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Faraday Discussions

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DISCUSSIONS

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Other Nanoparticles: general discussion

Zoe Pikramenou, Catriona McCallion, Sara Carreira, Peter Dobson, Katherine Brown, Yuri Antonio Diaz Fernandez, Maha Abdollah, Dejian Zhou, Dan Sun, Sandhya Moise, Lucio Litti, Lanry L. Yung, Stefan Borsley, Nadiya Dragneva, Annette Barchanski, Mostafa El-Sayed, Amelie Heuer-Jungemann, Roger M. Pallares, Edman Tsang, Nicolas Barry, Scott Mitchell, Nguyen T. K. Thanh, Maya Thanou, Ivan Parkin, Paresh Ray and Richard Jones

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Sara Carreira opened the discussion of the paper by Nicolas Barry: Why are your **RuMs** and **OsMs** so specific to cancer cells without using any targeting moiety?

Nicolas Barry replied: We believe that the specificity comes from the size of the particles – passive targeting. Clinically-validated therapeutic and imaging NPs usually target cancer cells in a passive way. This is achieved by taking advantage of the enhanced permeability and retention (EPR) effect in tumor tissues. Tumor vasculature is highly disorganised, compared to the vasculature in normal tissues, and the vascular endothelium in tumors proliferates rapidly and discontinuously. This results in a higher number of fenestrations and open junctions (from 200 nm to 1.2 μm) than in normal vessels. Particles with a typical size of a few nanometres can therefore passively cross the tumor endothelial barrier through fenestrations, and accumulate at particular sites through blood hemodynamic forces and diffusion mechanisms.

One of our objectives is to increase the size of our particles from 15 nm to a few hundred nanometres, in order to maximize this passive targeting. We also wish to introduce an active targeting moiety (*e.g.* specific peptides, antibodies) on the corona of the particles to increase this selectivity.

Peter Dobson asked: Have you looked to see if any of your compounds are luminescent?

Nicolas Barry responded: There are numerous examples of ruthenium compounds that are luminescent. Usually, arene Ru(II) complexes are not luminescent owing to the arene–metal interactions. Nonetheless, it is possible to introduce a luminescent ligand (such as a pyrene derivative) by functionalizing the 16-electron complexes (to make an 18-electron complex).

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Peter Dobson added: The reason for my previous question is that there is growing interest in making screens for visual detection of neutrons, and I wondered if your compounds might be used for this purpose?

Nicolas Barry answered: This is an interesting remark, especially since our compounds are also boron-rich (boron–neutron interactions being of potential interest).

Ivan Parkin commented: Do you have any idea where the anticancer properties of these materials orginates from?

Nicolas Barry responded: The polymer used for the assembly of the metallated micelles belongs to the Pluronic® family, a class of block co-polymers widely used for the design of drug delivery systems, or used as pharmaceutical ingredients and biological response modifiers. In the absence of an encapsulated complex, the micelles made of Pluronic® P123 do not exhibit any toxicity towards the two cell lines studied in this paper. On the other hand, the carborane-containing organometallic complexes are highly cytotoxic. Although entrapment of the 16-electron complexes in Pluronic® micelles leads to a reduction in their anticancer potency, the micelles exhibit enhanced selectivity towards cancer cells compared to normal cells. These results therefore suggest that the anticancer properties of these materials originate from the organometallic complexes, but that the micellar formulation has a direct impact on their biological mode(s) of action.

Ivan Parkin asked: Why did you initially choose to study these materials?

Nicolas Barry replied: It is clear that dicarba-*closo*-dodecarborane derivatives can give rise to interesting and unusual properties as ligands in organometallic complexes. These clusters are remarkably stable in biological media and can be recognised by various bio-targets. They can be easily functionalised *via* organic reactions on the CH vertices and they have useful probe properties. Due to the high number of boron atoms in their globular structures, they have been extensively studied as potential boron neutron capture theory (BNCT) agents. Different clinical trials have been carried out in Japan, Europe and the United States, and sodium borocaptate ($Na_2B_{12}H_{11}SH$) has been used clinically. Nevertheless, selective and effective delivery of boron agents is still a critical issue. For this reason, it is of central importance to explore new concepts that are able to take advantage of these unique pharmacophores. The combination of dicarba-*closo*-dodecarboranes with half-sandwich complexes of ruthenium, osmium, rhodium and iridium could provide the expected breakthrough in dicarba-*closo*-dodecarborane biochemistry. However, these complexes being highly hydrophobic, their biological applications are impaired by their lack of solubility in water. Here we have formulated these complexes in micelles made of polymer, and reported their encapsulation in Pluronic® polymer micelles dispersed in water.

