Faraday Discussions
Cite this: DOI: 10.1039/C4FD90079K

Optical nanoparticles: general discussion

Peter Dobson, Lanry L. Yung, Liane Rossi, Zoe Pikramenou, Sara Carreira, Paresh Ray, Catherine Amiens, Katherine Brown, Maha Abdollah, Dejian Zhou, Mauro Prato, Sandhya Moise, Lucio Litti, Matthew Todd, Kristian Göeken, Stefan Borsley, Amelie Heuer-Jungemann, Oliver Reiser, Peter Harvey, Thomas Carter, Maya Thanou, Dalibor Soukup, Anna Lesniak, Hedi Mattoussi and Siti Fatimah Abdul Ghani

DOI: 10.1039/C4FD90079K

Stefan Borsley opened the discussion of the paper by Hedi Mattoussi : Are there any advantages to using this polymeric His binding system over traditional thiol-bound ligands?

Hedi Mattoussi responded: The use of a multi-coordinating polymer ligand having several histidine groups for surface coordination may be beneficial for gold nanorods (AuNRs). These NRs tend to have large surface areas, and a polymer structure provides stronger binding and better interactions with the hydrophilic surrounding medium. Multi-thiol polymer coating would also provide stronger affinity, nonetheless. I should also stress the importance of using a PEG-rich polymer for these systems.

Dalibor Soukup asked: In your presentation you have shown that your particles are very stable in media. However, I was wondering if it is pure medium without serum or a complete medium with serum, as serum proteins can spontaneously adsorb on the surface of highly charged nanoparticles? This would increase the hydrodynamic size of particles and in turn affect the stability of nanoparticles. Typically, in order to avoid this, PEG-functionalized nanoparticles have been introduced, however, are there any other chemical coatings known that could do the same?

Hedi Mattoussi answered: The polymer-coated nanoparticles we discussed are stable in complete growth media. I should add that protein corona formation, which stems from adsorbed serum proteins on the nanoparticles (often discussed these days), takes place with electrostatically-stabilized nanoparticles, such as the very commonly used citrate-stabilized gold nanoparticles (AuNPs). One of the most effective routes to reducing those problems is to use PEG or zwitterion coating. Such coating circumvents reliance on charge stabilization and eliminates protein adsorption on the nanoparticles.

Sandhya Moise queried: Is it advantageous to have a protein corona form on the nanoparticle surface and, if so, when?

Hedi Mattoussi answered: The presence of a lateral PEG shell on His–PIMA–PEG-capped nanoparticles prevents protein corona formation by simply eliminating nonspecific interactions. These are the primary promoter of protein adsorption on electrostatically-stabilized nanoparticles, such as citrate-capped AuNPs. Corona formation can stabilize charged nanoparticles, but in general it is a problem when applications require control over nanoparticle size and/or coupling to target molecules.

Oliver Reiser remarked: When you add ligands you showed that this should have potential for chelating metals to your iron nanoparticles – do you observe the formation of discrete iron complexes dissociating into solutions?

Hedi Mattoussi replied: We have not observed metal complexes dissociating from the nanoparticle surfaces. If such complex dissociation occurred, it would certainly be very weak, as the overall size of the nanoparticles before and after ligand exchange remained the same, as verified by TEM data collected from dispersions of these materials. Also, the measured hydrodynamic size of these nanoparticles stayed constant even after several months of storage, indicating no deterioration or etching of the inorganic cores by the ligands.

Dejian Zhou enquired: Are these poly-His–PEG ligand-capped nanoparticles still able to bind to His-tagged proteins?

Hedi Mattoussi answered: The His–PIMA–PEG-capped AuNPs and AuNRs do not bind to His-tagged proteins, a problem primarily attributed to the steric hindrance imposed by the PEG shell on a full size protein. Nonetheless, if the protein is engineered with a longer linker between the polyhistidine tag and the protein itself, self-assembly can take place. This mode of coupling requires direct interactions between the histidine groups and the metal surface of the nanoparticles.

Maya Thanou said: To what extent does the polymer added affect the linkage – is the polymer (molecular weight) critical?

Hedi Mattoussi replied: No, the polymer molecular weight is not critical in itself. What matters most is the presence of several metal-coordinating groups to allow strong anchoring on the nanoparticle surface. Introducing specific hydrophilic moieties (*e.g.* PEG) enhances its affinity to water, while maintaining strong binding on the metal surface. It is, however, better to use a small molecular weight polymer to avoid a large hydrodynamic size for the hydrophilic nanoparticles.

Lucio Litti asked: Did you perform some experiments on the yield of ligand exchange between your multi-His polymers and mono-thiolated molecules on the surface of gold nanoparticles?

