, *Nat. Mater.*, 2017, **15**, 1203–1211.
- 23 P. B. Flynn, W. G. Graham and B. F. Gilmore, *Lett. Appl. Microbiol.*, 2019, **68**, 344–349.
- 24 L. J. Xu, Z. C. Lei, J. Li, C. Zong, C. J. Yang and B. Ren, *J. Am. Chem. Soc.*, 2015, **137**, 5149–5154.
- 25 F. Monticcolo, E. Palomba, P. Termolino, P. Chiaiese, E. de Alteriis, S. Mazzoleni and M. L. Chiusano, *Front. Plant Sci.*, 2020, **11**, 1–15.
- 26 Y. Tanaka, E. H. Khoo, N. A. B. M. Salleh, S. L. Teo, S. Y. Ow, L. Sutarlie and X. Su, *Analyst*, 2021, **146**, 6924–6934.
- 27 F. Sun, D. D. Galvan, P. Jain and Q. Yu, *Chem. Commun.*, 2017, **53**, 4550–4561.
- 28 W. Lai, Q. Wang, L. Li, Z. Hu, J. Chen and Q. Fang, *Colloids Surf., B*, 2017, **152**, 317–325.
- 29 A. Shiohara, Y. Wang and L. M. Liz-Marzán, *J. Photochem. Photobiol., C*, 2014, **21**, 2–25.
- 30 Z. Ye, C. Li, M. Celentano, M. Lindley, T. O'Reilly, A. J. Greer, Y. Huang, C. Hardacre, S. J. Haigh, Y. Xu and S. E. J. Bell, *JACS Au*, 2022, **2**, 178–187.
- 31 A. Tukova, I. C. Kuschnerus, A. Garcia-Bennett, Y. Wang and A. Rodger, *Nanomaterials*, 2021, **11**, 1–17.