Zoe Pikramenou enquired: Can you identify the interactions between your metal complex and the polymer by NMR?

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Nicolas Barry responded: We cannot identify the interactions between the metal complexes and the polymer by proton or carbon NMR since the resonances of the complexes are too broad and unresolved to be visible. The encapsulation does not lead to noticeable shifts in the polymer signals. We are, however, in the process of studying the micelles by diffusion NMR (DOSY).

Edman Tsang said: Do you know how these complexes locate in the core?

Nicolas Barry answered: The complexes are encapsulated in the PPO core of the micelles, through hydrophobic interactions between the hydrophobic blocks of the Pluronic® polymers and the organometallic complexes. There are *ca.* 50 complexes per micelles (and each micelle is made of *ca.* 50 unimers).

Dejian Zhou queried: When your micelles are taken up by cells, where are they located intracellularly?

Nicolas Barry answered: This is a question we urgently wish to address. At the moment, we do not know where the micelles are located, nor do we know about their speciation in cells. Are the organometallic complexes released from the micellar cores, where are they released, and at what rate? We are currently trying to image the micelles in cells (which is possible due to the high contrast of Os and Ru in microscopy) to try and address this question.

Dejian Zhou added: How stable are the micelles? After they are taken up by cells, are they going to gradually degrade? This is important for biosafety.

Nicolas Barry responded: We showed by SAXS studies that the micelles are stable in aqueous solution and in RPMI media at various concentrations (1 to 10 mg mL^{-1}) and at various temperature (10 to 50 degrees C). However, we do not know how stable the micelles are in cells. Are they going to gradually degrade? Are they going to release the complexes and, if so, where? These are important questions we need to answer.

Sara Carreira asked: Do you know why the **RuMs** are more potent than the **OsMs**? Is there possibly a difference in electronic structure between the two elements that could affect toxicity?

Nicolas Barry answered: No, we don't know. However, osmium, the heavier congener of ruthenium and a third row transition metal, commonly exhibits slower kinetics than ruthenium, and is often considered to be relatively inert.

Ivan Parkin opened the discussion of the paper by Richard Jones: Can you say something about the generic properties of these materials? Perhaps in "Chem 101" terms in the form of hard acids and bases? Cadmium (2+) would be expected to be a soft acid, whilst lanthanides would be hard acids (3+).

Richard Jones responded: I'm not sure I understand the question about generic properties. Yes, Cd^{2+} is generally considered a soft acid and Ln^{3+} a hard acid, but they both coordinate to the available N and O donor atoms of the

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ligands. The overall structure is effectively a giant coordination compound (complex) with chemical and physical properties that you would expect for these ions bound by organic based ligands – soluble in polar organic solvents (MeOH, MeCN *etc.*), crystalline, fairly high melting points and stable in air.

Ivan Parkin said: Is there a particular architecture that results in the lanthanides and cadmium occupying specific sites in the lattice?

Richard Jones answered: Careful examination of the crystal structures reveals a 1 : 3 ratio of $Ln^{3+}$to Cd^{2+}. In compound **1**, for example, the 1 : 3 motif is repeated three times as you progress around the top and bottom rings of metal ions. The coordination environments are quite complex and involve donor atoms from both the ligands, as well as acetates. The lanthanides (in **1**) are 8-coordinate, while the Cd ions have lower coordination numbers (7 and 6). Other than that, there does not seem to be any particularly well-recognized architecture that results in metals occupying specific sites.

Ivan Parkin remarked: How sure are you of the absolute configurations of the structures? The lanthanides and the cadmium have similar electron densities and might be imagined to have a variety of site occupancies within the crystal.

Richard Jones responded: Yes, you are absolutely correct when you say that the Ln and Cd ions have similar electron densities, and so site occupancies could be a concern. When we first discovered these structures we obtained good reproducible ICPMS data on the Ln : Cd ratios and have confirmed that the Ln : Cd ratio is 1 : 3 for the bulk materials. While it is possible that some Ln and Cd ions could partially occupy the same sites in a structure, the best refinements (lowest R values) of single crystal X-ray data have the ions in the locations published.

