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Faraday Discussions
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DISCUSSIONS View Article Online View Journal

Magnetic Nanoparticles: general discussion

Peter Dobson, Lanry L. Yung, Liane Rossi, Sara Carreira, Paresh Ray, Catherine Amiens, Katherine Brown, Dejian Zhou, Asterios Gavriilidis, Nguyen T. K. Thanh, Sandhya Moise, Lucio Litti, Hedi Mattoussi, Matthew Todd, Scott Mitchell, Stefan Borsley, Amelie Heuer-Jungemann, Oliver Reiser, Edman Tsang, Maya Thanou, Tom Berkleman, Dalibor Soukup, Kerry Chester, Ivan Parkin, Yuri Antonio Diaz Fernandez and Benjamin P. Burke

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Oliver Reiser opened the discussion of the paper by Peter Dobson: Is a size of below 20 nm critical to achieve cell penetration by nanoparticles?

Peter Dobson responded: No, I chose this as a typical size. I believe that the most important point is the surface chemistry and that particle size could be a secondary factor. On the other hand, smaller particles of <20 nm will induce a distortion of the outer cell membrane enabling the particle to penetrate by endocytosis, but this requires a strong affinity brought about by the nanoparticle–cell surface interaction.

Ivan Parkin remarked: The antimicrobial properties of nanodiamond may have been misrepresented in the literature as the commercial solutions contain a variety of antimicrobial agents. Studies must make sure that these are not present if antimicrobial analyses are to be performed.

Peter Dobson replied: I fully agree, but on the other hand nanodiamond has got unique energy levels with respect to water and related systems.[1]

1 C. E. Nebel, *Nature Materials*, 2013, **12**, 780.

Dejian Zhou remarked: What is your favourite inorganic drug nanocarrier and why?

Peter Dobson responded: I think this has to be porous nanoparticles of silica. They are relatively easy to make and to load. The problems come in allowing them to circulate and target the right cells and then release their payload. I select this choice also because there do not appear to be any adverse reactions to small quantities of silica in the body. Silica is also close to ideal for the functionalisation

protocols that will need to be adopted. So, to summarise: porous silica is my favourite, but I am less sure about rendering them with the right functionality for circulation, attachment and drug release.

Dejian Zhou commented: How do you achieve controlled release of drugs from mesoporous silica nanoparticles to only release the drug load when it reaches the target, without release occurring during drug transport? We have recently developed a pH-responsive DNA-based drug carrier that displays reversible pH-triggered conformational changes between a four-stranded I-motif and single-stranded structure.[1] Do you think that this DNA system could be used for controlled release of mesoporous silica nanoparticles?

1 L. Song, V. H. B. Ho, C. Chen, Z. Yang, D. Liu, R. Chen and D. Zhou, *Adv. Healthcare Mater.*, 2013, **2**, 275–280.

Peter Dobson answered: I have little to add because triggering release is not going to be easy. If the proposed pH-triggered method works under physiological pH conditions then it is worth a try. My first understanding of the work published by Song *et al.* is that a large pH swing from 7.6 to 5.1 is needed.[1] I am not sure, but believe the local pH at a tumour site is closer to 7. Another factor of concern is the increased complexity that is being introduced. Is it because the DNA-based idea does not carry a sufficient amount of drug?

1 L. Song, V. H. B. Ho, C. Chen, Z. Yang, D. Liu, R. Chen and D. Zhou, *Adv. Healthcare Mater.*, 2013, **2**, 275–280.

Nguyen T. K. Thanh opened the discussion of the paper by Edman Tsang: How did you inject the magnetic nanoparticles into the rat brain?

Edman Tsang responded: Before the micro-surgery, the SD (Sprague Dawley) rats were first anesthetized with sodium pentobarbital (60 mg kg^{-1}; i.p.; Saggittal). A small 1 cm midline sagittal skin incision was cut approximately on the scalp to expose the skull. Two holes (Bregma: +0.05 cm, Medline: $\pm$0.1 cm) with a diameter of 0.2 cm were stereotaxically drilled in the skull for the injection of particles into the left hand side of the sub-ventricular zone (SVZ). The particles (5 μl, 2000 μg ml^{-1}) were then stereotaxically administered into the target sites (Dura: −0.5 cm; 1 μl min^{-1}) and were allowed to incubate for different amounts of time (0 hour [$n = 5$], 1 hour [$n = 5$], 3 hours [$n = 5$], 6 hours [$n = 5$] and 24 hours [$n = 5$]).

Nguyen T. K. Thanh remarked: Could you please comment on the translational aspect of this technology? How close is it to the clinical trial? What else is needed to be done before neural stem cell therapies can be a reality? What type of rats did you use; you mentioned Institute of Cancer Research rats?

Edman Tsang answered: Magnetic separation of biological species, magnetic hyperthermia and magnetic controlled drug release using modified magnetic nanoparticles have already found clinical applications. The use of iron oxide based magnetic nanoparticles in particular is deemed acceptable with FDA approval. However, the technique involving the use of such particles to extract

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neuron stem cells from a live brain *in vivo* has only recently been disclosed by our group. We are at the stage of refining and optimizing important parameters such as minimization of unnecessary damage to underneath brain tissue and reduction in the retention of magnetic particles in the living brain, *etc.* These are important parameters that we need to address before any further clinical trial is undertaken. We are therefore unable to put forward a timeline but so far the results look encouraging. The type of rat: SD (Sprague Dawley) rats (male and female), 150–180 g. The animal experiments have been carried out with our research partners: one based at Hong Kong Baptist University and the other at the Taiwan Mouse Clinic.

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Dejian Zhou said: Where are the magnetic nanoparticles located? On the inside or outside of the cells?

Edman Tsang replied: Initially the magnetic nanoparticles are attached to the exterior of the cells due to the surface recognition groups ($CD133^{+}$). However, confocal microscopy showed that some magnetic particles are internalised into the cells depending on various factors such as incubation time and particle concentration.

Dejian Zhou said: Have you determined whether the magnetic nanoparticles are located inside or outside of the cell surface? This can be done by cryo-TEM imaging by which we have recently shown that nanoparticles are mostly trapped in endosome-like intracellular compartments after cell uptake.[1]

1 L. Song, V. H. B. Ho, C. Chen, Z. Yang, D. Liu, R. Chen and D. Zhou, *Adv. Healthcare Mat.*, 2013, **2**, 275–280.

Edman Tsang responded: Thank you for the suggestion of using cryo-TEM imaging. Although the confocal microscopy shows evidence for the internalization of these particles into the cells the image resolution is not satisfactory. The mentioned technique could be useful for further study in this area.

Dejian Zhou remarked: When cells differentiate, where do the nanoparticles end up?

Edman Tsang answered: This is an interesting question. The simple answer is that we do not know as we have not yet monitored the differentiation process of cells in the presence of the magnetic nanoparticles. Their presence and their location could be important regarding cell differentiation.

Asterios Gavriilidis asked: How do the microemulsion properties affect nanocomposite shell thickness?