Zoe Pikramenou asked: Do you get different structures for the different lanthanides, such as Eu and Yb?

Richard Jones replied: Generally speaking, we get very similar structures when we go from one lanthanide to another. However, we do get drums of different dimensions and compositions when we vary the nature of the lanthanide salt starting material (*e.g.* acetate *vs.* chloride) or when we vary the length of the flexible linker of the ligand. See, for example, Yang *et al.*[1] and Yang *et al.*[2]

1 X. Yang, D. Schipper, R. A. Jones, L. A. Lytwak, B. J. Holliday and S. Huang, *J. Am. Chem. Soc.*, 2013, **135**, 8468–8471.
2 X. Yang, D. Schipper, L. Zhang, K. Yang, S. Huang, J. Jiang, C. Su and R. A. Jones, *Nanoscale*, 2014, **6**, 10569–10573.

Sara Carreira enquired: Could you tune the size of the cavity in your nanodrum and therefore make it possible to load drugs?

Richard Jones answered: This would be a very interesting and potentially useful approach to drug delivery. However, at present the size of the cavity is fairly small and probably not large enough to load drugs. We are exploring ways to tune the size of the cavity.

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Scott Mitchell said: Out of curiosity, have you performed any theoretical studies on the stability of the clusters in solution? The presence of multiple lanthanide centres obviously makes calculations more demanding, but perhaps you might be able to obtain at least some indication of the solution stability of your nano-drums using density functional theory, if you can find someone who is undaunted by modelling molecules with so many atoms.

Richard Jones replied: Thank you, Scott, we appreciate the question. The drums have 32 metals and their molecular weights are well over 12,000 amu. Our DFT programs are currently not capable of handling this size of molecule with any level of accuracy. We would need far more computer power, more sophisticated programs and, as you say, someone with expertise in modelling such large structures.

Edman Tsang remarked: These lanthanides are very sensitive to chemical environment – is there any design which could maintain their photophysical properties?

Richard Jones answered: Thanks for your question. We are working on multidentate ligand systems designed to encapsulate them firmly and to shield the lanthanides from external solvents, such as water, which can quench the luminescence.

Sandhya Moise asked: How do the nano-drums interact with cells?

Richard Jones replied: We do not know at this time, but given that the dimensions of the nano-drums described here are approximately 30 Å, uptake is likely to involve one or more endocytotic pathways.

Sandhya Moise noted: It may be possible to use total internal reflection fluorescence (TIRF) to study the interactions of the nano-drums with cell membranes.

Nicolas Barry enquired: Have you tried to collect your crystals from the TEM?

Richard Jones replied: Thank you. We have not tried this and to be completely honest I did not know that this might be possible. I will ask our TEM experts how we might go about attempting this experiment.

Sara Carreira asked: Is the "rarity" of these lanthanides reflected in their price?

Richard Jones replied: Despite their name, rare earth elements (with the exception of radioactive promethium) are relatively plentiful in the Earth's crust. Although they are expensive to isolate by modern techniques, and future geopolitical issues may affect the price of the bulk ores, the amount of material used in a typical bioassay is very small. The lanthanides we use in these studies may offer a more affordable alternative to precious metals, such as gold.

Ivan Parkin added: The rare earths are in general not that "rare". The issue is that they are often widely distributed, making extraction costs high.

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Peter Dobson also noted: Rare earth materials are generally not "rare" in the sense that they are scarce. They are very plentiful, and the fluorescent tubes in this lecture room probably contain enough rare earths to make a large batch of the nano-drum-type materials studied here.

The situation is made clear in ref. 1.

1 M. Humphries, *Rare Earth Elements: The Global Supply Chain*, http://fas.org/sgp/crs/natsec/R41347.pdf

Nguyen T. K. Thanh addressed Peter Dobson: Thank you Peter, it is a very useful and interesting document. I also later received ref. 1 from a colleague, which is quite interesting as well.

Looks like, for now, we can safely assume we have enough resources to make nanomaterials. The final decision would be based on the cost of the materials and the economic benefit from each application. Thank you for the discussion.

1 F. W. Clarke and H. S. Washington, The composition of the Earth's crust, 1924, *U. S. G. S. Prof. Paper.*, 127.

Ivan Parkin added : According to Wikipedia, 95% of the world's rare earth supply comes from China.

Dejian Zhou continued the discussion of Richard Jones' paper: When you want to biofunctionalise the nanodrums, do you make the nano-drums first and then biofunctionalise them, or do you conjugate the ligands first and let them self-assembly?

Richard Jones responded: That's a really good question. We intend to explore and evaluate both routes to bio-functionalized nanoparticles.

Dejian Zhou commented: Biomolecules are typically much bigger than your nano-drums. If you attach a biomolecule to the nanodrum, will that affect the assembly properties?

Richard Jones answered: We plan to carry out conjugation of assembled nano-drums if possible. However, the self-assembly properties could change, although at this point we do not know whether alternative assemblies would necessarily affect the utility of our compounds.

Dejian Zhou asked: What are the coordination numbers for the lanthanides in the complexes? If they are coordination saturated then it would be difficult to bind other species.

Richard Jones responded: The coordination numbers for the lanthanides are high. However, we are proposing to bind other species (*i.e.* biomolecules) *via* functionalized ligand systems, not directly to the Ln ions.

Stefan Borsley said: Have you tried reducing the imines? If so, does this lead to increased stability of the nanodrums?

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Richard Jones replied: Yes, the imine portion of the flexible Schiff base ligand can be reduced easily with $NaBH_4$. We are currently exploring the use of saturated ligands for the synthesis of self-assembled nano-drums.

Peter Dobson asked: How stable are these nanodrum compounds to heating?

Richard Jones answered: The nano-drums we have isolated and characterized so far appear to be stable to moderate heating and we do not anticipate that thermal degradation would be a problem for these compounds in biological applications. Some indication of their thermal stability can be gleaned from melting point data. The nano-drums do not melt when heated, but decompose. Their decomposition points range from 176 to 212 °C. (See also supplementary data in ref. 1 and 2.)

1 X. Yang, D. Schipper, R. A. Jones, L. A. Lytwak, B. J. Holliday and S. Huang, *J. Am. Chem. Soc.*, 2013, **135**, 8468–8471.
2 X. Yang, D. Schipper, L. Zhang, K. Yang, S. Huang, J. Jiang, C. Su and R. A. Jones, *Nanoscale*, 2014, **6**, 10569–10573.

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Lanry L. Yung enquired: Since it was shown in the talk that the nano-drum assembly forms spontaneously and is very stable, has the dissociation constant of the assembly been measured? If not, is there any difficulty in measuring it?

Richard Jones responded: As the nano-drums form during crystallization, we have no information on the dissociation constant of the complex, although we predict that it will be very small as the nano-drums are very stable. We agree that understanding the assembly mechanism of these compounds would be very valuable for developing additional strategies for controlling the self-assembled properties of these compounds. This is an area of research we are interested in exploring further.

Amelie Heuer-Jungemann opened the discussion of the paper by Paresh Ray: Do you see a future for graphene-based materials for *in vivo* applications, despite the fact that their size and surface chemistry still cannot be controlled very well?

Paresh Ray answered: All the reported results are from experiments within the past year, whereas it will take one to two decades to discover the future of graphene-based materials. The synthesis procedures need to evolve to allow control of the size and surface chemistry of the material. One needs to understand their interactions with the environment and with biomolecules, before the materials can be used for real life applications.

Peter Dobson remarked: I don't understand what you are tuning with graphene oxide. It is not like a semiconductor quantum dot and these are flakes with variable lateral dimensions.

Paresh Ray responded: Thank you for your comment. These are carbon dots, not quantum dots. The quantum effects that we have observed in semiconductor nanoparticles are not significant in carbon dots. In graphene oxide, the emission

wavelength can be tuned by changing the excitation wavelength, which is mainly due to the huge red-edge effect and excited state proton transfer, as reported by several groups.[1–3]

1 C. T.Chien, S. S. Li, W. J. Lai, Y. C. Yeh, H. A. Chen, I. S. Chen, L. C. Chen, K. H. Chen, T. Nemoto and S. Isoda, Tunable Photoluminescence from Graphene Oxide, *Angew. Chem., Int. Ed.*, 2012, **51**, 6662–6666.
2 J. Shang, L. Ma, J. Li, W. Ai, T. Yu and G. G. Gurzadyan, The Origin of Fluorescence from Graphene Oxide, *Sci. Rep.*, 2012, **2**, 792.
3 S. K. Cushing, M. Li, F. Huang and N. Wu, Origin of Strong Excitation Wavelength Dependent Fluorescence of Graphene Oxide, *ACS Nano*, 2014, **8**, 1002–1013.