Edman Tsang responded: Basically, the thickness of the silica shell increases as the amount of TEOS increases; a situation in which core-free silica particles appear when the concentration of TEOS is too high should be avoided. Also, there are other minor factors that can affect the shell thickness *e.g.* the ratio of base to amphiphilic surfactant, reaction time, the size of aqueous domains and the

number of hydrophobic NPs added into this system, *etc.* For detailed information please refer to the paper titled "Fe_3O_4@SiO_2 Core/Shell Nanoparticles: The Silica Coating Regulations with a Single Core for Different Core Sizes and Shell Thicknesses".[1]

1 H. L. Ding *et al.*, *Chem. Mater.*, 2012, **24**, 4572–4580.

Asterios Gavriilidis remarked: What is the reason for some nanoparticles having two iron cores?

Edman Tsang answered: There are many possible reasons leading to two iron cores in the same shell, which has been commonly observed when core–shell particles are synthesized *via* a reverse micro-emulsion technique. However, we believe that the main reason in our case is that the number of magnetic nanoparticles added (excess) did not match with the number of aqueous domains (micelles) in the reverse micro-emulsion system, hence two iron cores shared the same shell. It should be noted that it is very difficult experimentally to match their numbers.[1] However, by optimizing our system, we managed to produce 90% of our Fe_3O_4@SiO_2 particles with a single core (please refer to the low magnification TEM image Fig. S2 in the supporting information of our paper (*Faraday Discuss.*, 2014, DOI:10.1039/C4FD00132J)).

1 H. L. Ding *et al.*, *Chem. Mater.*, 2012, **24**, 4572–4580.

Sara Carreira said: How many of the magnetic NPs attach to the surface of the neural stem cells *in vivo*? Can you at least estimate it, maybe from the number of CD133 receptors present on the cell surface?

Edman Tsang responded: We are at present unable to give the number of magnetic NPs that attach to each neural stem cell (NSC). It is evident that the process of attachment of ependymal cells by magnetic NPs is rather dynamic in a living subject. They continue to attach and detach in a flowing fluid and can also be taken up by inner cells. As we mentioned in the article, several processes have to be considered: (1) the diffusion and adsorption of particles on NSCs, (2) the removal of particles by cerebrospinal fluid (CSF), (3) dynamically binding/(4) unbinding to NSCs and (5) internalization of bound particles by NSCs. Accordingly, the initial decrease in the T_2 CNR signal can be attributed to the higher rate of particle removal (processes 2 and 4) compared to the counter processes (1), (3) and (5). In order to effectively isolate the NSCs magnetically we injected excess magnetic NPs for maximal attachment, however, we are not yet able to estimate the number of magnetic NPs on a single NSC.

Katherine Brown said: Are you able to determine or quantify the amount of active antibody on your nanoparticles?

Edman Tsang replied: We incubated excess antibodies to the given weight of nanoparticles after chemical functionalization (see the Experimental section of our paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00132J)) in order to maximize the carrying capacity of the nanoparticles and reduce multiple attachment to

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two or more nanoparticles. However, we did not determine the exact amount of grafted antibody per nanoparticle. No doubt that different morphological forms of bound antibodies of different activities arise. We are currently working to quantify their attachment, morphology and their activity.

Hedi Mattoussi asked: Is there a minimum required number of antibodies attached to a nanoparticle to promote strong binding onto the target cell membranes?

Edman Tsang answered: In our experiment, we aimed to attach as many antibodies as possible to a single nanoparticle. This not only enhances its chance of binding to the CD133 receptors of a neuron stem cell (NSC) but it also strengthens the interactions between the composite particle carrying the antibodies and NSCs through multiple antibody–antigen bonds (surface recognition groups). As a result, we have not yet studied the minimum number of antibodies per particle for the binding of NSCs. It is noted that the binding force between such particles and NSCs could depend on their relative numbers and morphologies, which are rather difficult to assess.

Matthew Todd asked: If the aim of using a nanoparticle appended to an antibody is to extract cells, don't you ideally want one antibody per particle? That way you will have multiple nanoparticles attached per cell, increasing the chance of extraction. On the other hand, if you make particles each appended to multiple antibodies, you will more likely achieve aggregation behaviour, since one particle could (cross-)link to multiple cells.

Edman Tsang answered: Thank you for your interesting suggestion. However, we feel that it is rather difficult to engineer one single molecule on a single particle surface since chemical modification is normally carried out on a generic surface with many related energetic surface sites. Although there are reported methods to immobilize a single guest molecule on a particle surface, the process is always time consuming and complicated.[1] This is achieved by limiting the chemical functionalities on a single particle; the stability of this antibody–particle ensemble through limited surface bonding is still open to question. Thus for us, a more practical way is to graft more than one guest molecule onto a single particle. We understand the concern of aggregation, however, the relative size of the magnetic nanoparticle is about 50 nm and the size of the $CD133^+$ cell (antigen) is tens of microns. Thus, there will not be many cells that can be spatially attached to the magnetic nanoparticle–antibody composite. Besides, the antibody–antigen composite is highly specific and complimentary recognition groups must be present between them before they can be attached in a specific orientation. We feel the chance of the same bound antibody becoming fixed with one antigen to bind with another bound antibody and leading to extensive aggregation is not high.

1 M.-L. Ho *et al.*, *J. Phys. Chem. C*, 2009, **113**, 1686–1693.

Nguyen T. K. Thanh said: Your work is so fascinating, so I hope you do not mind another question.

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You said that you would like to isolate a single cell, but that would be very hard with magnetic separation, how will you tackle this?

Edman Tsang answered: We are pleased that this new work is appreciated by our fellow researchers. Yes, it will be a very challenging goal. Firstly, according to our results, the neuron stem cells (NSCs) appear to be located in specific areas of a living rat brain rather than being evenly distributed on all brain tissue. Specifically, they are located by MRI in the epithelial regions of ventricles and SVZ areas. However, there is no information on how these NSCs are distributed depth-wise. On the other hand, many brain diseases are thought to relate to their deprival in some functional areas. It will therefore be exciting to move the NSCs from rich areas to deficient areas in future nano-surgery. At this stage, we are unfortunately unable to image them with sufficient resolution in order to differentiate them on a single cell basis. To move this research forward, we believe the first stage is to achieve single cell imaging of our optimized particles (t1/t2 optimization). Then we would be able to relocate each of them by magnetic means in a highly precise way (computer aided technology). We agree that the magnetic separation would depend on the overall magnetic susceptibility of the particles (the total volume of the magnetic phase). In order to achieve this goal, we would need to design the particles with a high response as well as a small but strong magnetic probe for computer operated relocation for the required precision.

Peter Dobson opened the discussion of the paper by Oliver Reiser: I believe that the particles you refer to in your work are ferromagnetic so the magnetic moments will be much larger than the superparamagnetic iron oxide particles that have been referred to in earlier papers in the meeting.

Oliver Reiser responded: Yes, this is correct. The "naked" carbon coated nanoparticles have a magnetic moment of approx. 140–150 emu g^{-1}, which will decrease once you attach molecules, for example polymers. We feel that this can be an advantage since you are able to generate highly functionalized materials that still have a considerable high magnetic moment, but the disadvantage is that agglomeration, especially with the unfunctionalized particles, is more severe.

Peter Dobson commented: Because the particles are ferromagnetic with a fairly high magnetic moment, they will be difficult to disperse I believe?

Oliver Reiser replied: The unfunctionalized particles are not dispersible, *e.g.* in water. With the appropriate coating, *i.e.* the PEI coating shown here (and described in our paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00108G) the particles are perfectly dispersible in water.

Edman Tsang asked: In the encapsulation of a metal phase by carbon nanotubes and related structures, there is a defective region in the carbon structure which may lead to leaching of the metal phase into corrosive solution. Has this been observed in your experiments? Are any measures to reduce such metal leaching in place?

Oliver Reiser responded: Thank you for this important comment. We constantly discuss this with our collaborators at the ETHZ who manufacture the

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particles. In fact, we have noticed differences in quality in various batches that we have received from them (you can easily check by adding EDTA to see if you extract cobalt). TURBOBEEDS who sells the particles is therefore performing quality checks to make sure that they not distribute particles which have such effects that would cause metal leaching. All in all, credit to the procedure developed at the ETHZ as the coating is generally uniform to prevent metal leaching.[1]

1 R. N. Grass, E. K. Athanassiou, W. J. Stark, *Angew. Chem.*, 2007, **46**, 4909.

Edman Tsang commented: Is there any issue in the material preparation that means that encapsulated metal particles are not totally separated from each other. Would this create a problem in magnetic separation if they were actually aggregated during graphitization?

Oliver Reiser replied: Thank you for the question, all credit goes to our collaborators at ETHZ who manufacture the particles.[1] Based on TEM analysis of these particles, single entities and non-aggregated ones are formed during graphitization, so we did not run into this problem. Magnetic separation works very well with these particles.

1 R. N. Grass, E. K. Athanassiou, W. J. Stark, *Angew. Chem.*, 2007, **46**, 4909.

Dejian Zhou said: Are these magnetic nanoparticles cytotoxic? The stability of the amine grafted magnetic nanoparticles can be further stabilised by PEGylation. We have found that PEGylated nanoparticles can sustain freeze-drying without undergoing aggregation.

Oliver Reiser responded: Thanks for the comment and suggestion. We do not yet know the cytotoxicity of the particles, but it is currently under investigation by our colleagues in bioanalytics. Thank you also for pointing out the PEGylation – we need to look into this more. We have some preliminary results though that PEGylated PEI nanoparticles are less stable than the PEI only particles.

Liane Rossi commented: Regarding the stabilization of the cobalt nanoparticles against oxidation: is it related to the carbon-coating or a matter of particle size?

Oliver Reiser responded: The stabilization is due to the thin (3–5 layers) graphene-type layer around the particles.

Liane Rossi commented: What characterization techniques were used to show that the cobalt nanoparticles are metallic and do not oxidize after exposure to air?

Oliver Reiser replied: The following work was not done by us but by our collaborators (Prof. W. Stark, ETH Zurich; Turbobeeds Inc.) who manufacture the nanoparticles:

A combination of three methods was used:

a) X-ray diffraction to validate the identity of the metallic crystalline phase. No oxidic phases could be identified.

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b) Magnetic measurements; cobalt oxide is not ferromagnetic, so any saturation magnetization has to be allocated to the metallic phase. The saturation magnetization of the carbon coated cobalt nanomaterials is very close to the saturation magnetization of bulk cobalt.

c) Thermogravimetry: Due to the weight gain upon oxidation of the metallic material at elevated temperatures (at 400 °C) to the oxides, the initial metal content can be calculated.

Nguyen T. K. Thanh commented: Did they determine that it is a graphene layer on the surface of nanoparticles, and not any other form of carbon?

Oliver Reiser replied: Yes, our collaborators at the ETHZ did Solid State NMR, Raman, IR and also measured electronic transport, everything – as well as the chemical reactivity we see – is consistent with a graphene-like surface.[1]

1 W. Stark *et al.*, *Acc. Chem. Res.*, 2013, **47**, 2297.

Matthew Todd commented: Covalent attachment to a nanoparticle means that the attached compound is fixed to the surface. π-π attachment is interesting because the appended species could move over the surface. This could, for example, permit initial binding interaction followed by the slower development of polyvalency in binding. Do you see evidence of such movement on the surface?

Oliver Reiser answered: We have deposited Pd-nanoparticles on the carbon surface of the magnetic nanoparticles, and indeed, we see movement here in ref. 1. We also see that the attachment through π-stacking (pyrene) units is reversible with temperature, so indeed I would expect to have movement on the surface.

1 Q. M. Kainz *et al.*, *Adv. Funct. Mat.*, 2014, **24**, 2020.

Nguyen T. K. Thanh asked: In Fig. 2 (in *Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00108G) you mentioned: "The dispersibility of 12 was by far superior in dichloromethane than in water."

There are some ligand-ligand interactions, and 12 might not form a well-packed passivating layer due to side chain packing/bonding properties.

We have studied the effect of 58 different peptide sequences on the electrolyte-induced aggregation of the nanoparticles in our paper.[1]

1 R. Levy, N. T. K. Thanh, R. C. Doty, I. Hussain, R. J. Nichols, D. J. Schiffrin, M. Brust, D. G. Fernig, *J. Am. Chem. Soc.*, 2004, **126**, 10076–10084.

The stabilities conferred by the peptide ligands depended on their length, hydrophobicity, and charge.

Oliver Reiser replied: Yes, very nice work, and I completely agree with your statement that the stability of the dispersions is dependent on length, hydrophobicity, and the charge of the outer layer. What we wanted to show with Fig. 2 in our paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00108G) is that (1) typical coatings like MeOPEG 2000 that have been shown to stabilize metaloxide nanoparticles are not sufficient for the highly magnetic metal particles we have studied

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and (2) that seemingly very similar coatings (*e.g.* compare 12 and 16) can still confer quite different stabilities to the nanoparticles.

Nguyen T. K. Thanh asked: If the nanoparticles are polydisperse, different sized particles would not only have different physical properties, but they would also have different surface areas. Therefore, the coating might not be so effective, as different curvatures require different coatings (*e.g.* molecular structures). Moreover, they would have different numbers of active molecules, such as antibodies. So, the bioactivities are also different; this would cause non-reproducibility of subsequent biological assays.

Oliver Reiser replied: Thank you, this is a very good comment, and we will need to address this when we move to biological assays. The nanoparticles have by and large a diameter of 50 nm, but indeed, smaller particles are also present.

Hedi Mattoussi remarked: We know that cobalt metal is easily oxidized by simple exposure to air and/or water.

Does the graphene protective layer used in your Co nanoparticles provide enough shielding to avoid the issue of cobalt oxidation?

Oliver Reiser answered: Yes, while the oxidation of metallic cobalt surfaces even occurs at room temperature the carbon layer shields the surface from this oxidation. This can be evidenced by differential calorimetry combined with thermogravimetry, where no material oxidation (= weight gain and energy release) is observed at temperatures <180 °C. Similarly, the oxidative properties in water over a wide pH range have been quantified by leaching studies over the course of several weeks[1] and support the excellent shielding properties of the carbon layers. Our collborators at ETH Zurich and Turbobeeds who manufacture the particles have been using the materials for more than seven years, and still have old samples – they can find no indication of cobalt oxidation during storage at ambient conditions over this time frame.