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Nadiya Dragneva asked: It seems like the modified Hummers method is the most popular method for graphene oxide production. Are there any defects present in your structure as a result of using this method?

Paresh Ray replied: Yes, Hummers method produced defects in the structure and, as a result, we have reported a strong defect band in the Raman spectra from graphene oxide. The observed wavelength dependent photo-luminescence in graphene oxide is associated with defects in single- and/or multiple-layers. Scientists believe that defects and their effects on electronic transitions are the main cause for the bright photoluminescence emissions that we have used for sensing.

Nadiya Dragneva queried: What kind of defects (vacancies, impurities *etc.*) can be found in your graphene oxide structure?

Paresh Ray responded: Graphene oxide has oxygenated functional groups, combined with non-oxidized regions, where most of the carbon atoms preserve sp^2 hybridization. On the other hand, in the oxidized region, the oxygen atoms attach to graphene sites randomly and convert sp^2 carbon bonds in graphene to sp^3 bonds. Several reports also indicate vacancies or line defects, which are due to reconstruction of the graphenic lattice.

Nadiya Dragneva remarked: Have you tried reduced graphene oxide? How may this affect the cytotoxic or luminesence properties of the resulting hybrid structure?

Paresh Ray replied: We have not tried to reduce graphene oxide and, as a result, we do not know how the cytotoxic and luminescence properties change with reduction.

Nadiya Dragneva commented: Could you please explain more about 3D graphene oxide? Is it more like graphite oxide (multilayered graphene)? Are there any unique properties of 3D graphene oxide?

Paresh Ray answered: The 3D graphene oxide has been prepared by cross-linking of 2D graphene oxide. Due to its highly porous structure, 3D graphene oxide will be valuable for membrane applications, such as separating bacteria and cancer cells from a blood sample.

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Lucio Litti remarked: What is the efficiency of your nano-systems and your protocols for targeting circulating tumor cells (CTCs), in terms of selectivity and number of CTCs recognized to total number of cells in the sample?

Paresh Ray answered: We have demonstrated that AGE-aptamer attached magnetic graphene oxide can be used for the selective separation of G361 malignant melanoma cells from infected blood sample. We have tested with different cancer cells and found that our developed nano-system is highly selective in separating cancer cells from infected blood. Our result shows 97% malignant melanoma G361 cell separation from infected blood, when the number of cells was 100 cells mL^{-1}.

Lucio Litti asked: Have you tried to compare your method to other FDA-approved methods, such as Veridex, for example? In your opinion, what are the principal limitations of these methods and what are the advantages of your approach compared to them?

Paresh Ray answered: We have not compare our assay results with FDA-approved methods. In future, we plan to optimise our system in a clinical environment and we will then compare our assay result with FDA-approved technology.

Nguyen T. K. Thanh queried: What is the mechanism of fluorescence of graphene oxide? How high a quantum yield do you get from your system?

Paresh Ray responded: The origin of the fluorescence can be attributed to electron–hole recombination from the lowest conduction band and the wide-range of the valence band. The emission band between 400 and 800 nm for graphene oxide is mainly due to the presence of functionalized groups C–O, C=O and O=C–OH on the graphene sheet. Our experimental data indicate excitation wavelength dependent fluorescence and multiexponential fluorescence decay kinetics. Water-soluble graphene oxide exhibits a quantum yield of 0.28 with respect to fluorescein as a standard.

Catriona McCallion commented: It has been shown that the structure of graphene oxide, when prepared using the Hummers method or a related protocol, actually consists of a poorly oxidised graphene sheet covered with oxidative debris, which can be removed by washing with a base, such as NaOH.[1] It has also been shown that it is the oxidative debris that imparts the fluorescence properties and that the graphene oxide is no longer fluorescent after the oxidative debris has been removed.[2] Have you tried base washing your graphene oxide to see if it affects its fluorescence properties at all?

1 J. P. Rourke, P. A. Pandey, J. J. Moore, M. Bates, I. A. Kinloch, R. J. Young and N. R. Wilson, The real graphene oxide revealed: stripping the oxidative debris from the graphene-like sheets, *Angew. Chem., Int. Ed*, 2011, **50**, 3173–3177.
2 H. R. Thomas, C. Vallés, R. J. Young, I. A. Kinloch, N. R. Wilson and J. P. Rourke, Identifying the fluorescence of graphene oxide, *J. Mater. Chem C*, 2013, **1**, 338–342.