1 A. Schaetz *et al.*, *Chem. Eur. J.*, 2011, **17**, 10566–10573.

Catherine Amiens said: When you functionalised the graphene layer around the cobalt nanoparticles, you either use or create defects. Does it alter the protection that this graphene layer affords against oxidation of the cobalt core?

Oliver Reiser answered: The functionalization takes place only on the first carbon layer exposed to the surface. Since there are multiple carbon layers around the nanoparticle (3–5), the stabilization is not affected.

Catherine Amiens commented: To estimate the mobility of the ligands physisorbed on the graphitic surface, you could work with ligands bearing radicals at the end of their chains (such as TEMPO).

This method has been followed by V. Chechik to demonstrate the mobility of thiols on nanoparticles.[1]

1 P. Ionita, A. Volkov, G. Jeschke, V. Chechik, *Anal. Chem.*, 2008, **80**(1), 95–106.

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Oliver Reiser replied: Thank you for this excellent suggestion, we will definitely follow up on this, especially, since we had have already experience with (covalently bound) TEMPO to these nanoparticles.[1]

1 A. Schaetz *et al.*, *Chem. Eur. J.*, 2008, **14**, 8262.

Edman Tsang asked: The total encapsulation of graphitic layers on Co particles is rather challenging to achieve. The Co particle may have different shapes and crystallographic facets. The graphitic layers composed of sp^2 carbon atoms are also spatially rigid, and hence will not be able to join up seamlessly around the particle to offer the perfect encapsulation. My main question is are there substantial defect regions between the junctions of the graphitic layers, particularly at the interface between the metal facets that cause metal leaching into the solution?

Oliver Reiser answered: Thank you, this is a point well taken, and we constantly discuss this with our collaborators at the ETHZ who manufacture the particles. In fact, we have noticed differences in quality in various batches we have received from them (you can easily check by adding EDTA to see if you extract cobalt). TURBOBEEDS, who sells the particles, is therefore performing quality checks to make sure that they not distribute particles that would cause metal leaching. All in all – credit to the procedure developed at the ETHZ the coating is generally uniform to prevent metal leaching.[1]

1 R. N. Grass, E. K. Athanassiou, W. J. Stark, *Angew. Chem.*, 2007, **46**, 4909.

Maya Thanou opened the discussion of the paper by Kerry Chester: Are there any potential risks in the use of dextran sulfate as a plasma expander?

Kerry Chester answered: To the best of our knowledge, unsulfated dextrans are the agents clinically used as plasma substituents.[1,2] Dextran sulfate 500 has been used clinically as an anti-coagulant (as it is a synthetic analogue to heparin)[3] and an antilipemic, as well as an antiviral against human immunodeficiency virus (HIV);[4] it was shown that dextran sulfate inhibits the binding of viruses to target cells and was used as an anti-HIV agent in patients with AIDS. However, in the doses applied it showed limited anti-viral efficacy as well as some toxicities (like reversible thrombocytopenia and alopecia) and was not taken further as an antiviral agent.[5]

1 C. Y. Quon, Clinical pharmacokinetics and pharmacodynamics of colloidal plasma volume expanders, *Journal of Cardiothoracic Anesthesia*, 1988, **2**, 13–23.
2 R. McCahon and J. Hardman, Pharmacology of plasma expanders, *Anaesthesia and Intensive Care Medicine*, 2010, **11**, 75–77.
3 K. Walton, Experiments with dextran sulphate as an anticoagulant, *Proc. R. Soc. Med.*, 1951, **44**, 563–4.
4 H. Mitsuya, D. J. Looney, S. Kuno, R. Ueno, F. Wong-Staal and S. Broder, Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4+ cells, *Science*, 1988, **240**, 646.
5 C. Flexner, P. A. Barditch-Crovo, D. M. Kornhauser, H. Farzadegan, L. J. Nerhood, R. E. Chaisson, K. M. Bell, K. J. Lorentsen, C. W. Hendrix and B. G. Petty, Pharmacokinetics, toxicity, and activity of intravenous dextran sulfate in human immunodeficiency virus infection.[1]
1 C. Flexner *et al.*, *Antimicrob. Agents Chemother.*, 1991, **35**, 2544.

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Maya Thanou remarked: Near IR fluorescence may be affected (quenched) depending on the distance from the core NP and or the density of grafting. Do you observe such a phenomenon?

Kerry Chester replied: This is an interesting point, but we have not attempted to measure quenching.

Nguyen T. K. Thanh remarked: In your paper you wrote: "We therefore developed methods to functionalize SPIONs with near-infrared (NIR) dyes in order to trace their biodistribution" In Fig 6 in your paper, it says "NIR signal of SPIONs in blood measured on Odyssey scanner". Could you please clarify how you trace their "biodistibution"?

Kerry Chester replied: We only measured the blood but *in vivo* imaging can be done using a mouse pod attached to the LiCor Odyssey scanner: http://www.licor.com/bio/products/accessories/odyssey/mousepod/. Examples of some papers that used the scanner to trace and quantify dye labelled conjugates *in vivo* can be found in refs. 1–4.

1 J. L. Kovar, M. A. Simpson, A. Schutz-Geschwender, D. M. Olive, A systematic approach to the development of fluorescent contrast agents for optical imaging of mouse cancer models, Biochemistry, Faculty Publications, 2007, **9**.
2 J. L. Kovar, W. Volcheck, E. Sevick-Muraca, M. A. Simpson and D. M. Olive, Characterization and performance of a near-infrared 2-deoxyglucose optical imaging agent for mouse cancer models, *Anal. Biochem.*, 2009, **384**, 254–262.
3 J. L. Kovar, W. Volcheck, J. Chen and M. A. Simpson, Purification method directly influences effectiveness of an epidermal growth factor-coupled targeting agent for noninvasive tumor detection in mice, *Anal. Biochem.*, 2007, **361**, 47–54.
4 L. Sampath, S. Kwon, S. Ke, W. Wang, R. Schiff, M. E. Mawad and E. M. Sevick-Muraca, Dual-labeled trastuzumab-based imaging agent for the detection of human epidermal growth factor receptor 2 overexpression in breast cancer, *J. Nucl. Med.*, 2007, **48**, 1501–1510.

Maya Thanou asked: At what time post-injection can you observe NIRF in the blood samples?

Kerry Chester replied: We have only investigated the NIR signal 1 hour post injection but it will be interesting to further investigate the fate of the NIR labelled SPIONs at different time points.

Hedi Mattoussi questioned: (1) Does the sulfate agent used in your experiment block binding to cells by interacting with the receptor(s) or by interacting with the nanoparticle itself?

(2) Have you considered using polyethyelne glycol (PEG) to coat the nanoparticle surfaces as a means of reducing nonspecific interactions?

Kerry Chester answered: (1) We have not carried out a formal experiment but our working hypothesis is that dextran sulfate 500 interacts with the receptors and not the SPIONs themselves. Ferucarbotran is rapidly cleared from circulation mainly (but not exclusively) by the scavenger receptors present on the liver Kupffer cells. Dextran sulfate 500 is a known blocker of this type of receptor and we achieve blocking more effectively when we give dextran sulfate 500 at 2 and

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24 hours prior to Ferucarbotran than when we give both agents simultaneously. Furthermore, both Ferucarbotran and dextran sulfate 500 are negatively charged, suggesting that direct interactions with each other are less likely. Please refer to the first and third paragraph of the paper discussion for references.