Paresh Ray answered: We have not washed our graphene oxide with NaOH and, as a result, I can not comment about the reported results.

However, we have measured the fluorescence below pH 10 in the presence of NaOH solution. We have observed huge fluorescence even at pH 10 to 12. As we have discussed, the fluorescence intensity and position can both be tuned by changing the pH or by changing the excitation wavelength. As a result, one may need to excite at different wavelength to obtain good fluorescence intensity at a higher pH, or after washing by NaOH.

Dejian Zhou queried: The use of different excitation wavelength results in different emission – do you see any lifetime-dependency on the excitation wavelength?

Paresh Ray replied: Several reports using time resolved experiments clearly show multiexponential decay features for graphene oxide fluorescence. The reported data indicate that the graphene oxide emission peaks shifted due to the decrease in lifetime. Experimental results shows that with the change in excitation, the emission lifetime decreases from the lower to higher excited states.

1 C. T.Chien, S. S. Li, W. J. Lai, Y. C. Yeh, H. A. Chen, I. S. Chen, L. C. Chen, K. H. Chen, T. Nemoto and S. Isoda, Tunable Photoluminescence from Graphene Oxide, *Angew. Chem., Int. Ed.*, 2012, **51**, 6662–6666.
2 Z. Luo, P. M. Vora, E. J. Mele, A. T. C. Johnson and J. M. Kikkawa, Photoluminescence and band gap modulation in graphene oxide, *Appl. Phys. Lett.*, 2009, **94**, 111909.
3 S. K. Cushing, M. Li, F. Huang and N. Wu, Origin of Strong Excitation Wavelength Dependent Fluorescence of Graphene Oxide, *ACS Nano*, 2014, **8**, 1002–1013.
4 J. Shang, L. Ma, J. Li, W. Ai, T. Yu and G. G. Gurzadyan, The Origin of Fluorescence from Graphene Oxide, *Sci. Rep.*, 2012, **2**, 792.

Dejian Zhou asked: What are the typical quantum yields of your graphene dots?

Paresh Ray responded: Reported typical quantum yields of the graphene dots are from 0.1 to 0.15.

Nguyen T. K. Thanh said: What is the excitation and emission range for graphene oxide – can you extend it by doping?

Paresh Ray responded: The excitation range for graphene oxide lies between 280 and 550 nm and the emission band varies from 360 to 660 nm. Several reports indicate that the emission range can be extended by doping with nitrogen, boron or other atoms.

Annette Barchanski opened the discussion of the paper by Ivan Parkin: Do you have any information about the particle distribution in your polymer?

Ivan Parkin answered: We have not investigated this in detail for this system. However, for related zinc oxide nanoparticles we have found that, although there are particles throughout the polymer, most of them reside close to the surface, in the first few microns.

Maya Thanou said: What is the reaction between the surface and the bacteria? How are the bacteria killed? Is the surface altered afterwards?

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Ivan Parkin answered: The surface seems unaltered afterwards. The killing method is a mixture of oleic acid disrupting the bacteria and some free radical-based killing, engendered by the titanium dioxide when it is illuminated with UV light.

Maha Abdollah commented: How long will the surfaces covered with these polymers last and do they need to be stimulated with light every time before use?

Ivan Parkin answered: We have not studied this for the current system. In previous work using nanoparticles and dyes, we have shown that they are effective for many years when exposed to room lighting. However, in direct sunlight the dyes can degrade in two weeks.

Sacha Noimark, with whom I discussed the matter, stated that the antimicrobial properties are activated by the generation of reactive oxygen species at the sample surface, instead of through a "leaching" mechanism by which the antimicrobial agent is released from the polymer. It is therefore expected that these surfaces should maintain antimicrobial efficacy for many years. Illumination is required to maintain low surface bacterial contamination levels, however, these polymers have also demonstrated potent antimicrobial activity under dark conditions against *Staphylococcus aureus*.

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Yuri Antonio Diaz Fernandez commented: Thank you very much for presenting these interesting results. I was particularly impressed by the antimicrobial effect of this material under dark conditions. Could you please comment on the fate of oleic acid? Is oleic acid released from the material? Have you characterized the extent and the kinetics of this release? Do you have any idea of the mechanism for antimicrobial activity in absence of light and on the long-term duration of this effect?