(2) Yes, we have considered PEG but have not yet explored this option for clinical use for a number of reasons, including the complexity, heterogeneity and biodegradability of PEGylated products and our experience that, in some instances, PEG modification of SPIONs can increase unspecific or untargeted internalisation and uptake (see reference 37 in the paper).

Maya Thanou enquired: For NP injected directly into the brain, is a stealth coating required?

Kerry Chester responded: Within the brain tumour microenvironment there are a range of cell types which include: tumour core, microglial cells (brain macrophages), astrocytes, oligodendrocytes and neurons. All these cell types could take up the SPIONs, specifically the macrophages. Therefore, despite the possible evasion of the RES by direct intratumoural injection of SPIONs, stealth is still advisable.

Dejian Zhou opened the discussion of the paper by Benjamin P. Burke: Where are the gallium ions loaded on the magnetic nanorods?

Benjamin P. Burke answered: This is a question that we are very interested in answering but have not yet identified an experiment to satisfy our curiosity. The issue lies in the potential difference between the larger amounts of gallium(III) used in a standard characterisation experiment and the picomolar amounts of radioactive gallium ions. It has proved challenging to assign data in some spectroscopic methods used to analyse the non-radioactive gallium(III) species, due to overlap of Fe–Si and Fe–O stretches. Even if an assignment could be made it may not be an analogous mechanism for the much smaller amounts of radioactive gallium.

It is interesting to note that the formation of stable multinuclear gallium oxide cations has been observed in zeolite pores.[1] This could be of relevance to the observed high stability interaction with the silica surface.

1 E. A. Pidko *et al.*, *Phys. Chem. Chem. Phys.*, 2009, **11**, 2893–2902.

Tom Berkleman asked: Could you be seeing deposition of gallium oxide, perhaps templated or promoted by your silica layer?

Benjamin P. Burke replied: This is one possible mechanism. However, it seems unlikely that it would be a coating, as the radiosynthesis is carried out at acidic pH and the subsequent stability is high. We think that it is more likely to be a gallium species (which could be oxides or hydroxides) formed in pores on the silica surface. This hypothesis requires further investigation (see also the answer to the previous question).

Sara Carreira asked: What was your motivation for using rod-shaped particles rather than spherical ones? Are there any advantages of using that shape?

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Maya Thanou asked: Rods interfere with the cell membrane in a different way to spherical nanoparticles. What is the rationale of using rods for this application?

Benjamin P. Burke answered both questions: Rod shaped particles were selected in part for reasons of curiosity. Iron oxide based PET/MR imaging agents have been developed a handful of times previously, but always using nanospheres. We wanted to see if we could not only develop a novel method of radiolabelling for PET, but also using a previously untested core material shape. We anticipate that similar procedures will be equally valid with spherical iron oxide nanoparticles. The literature is less well developed for iron oxide nanorods than it is for nanospheres, however preliminary studies offer possible advantages in magnetic susceptibility and cell penetration.

Nguyen T. K. Thanh asked: What is the crystal phase of your iron oxide nanorods?

Benjamin P. Burke answered: The preparation of iron oxide nanorods in this publication utilises literature methods (references 42 and 43 in our paper) which are known to form magnetite.

Nguyen T. K. Thanh remarked: What do you think about the regulatory issues of such a novel ligand for clinical use?

Benjamin P. Burke responded: Novel MRI contrast agents have to meet the standard regulations and approval processes for the use of new agents in humans. The key advantage of this work is its potential applicability in the modification of currently licensed agents to a form of PET/MRI multimodal imaging constructs.

Nguyen T. K. Thanh asked: How do you quantify the radioactive dose if your particles are very different sizes?

Benjamin P. Burke replied: The radioactive dose is quantified as activity (MBq)/Fe weight (g) and is therefore not size dependent.

Edman Tsang remarked: Are there any advantages or disadvantages of your approach compared to other labelling techniques?

Benjamin P. Burke answered: The advantages come from the simplicity of the approach: the preparation is facile as no chelator is attached to the surface, only short heating times are needed and it is a final step radiolabelling process. The tracer can therefore be produced rapidly (which is an important issue with short lived radioisotopes). The biggest disadvantage is the temperature required for the radiolabelling reaction, which could lead to stability issues with some surface coatings.

Edman Tsang asked: Would any radioactive species leach out to the external environment when applied ?

Benjamin P. Burke replied: During *in vitro* experiments using human serum we see no evidence of radioactivity being transchelated to transferrin at up to 3

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hours, which is the maximum applicable time for acquiring image data using the short lived ^{68}Ga isotope.

Matthew Todd remarked: The outlier result from the Ga labelling in Figure 3 is the increase in size for 5. Does this imply that the metal is loosely chelated by something other than the macrocycle, and in a way that leads to aggregation of several rods through chelation of a metal by more than one rod?

Benjamin P. Burke replied: Although the proposed mechanism is possible, we believe it to be temperature related. Reactions with ^{68}Ga at room temperature do not seem to cause aggregation. In our opinion the most likely explanation is temperature based aggregation of PEG chains, which is documented.

Amelie Heuer-Jungemann opened the discussion of the paper by Nguyen TK Thanh: Have you encountered any problems during the coupling, *e.g.* aggregation due to one glutaraldehyde molecule coupling to two different particles?

Lanry L. Yung asked: Can you give any information about the experimental design, with regard to minimizing particle crosslinking when functionalizing the surface of the particles with glutaraldehyde?

Edman Tsang asked: How can you avoid the problem of condensation between amine and aldehyde onto the same particle?

Nguyen T. K. Thanh responded to all three questions: In our experiment we used huge a excess of glutaraldehyde (GA). Density of GA per nm^2: 4.10×10^8. therefore each particle is immediately coated with GA, so we did not observe any cross linking.

Paresh Ray queried: How many antibodies are attached to each particle?

Nguyen T. K. Thanh answered: We do not normally calculate the number of antibodies attached to each particle, for further information please see:

B. Kozissnik, L. A. W. Green, K. Chester and N. T. K. Thanh, Strategy for functionalisation of magnetic nanoparicles for biological targets, in Magnetic nanoparticles: from fabrication and clinical applications, ed. N. T. K. Thanh, CRC Press, Taylor and Francis, Boca Raton, London & New York, 2012, pp. 129–150.

Tom Berkleman said: I would like to point out that with the high concentration of glutaraldehyde used, it would be in orders of magnitude excess over the amine functionality on the surface of the nanoparticle. You actually wouldn't expect much crosslinking.

Nguyen T. K. Thanh answered: We agree.

Scott Mitchell asked: In terms of control experiments for the interactions with *V. cholerae* bacteria, I was wondering what the TEM images of the IONPs@CHI look like. Do the IONPs@CHI interact in the same way as the complex of ION-P@CHI@GA@PrA@Ab? Surely this is an essential control that must be shown to

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test the hypothesis of the presence and importance of the Ab conjugated to the polymer matrix? It is an important study to show: Abs are expensive, so in this case are they really necessary?

Furthermore, what about the toxicity of the IONPs@CHI and IONP@CHI@GA@PrA@Ab? The growth of the bacteria appears to be affected in the TEM images. Did the authors carry out Resazurin cell viability assays (or similar) to quantify the toxicity of the particles, or lack thereof?

Nguyen T. K. Thanh responded: It is clearly stated in the paper: "For positive controls, the solutions containing IONP@CHI and ION- P@CHI@GA@PrA were incubated with *V. cholerae* bacteria diluted in water from an initial concentration of 103 cfu mL^{-1}. The same procedures used for testing the IONP@CHI@GA@PrA@Ab complex were followed. A significant number of nanoparticles were found clustered on the grid, but no *V. cholerae* bacterial cells were found (data not shown)." I do not see the relevance of cell viability assays in this work.

Dejian Zhou said: How stable is your magnetic nanoparticle-protein A conjugate? Are the results repeatable after being stored for an extended period? This is important in terms of practical applications. For example, we have found that our covalently conjugated magnetic nanoparticle-DNA conjugate is stable for about 6 months,[1] which is often considered the minimum stability requirement for commerical reagents.

1 Y. Zhang, C. Pilapong, Y. Guo, Z. Ling, P. Quirke and D. Zhou, *Anal. Chem.*, 2013, **85**, 9238.

Nguyen T. K. Thanh replied: To retain the bioactivity of protein A molecules, their stability can be maintained for years if kept at −20 °C. In our experiments, the conjugate was tested every month; during the first 3 months in storage at 4 °C we did not see any difference in the tested results between different time points. We expect it to remain stable for even longer.

Dejian Zhou remarked: Detection of bacteria in your method takes place by SEM or TEM – can you quantify the number of bacteria? What is the dynamic range of detection?

Nguyen T. K. Thanh responded: In our method, the number of bacteria can be quantified by TEM or SEM. We reported that the conjugate could easily separate *V. cholerae* bacteria from water samples at concentrations as low as 10 cfu mL^{-1} by TEM observation.

Liane Rossi asked: Did you reduce the imine group obtained by the condensation of the aldehyde group of glutaraldehyde and the terminal amino group on the nanoparticle surface? Please add this information in the experimental section.

Nguyen T. K. Thanh answered: No, we did not.

Lucio Litti commented: You used imine formation to cross-link the nanoparticle and protein A. As you know, Schiff bases are pH-sensitive and can

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undergo hydrolysis. Did you verify the stability of your systems in terms of protein A antibody release in aqueous media? On the other hand, you could use the complete hydrolysis of your Schiff bases for a quantitative estimation of the total number of antibodies loaded onto your nanoparticles.

Nguyen T. K. Thanh replied: We used an excess of glutaraldehyde in the conjugation step. The imine hydrolysis is assisted through the use of an acid catalyst. Under our conditions, the final products IONP@CHI@GA@PrA@antibody were formed, and evidently they were selectively bound to the bacteria. We tested our conjugates after three months and they were still stable.

Kerry Chester opened the discussion of the paper by Ivan Parkin: In Figure 2 in the paper there appear to be particles that are rod-shaped and others that are spherical, why is this?

Ivan Parkin replied: This is because they are of different types of material – one is due to the iron oxide (spherical) and the other to rhenanite (rods).

Kerry Chester asked: Is there a way to control the synthesis process to obtain uniform particles?

Ivan Parkin answered: We are looking into this but do not have a synthesis for mono-dispersed particles. We will be exploring a microfluidics approach outlined in Scheme 1 in our paper "Sample (I) is the route to iron oxide nanoparticles proposed by Park *et al.*[3]"

Catherine Amiens commented: In your paper you mentioned that nanorods are epitaxially grown. What do you mean exactly? Do the iron oxide rods grow on top of the $NaCaPO_4$ ones? What is the epitaxial relationship in this case?

Ivan Parkin replied: The paper has since been rectified, as the rods were not epitaxially grown, rather the particular blend of surfactants afforded by the shark liver oil/olive oil mix was enough to promote directional growth of $NaCaPO_4$ (β-rhenanite) rods. As for iron oxide, there were spherical nanoparticles observed in the sample, and a little iron content was observed for the rods by EDX spectroscopy, so the possibility of the growth of the rods from iron oxide nanoparticles remains a distinct possibility.

Catherine Amiens asked: Do you mean that in the preparation you observe $NaCaPO_4$ rods and iron oxide rods at the same time?

Ivan Parkin responded: Just the one type of rod was observed, with a portion of iron oxide nanoparticles. The composition of said rods was difficult to determine, but is probably $NaCaPO_4$ (β-rhenanite). However the possibility that the rods grew from a seed iron oxide nanoparticle is possible.

Catherine Amiens commented: Can you please give the mean size of the nanoparticles used during hyperthermia experiments. It would be useful for comparison purposes.

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Ivan Parkin replied: From this and previous work, the optimum size for nanoparticles for hyperthermia are single magnetic domain particles of around 25 nm in size. However this is related to the frequency used in the hyperthermia system. In theory it should be possible to sweep the frequency of the hyperthermia apparatus and find an optimum resonance value that could be used for a wide range of nanoparticle sizes. However this does not seem to be easy to achieve and hence we have focussed our efforts on making near monodisperse particles around the best heating values. In this paper, nanoparticle sizes for magnetic hyperthermia were recorded as follows: Standard synthesis ~8.5 nm, iron-palmitate decomposed in 1-octadecene ~10 nm and iron-palmitate decomposed in 1-octadecene ~12 nm nanoparticles with rods in the order of ~150 nm in length.

Matthew Todd commented: You are applying a field and generating motion. Presumably you can run this the other way – move a liquid in which the same nanoparticles are suspended and generate a field? *I.e.* conversion of mechanical power to electrical?

Ivan Parkin responded: The nanoparticles used in this paper are super-paramagnetic so do not have a net magnetic moment under normal conditions. This may be possible with ferrofluids of greater particle size.

Nguyen T. K. Thanh asked: In Scheme 1 you state "Sample (I) is the route to iron oxide nanoparticles proposed by Park *et al.*,[3] Sample (II) is the synthesis of iron palmitate from high street sources and its subsequent decomposition in 1-octadecene and Sample (III) the decomposition of iron palmitate in shark liver oil." In the reprint, SQUID magnetometry shows:

"Samples (I), (II) and (III) have saturation magnetisation values of 52, 2.2 and 0.1 emu per gram, respectively". Could you please explain why Sample (III) has lost its saturation magnetisation by ~900 fold?

Ivan Parkin responded: We are not sure why this occurred – probably due to different sized crystallites.

Sandhya Moise asked: What is the dominant mechanism of heat generation for your nanoparticles during magnetic hyperthermia? What are the field conditions used?

Ivan Parkin answered: There are thought to be three mechanisms in operation – changes in Brownian motion, reversal by thermal activation and suppression of the anisotropic barrier by a magnetic field for single domain nanoparticles. For multidomain particles domain nucleation and domain wall switching can be important. I am not a physicist but I normally think about this heating effect in terms of a friction induced by the fact that the external field can switch faster than the particles, and the lag generates a heating effect. The field conditions are constant and set by the MACH instrument and are as follows: frequency = 945 kHz, field strength = 6.6 kA m^{-1} with a mass of 1 mg ml^{-1} of iron in the nanoparticles. The coil itself was 6 turns, internally cooled with water, 36 mm in length with 18 mm internal diameter.