Ivan Parkin responded: From this study, we have found that oleic acid has a fairly potent antimicrobial effect on its own. Indeed, we have now made films with embedded oleic acid and these do show marked kill in the dark. However, the kill rates we have obtained are not as high as for titanium dioxide particles coated with oleic acid. We presume that the oleic acid is used up in killing the bacteria, but we have not done any kinetic studies.

Yuri Antonio Diaz Fernandez asked: Considering the application of these materials in hospital environments, could you please comment on the efficiency of the photo-activated reaction under normal indoor light?

Ivan Parkin answered: The titanium dioxide–gold nanoparticles made here work in the dark and hence would also work (but not be enhanced by) in visible light. We think this is due in part to the oleic acid component. To get a photocatalytic effect, we would need to use UV-light. We have, however, published previous work on making visible light-activated titanium dioxide based coatings.[1–2] In those cases, we use N or S dopants in the TiO_2. They give the material a colour and allow it to function with indoor lighting.

1 C. W. Dunhill and I. P. Parkin, *Dalton Trans.*, 2011, **40**, 1635–1640.

2 C. W. Dunnill, Z. A. Aiken, J. Pratten, M. Wilson, D. J. Morgan, I. P. Parkin, *J. Photochem. Photobiol. A*, 2009, **207**, 244–253.

Yuri Antonio Diaz Fernandez asked: While combining noble metal nanoparticles with TiO_2, have you observed any antenna effect or catalytic enhancement due to the plasmonic structures? Are these effects correlated to the wavelength of the incident light?

Ivan Parkin replied: We have not found any specific plasmon effects for the nanoparticles under UV irradiation conditions and have not observed an interaction with the titanium dioxide. Normally, the smallest size of Au nanoparticles have the greatest effect in stimulating bacterial kill. Notably, from previous work we have shown that gold nanoparticles of mean size 2 nm have the greatest effect in enhancing bacteria kill, this size of particle is below the surface plasmon resonance limit for gold – that is the particles are colourless. We hence think the enhancements we see are due to catalytic effects, and also the fact that the gold nanoparticles can help stabilise the lifetime of the e^{-}–h^{+} exciton pair, as the metal can act as a reservoir for electron limiting recombination reactions.

Raul Quesada-Cabrera, with whom I discussed the matter, added that the investigation of plasmonic effects in gold-modified TiO_2 materials is planned as future work. The current work explored these materials in antimicrobial applications, aiming not only to increase the efficiency of these systems in the UV range, but to evaluate their performance against materials containing silver nanoparticles, which we have investigated extensively in our group. The examination of plasmonic effects will require action spectra studies and the optimisation of gold particle size. However, the latter experiments are very time-consuming, particularly when involving antimicrobial testing.

Katherine Brown enquired: Are your antimicrobial surfaces effective against the spore form of *Clostridium difficile*?

Ivan Parkin responded: Experiments are in progress at present against spore-forming organisms. However, these surfaces seem to be effective against a wide range of organisms, including the eight types of bacteria and virus mimics we have tested against.

Katherine Brown asked: Have you done a systematic comparison of how your antimicrobial coatings perform at concentrations of microbial pathogens found in actual hospital settings?

Ivan Parkin answered: Hospital contaminated surfaces tend to have much lower inoculum values than the ones we test. We have previously done clinical trials at the Eastman Dental Hospital and also Univeristy College London Hospitals using coated keyboards and settle plates. Both show a marked effect in these environments. We have also done studies at lower inoculum values and the coatings perform just as well. The reason we use such high bacterial counts in our studies is that it makes the data more statistically reliable.

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Mostafa El-Sayed said: Have you determined the action spectrum, *i.e.* the effect of using light of different wavelengths, but of the same intensity?

Ivan Parkin replied: Good question. We have not done many studies relating to action spectra herem but we have done so for photovoltaic devices – it looks like a good experiment to try. We have, however, done studies using filters to cut out the UV portion and to prove that we have created a visible light photocatalyst in the case of N-doped TiO_2.

Nguyen T. K. Thanh queried: In Fig 7 of your paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00113C), the proposed mechanism of photocatalytic antimicrobial activities involved both water and oxygen – which route is more dominant, please?

Ivan Parkin replied: It is hard to say, as we have not done these experiments for this system. Previous work has shown that both singlet oxygen and reactive oxygen species are important – hence both O_2 and H_2O are key.