Peter Dobson remarked: Regarding the mechanisms for hyperthermia, there are broadly two possibilities. For the larger single domain ferromagnetic particles the heating effects will be because of magnetic domain reversal; for the smaller superparamagnetic particles the heating will be associated with the movement of spins within the particle and this will manifest itself in high losses at certain frequencies. This requires a more detailed knowledge of the real and imaginary parts of the magnetic susceptibility of the materials.

Dejian Zhou opened the discussion of the paper by Catherine Amiens: Did you use any base to deprotonate your carboxylate ligands before ligand exchange?

Catherine Amiens answered: We tried to directly use sodium oleate but the exchange was still not effective.

Dejian Zhou asked: Both ligands contain just a single carboxylate to coordinate to the magnetic nanoparticle. The binding strength here may not be strong enough completely displace the original hyrdophobic ligands. It would be good to use chelating ligands to increase the binding strength, especially those with a dendritic shape, which can match the surface curvature of the nanoparticle much better and hence provide much more stable capping of the nanoparticle surface.[1,2]

1 Y. A. Wang, J. J. Li, H. Chen and X. Peng, *J. Am. Chem. Soc.*, 2002, **124**, 2293.
2 D. Zhou, Y. Li, E. A. H. Hall, C. Abell and D. Klenerman, *Nanoscale*, 2011, **3**, 201.

Catherine Amiens answered: This is a very good suggestion. We also plan to try ligands with stronger binding abilities towards the iron surface, such as phosphonate end groups.

Edman Tsang asked: Could you rationalize the chemical reactivity of FeBi? In one instance, Fe is preferentially oxidized but in another instance the reverse is taking place.

Catherine Amiens answered: As iron is located mainly at the surface of the nanoparticles it is not surprising that it should oxidize first. Then in time one can observe that the core of the nanoparticles is also modified. The long distances measured on the HREM images are indicative of oxidation, but we could not determine the type of oxide formed.

Edman Tsang asked: How much scrambling of the atoms within FeBi is taking place in your system?

Catherine Amiens responded: This is a crucial question. It is indeed very important to know if there is any redistribution of the Fe and Bi atoms, especially upon oxidation or after prolonged exposure to biological media. We are beginning work on this study, which is highly challenging due to the small size of the objects.

Edman Tsang said: Did you try EXAFS to characterize your samples?

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Catherine Amiens replied: This has been done in the case of the BiFe150 sample, and was reported in the paper by G. Mattei *et al.*[1] We plan to study the smaller nanoparticles during our next beamtime.

1 G. Mattei *et al.*, *J. Phys. Chem. C*, 2013, **117**, 1477.

Ivan Parkin remarked: Bismuth is very dense and often it is hard to prove if a composite material is formed or if it is a mixture of the elements. We have previously made BiP and it took over 12 separate techniques to provide proof that an actual compound, and not an intimate mixture of the elements, had been formed.

Edman Tsang asked: Have you seen any phase segregation upon oxidation?

Catherine Amiens responded: So far oxidation experiments have been carried out on already segregated nanoparticles only, *i.e.* on nanoparticles consisting of a bismuth core and iron shell.

In this case, HRTEM images clearly show that when working in the solid state, oxidation can be stopped at the very first stage when only the iron shell is oxidized. Upon prolonged oxidation (over weeks), full oxidation of the core is evidenced, however at this stage we cannot tell if the initial segregation between Fe and Bi is retained or if some bismuth atoms migrate towards the surface.

This is an important question that we want to investigate further, especially in the case of oxidation in aqueous solutions, as bismuth leaching would probably prevent the use of these nano-objects *in vivo*.

Lanry L. Yung said: Both Prof. Nguyen Thanh and Peter Dobson have mentioned the potential of applying nanotechnology for applications in infectious disease, such as pathogen separation and diagnostics (Prof. Thanh) and antimicrobial agents (Prof. Dobson). What are the key issues we should address from the materials and chemistry point of view?

Peter Dobson replied: There are several requirements for sensing bacteria and fall broadly into the following categories:

(1) To sense and quantify the presence of bacteria, and to some extent this is now being done in the food industry but it needs to be more widely adopted. The methods in current use measure oxygen consumption as an indicator of the presence of microbes, or, in some cases, the emission of other gaseous products.

(2) There needs to be methods of identifying the species of bacteria present and quantifying them. This should be very rapid and applied at "point of use".

(3) It is desirable to identify the strain of a bacterium and this will require a rapid miniature DNA sequencing technique.

Nguyen T. K. Thanh replied: For magnetic detection or separation of pathogens you need to have stable superparamagnetic nanoparticles with high magnetic moments. Also, the nanoparticles should have functional groups that are specific to the pathogens of interests and have sensitivities that allow early detection.

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Ivan Parkin observed: Surfaces can also be made antimicrobial by incorporation of dyes and gold nanoparticles.

Peter Dobson remarked: I was asked to comment on the scientific challenges. Firstly I suggest that nanoparticles could transform the way we try to design new antimicrobials; secondly there is still a fundamental question about the nature of the electron spins at the surface of magnetic particles, and if these differ from the spins in the bulk of the nanoparticle. These altered spins could be further modified by the binding of ligands onto the surface. This could have implications for the design of particles for use in hyperthermia or MRI.

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Dalibor Soukup remarked: Currently, interparticle dipole–dipole interactions seem to be a hot topic in the field of magnetic hyperthermia. Some papers show that they can enhance the heating efficiency of magnetic nanoparticles, some show the opposite. They can also reduce the magnetisation saturation, thus decreasing the heating efficiency in turn. What is your opinion on these dipole–dipole interactions with respect to magnetic hyperthermia? Routinely, magnetic nanoparticles are tested in solutions in which they are homogeneously distributed, however, once internalised in cells, they are usually stored in endosomes/lysosomes where the interparticle distance is much smaller than that in solutions, which favours dipole–dipole interactions. Do you think there is a need for better methods of testing that would show biologically relevant heating efficiencies?

Peter Dobson responded: This is a difficult question! Frankly I do not know. I have tried to find an answer, and a recent paper by M. E. Sadat *et al.* seems to give part of the answer to your question and they imply that there are very significant differences between "free" and "confined" nanoparticles.[1]

1 M. E. Sadat *et al.*, *Mat. Sci. Eng.*, 2014, **42**, 52.

Ivan Parkin remarked: In magnetic hyperthermia it is critical to have particles of exactly the correct size and shape for maximum effect.

Nguyen T. K. Thanh responded: I agree.

Catherine Amiens commented: Dispersibility is not always well described in the papers.

Most of the time one simply discusses the stability of the colloidal solutions on the macroscopic observation of the precipitation of nanoparticles with time in different conditions (variation of pH, ionic strength *etc.*). No microscopic (or even nanoscopic) description is made of the organisation of the nanoparticles in solution (real dispersion of small aggregates already forming), although this point is very important when discussing certain properties *e.g.* relaxivities in MRI. We need standard conditions to be able to compare results from one paper to the other and an agreement on the level of detail (macroscopic or otherwise).

Nguyen T. K. Thanh responded: We need cryo-TEM for the small aggregates you mention.

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Edman Tsang asked: Is there any subtle change in physiochemical properties with respect to the change in particle size?

Nguyen T. K. Thanh said: Reproducibility in nanoparticle synthesis is one of the biggest challenges. The physical properties of nanoparticles depend on their size, but also the number of ligands coated on the surface. The interaction between ligands is also dependent on the nanoparticle surface area. Therefore, it is very important to produce monodisperse nanoparticles. Currently most of the synthesis of nanoparticles is carried out in batch, subjects it to many factors that could affect the synthesis such as temperature, even at ambient temperature, mixing, and diffusion. Therefore the reproducibility is limited. On a fundamental level, each material will nucleate and grow depending on its reaction conditions and even a small change in conditions, such as pH, can lead to a completely different mechanism.[1]

1 N. T. K Thanh, N. Maclean and S. Mahiddine, *Chem. Rev.*, 2014, **114**, 7610–7630.

Lanry L. Yung commented: Since research knowledge often goes missing when a research student/postdoc leaves the group, I would be interested if anyone has any suggestions on how to keep knowledge within the research group.

Matthew Todd remarked: The development of new antimicrobial agents is a continual battle against resistance. It is easier to develop resistance to some drugs. Is there something unusual about the mechanism of bacterial killing employed by these surfaces (or indeed nanoparticles more generally) that might mean the bacteria would find it challenging to develop resistance?

Ivan Parkin responded: Yes. In general our work on light activated coatings as antimicrobials functions *via* the formation of reactive oxygen species and singlet oxygen. These typically function in a non-specific way to destroy the bacteria through thousands of possible pathways simultaneously. This is different from an antibiotic which typically has a very small number of pathways to attack the bacteria. Hence in the radical or singlet oxygen based approach it is very unlikely the bacteria could develop resistance.

Peter Dobson asked the delegates: How can one measure the temperature changes in hyperthermia? In fact, one might also call into question the very meaning of "temperature" at this nano-level.

Yuri Antonio Diaz Fernandez responded: I appreciate this topic being raised, since it is extremely relevant. The applicability of the definition of macroscopic temperature on the nanoscale is a fascinating and not always sufficiently addressed problem. To my understanding, this question can only be answered in the context of nonequilibrium thermodynamics and fluctuation theory, which, even after several years of development, are still works in progress, obscure to those from other disciplines. Those in the nanomaterial scientific community who have a particular interest in hyperthermia research, will benefit from establishing systematic discussion opportunities with mathematicians and experimental and theoretical physicists, bridging the gaps among these fields. Probably a Faraday Discussion on "Physical Chemistry Experimental Tools and

Theoretical Concepts Applied at the Nanoscale" could be the best starting point for this cross-disciplinary exchange. Regarding available methods to characterize hyperthermia, Pallavicini, Chirico *et al.* have recently demonstrated an indirect spectroscopic method able to probe the local temperature on the surface of different nanomaterials used for hyperthermia.[1]

1 S. Freddi *et al.*, *Nano Lett.*, 2013, **13**(5), 2004–2010.

Scott Mitchell also responded: Our group recently published a paper using DNA conjugated to iron oxide NPs as an indirect method for probing local temperature on the surface of magnetic nanoparticles during magnetic hyperthermia.[1]

1 J. T. Dias, M. Moros, P. del Pino, S. Rivera, V. Grazú and J. M. de la Fuente, *Angew. Chem.*, 2013, **125**, 11740–11743.

Maya Thanou raised the question: How would these magnetic nanoparticles (MNP) compare with already advanced or available MNPs, with respect to their ability to induce hyperthermia?

Ivan Parkin answered: The particles show good hyperthermia response but they are not as efficient as the best commercial materials.

Maya Thanou said: Hyperthermia (mild temperature increase) does not kill tumour cells, however it sensitizes them to other treatments. What other treatments would these be?

Ivan Parkin responded: We have not investigated this. Potential ideas we are developing include having a low melting point wax containing an anticancer drug.

Nguyen T. K. Thanh commented: The thermal energy needed for induction of cell death has been found to be close to the energy needed for protein denaturation, leading to the conclusion that the main cytotoxic effect of hyperthermia is based on the denaturation of membrane and cytoplasmic proteins. For more on this please see ref. 1.

1 A. Hervault and N. T. K. Thanh, *Nanoscale*, 2014, **6**, 11553–11573.

Maya Thanou asked: What is the required time of exposure of tumours to hyperthermic temperatures for sensitisation?

Nguyen T. K. Thanh answered: Please see a paper by M. Johannsen *et al.*

1 M. Johannsen, U. Gneveckow, L. Eckelt *et al.*, *Int. J. Hyperthermia*, 2005, **21**(7), 637–647.

Sandhya Moise addressed Ivan Parkin and Kerry Chester: During magnetic hyperthermia, the temperature rise is very local and falls with distance from the nanoparticle surface. Is bulk temperature measurement of nanoparticle suspensions accurate and representative of temperature changes encountered by the cells?

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Kerry Chester replied: Yes it is true that bulk measurements may not be the best comparison to *in vivo* or *in vitro* measurements, however depending upon the local concentration of nanoparticles within a cellular structure we have observed similar experimental bulk temperature rises *in vitro* and *in vivo*. In these cases all we can realistically say is that the temperature local to the cells has reached its equilibrium point. Constructing an experiment to measure nanoparticle temperatures would be very difficult and at best would have to be modelled/simulated.

Ivan Parkin responded: The local heating is one of the key reasons that hyperthermia works. The bulk temperature measurement will greatly underestimate the actual temperature at the surface of the nanoparticle. However it is a guide to indicate heating and a measure of relative effectiveness.

Stefan Borsley asked Nguyen TK Thanh: You are using imine formation with a short, flexible di-aldehyde (gluteraldehyde, GA) to bind to amine coated nanoparticles. This equilibrium process generally favours the carbonyl/amine side in water, as imine formation results in liberation of water. Imine formation is acid catalysed, so the pH of the solution will also influence the rate of the reaction. Bidentate binding of the GA to two amine ligands on the same nanoparticle also must be considered, this might be expected to be a favourable process due to the flexibility and proximity of the unbound end of the GA, essentially resulting in a higher effective molarity.

Given these concerns, do you have any evidence for determining the number of proteins bound per nanoparticle?

Stefan Borsley communicated: Further to this, the reversible nature of imine formation means that, once bound, proteins may be released as the environmental conditions change, favouring the equilibrium towards the aldehyde/amine side, a particular concern when diluting the samples. Have you verified that you do not get release of proteins from your protein-nanoparticle conjugates?

Nguyen T. K. Thanh communicated in reply: We used an excess of glutaraldehyde in the conjugation step. The imine hydrolysis is assisted through the use of an acid catalyst. Under our conditions, the final products IONP@CHI@GA@PrA@antibody were formed, so evidently they were bound to the bacteria selectively. We tested our conjugates after three months and they were still stable.

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