Peter Dobson commented: Due to the limited penetration of ultraviolet light, in order to stimulate TiO_2 inserted into the body to create reactive oxygen species, we have been trying to use X-rays to activate TiO_2 to kill tumours. We use rare earth-doped titania and the work has been published and patented as a possible way of enhancing radiotherapy.[1–2]

1 H. E. Townley, J. Kim and P. J. Dobson, *Nanoscale*, 2012, **4** 5043–5050.
2 H. E. Townley, E. Rapa, G. Wakefield and P. J. Dobson, *Nanomedicine*, 2012, **8**, 526–536.

Ivan Parkin responded: This is an interesting development that could provide a new method for reducing tumours. However, it is probably a long way from clinical application.

Roger M. Pallares asked: Do you expect any harmful effects to people exposed to the reactive oxygen species produced on the treated surfaces?

Ivan Parkin answered: Not really – mammalian cells are much more resistant to reactive oxygen species than bacteria. Skin also has a dead layer that provides protection against these radicals.

Dan Sun opened the discussion of the concluding remarks by Mostafa El-Sayed: What sort of temperature rise do you get from the gold nanoparticles in cancer cells? How did you measure the temperature?

Mostafa El-Sayed responded: In the cells we did not measure it, however, in the mice we did, as high temperature burns the skin. We tried to keep the temperature below 50 °C. We used a red light laser thermometer.

Dan Sun asked: How do you control the temperature of the gold nanoparticles inside the cell nuclei?

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Mostafa El-Sayed responded: By the intensity of the near-IR light and the amount of gold nanorods we inject.

Dan Sun added: How do you determine when the cell has been killed?

Mostafa El-Sayed responded: When we succeed in stopping the growth of the cancer spot.

Dan Sun asked: Have you tried to extend the application of this plasmonic effect to fields other than biomedical applications? What is the highest temperature the gold nanoparticle can produce?

Mostafa El-Sayed replied: Yes, we have used it in studying the activation energy of chemical reactions. Reagrding the highest temperature that can be achieved, this depends on the size of the rods and the light intensity.

Peter Dobson commented: Can you use the optical tweezers effect on your nanoparticles - you could manipulate the gold nanoparticles inside the cell?

Mostafa El-Sayed responded: This is a very good question. I am sure in the hands of a good physicist it could be done. To observe biochemical effects, one would have to move many particles.

Lucio Litti remarked: You showed your gold nanoparticles inside living cells. You were also able to recover SERS spectra from these particles. As you know, in order to penetrate the cell membrane and not damage the cell, the nanoparticles have to be small. At the same time, smaller nanoparticles are characterized by smaller scattering and so lower the SERS efficiency. What are the ideal dimensions, where the nanoparticles have good penetration in cells while still remaining good SERS substrates?

Mostafa El-Sayed responded: You are quite correct, the nanoparticles have to be small to penetrate into the cell, but as we shown very recently in a physical chemistry letter, the nanoparticles plasmonically (not chemically) aggregate when they localize around the nucleus and this aggregation gives them a high scattering probability.

Amelie Heuer-Jungemann queried: Your particles, which are presumably taken up by endocytosis, are targeted to the nucleus – how do they escape the endosomes in order to reach their target? If they do not escape the endosomes, but are transported to the perinuclear region simply by being in late endosomes or lysosomes, then do you even need to have the peptide coating?

Mostafa El-Sayed responded: Good question. What we think happens is that the particles do not make it to the nucleus surface, but get stuck around the nuclear membrane and aggregate, giving us strong Raman enhancement.

Sandhya Moise asked: Is the phenylalanine signal from the dying cells typical of all dying cells, or does it probably only occur due to the denaturation of proteins during heat generation from the gold nanoparticles?

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Mostafa El-Sayed replied: This is an excellent question. As you know phenylalanine is embedded in a very hydrophobic medium. Once death occurs, this opens up and it gets exposed to the plasmonic field which enhances its Raman signal. This could be due to the heat ,but we also saw it happening in apoptosis caused by addition of hydrogen peroxide.

Maha Abdollah remarked: Do the gold nanoparticles enter the nucleus or stay in the perinuclear region? If they stay around the nucleus, how do they cause DNA damage?

Mostafa El-Sayed responded: We really do not know where the gold nanoparticles are distributed. We are trying to see if we can determine the distribution now. I expect that most of them are outside, but some monomers could pass through the membrane and these are the ones that affect the DNA structure.

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