In your opinion, which one gives better and more stable coverage of the surface: a molecule with several weak ligands (such as your polymers) or one with only one stronger bond, such as thiolated molecules?

Hedi Mattoussi responded: We have not explored this idea of cap exchanging AuNPs capped with mono-thiol-ligands with the His–PIMA–PEG. We do believe that such polyhistidine-modified polymers work better with hydrophobic ligands or citrate- and CTAB-capped NPs/NRs. Conceptually, a polymer presenting 10-20 His groups along its backbone will bind tightly onto the NP/NR and should provide better long term colloidal stability than small molecules (mostly for AuNRs), due to the polymer nature of the coating. The latter provides higher affinity to water media, due to favorable Flory–Huggins interactions. We thus think that a polymer coating should provide better colloidal stability than a mono-dentate ligand, even if the latter presents a strong coordination group.

Sara Carreira opened the discussion of the paper by Siti Fatimah Abdul Ghani : Do you know the mechanism of the toxicity of the naked quantum dots and what the coating does to prevent that toxicity mechanism?

Siti Fatimah Abdul Ghani replied: Trioctylphosphine oxide (TOPO)-modified quantum dots (QDs) are poorly water-soluble and may contain solvent residues. As aggregates interact with cell membranes and compartments, this leads further to the rapid destabilisation of the core and metal leakage, which contributes to rapid cell death.

Water-soluble QDs modified with hydrophilic ligands, such as biphosphonates, enhance colloidal stability and aqueous dispersion, preventing aggregation and minimizing leakage within the cells.

Catherine Amiens asked: How do you think that the biphosphonate ligands are linked to the nanoparticles surface? Are both phosphonate end groups anchored onto the surface as suggested by the low zeta values of the solutions? In this case, what is the reason for the observed colloidal stability? No neat surface charge, hence no electrostatic stabilisation; no long chain ligands, hence no steric stabilisation: I do not understand the system.

Siti Fatimah Abdul Ghani responded: Although thorough studies need to be performed to identify the coordination of the biphosphonates on the quantum dots surface (ZnS), we believe that association of these molecules with the QD surface may be with either one or both phosphonate groups (or in an equilibrium). The zeta potential of the dispersions suggests that the particles appear neutral in solution and biphosphonates do not contribute to the nanoparticle charge. We hypothesize that stabilization is achieved because of the large number of biphosphonate ligands (as indicated by thermogravimetric analysis) associated with the surface of QDs, providing a thick hydrophilic “layer” that contributes to the colloidal stability.

Catherine Amiens queried: Did you try to use NMR spectroscopy to investigate the coordination of the biphosphonate ligands on the surface of the nanoparticles?

Siti Fatimah Abdul Ghani replied: We did not use NMR to investigate the coordination of the biphosphonate ligands on the surface of the nanoparticles. We have used ^{31}P-NMR to confirm ligand exchange reaction.

Amelie Heuer-Jungemann enquired: What is the advantage of your new ligand compared to other conventional ligands used?

Siti Fatimah Abdul Ghani replied: The biphosphonate ligands are safe (non-toxic) and efficient in stabilizing QDs. They provide a colloidally-stable water dispersion and can maintain this stability for days.

Amelie Heuer-Jungemann said: Did you try your ligand on different sizes of QDs?

Siti Fatimah Abdul Ghani responded: We have tried our ligands on CdSe/ZnS and $CuInS_2$/ZnS, which have a small difference in size. However, we have not tried this ligand on larger dots, *e.g.* polymer dots (80 nm)

Stefan Borsley commented: The TEM image presented of the biphosphonate-stabilised QDs appeared to show the presence of aggregates. Are these genuine aggregates or is this a drying effect? Have you tried verifying the colloidal stability of the particles in solution by another technique, such as dynamic light scattering (DLS)? I agree that your stability studies appear to show no change in the sample, however, this does not mean that the particle is not initially aggregated, only that the aggregation of the sample is not changing.

Siti Fatimah Abdul Ghani replied: The apparent aggregation is because of the drying effect. We have verified colloidal stability with DLS and Nanosight.

Maya Thanou added: DLS is used to measure size. However, if the NPs have fluorescent properties , these may affect the readings.

Mauro Prato asked: To perform cytotoxicity studies, you have tested an ovarian cancer cell line as a model. However, a major issue in general in tumor-targeting chemotherapy is the side effects on surrounding healthy tissues. Have you performed any cytotoxicity studies on normal healthy cells as controls? This could provide much more informative data on the potential toxicity of your molecules.

Siti Fatimah Abdul Ghani answered: This suggestion is correct. All nanomaterials should be tested on both cancer cell line and /or primary cell lines

Lanry L. Yung commented: As a materials scientist, we usually test the toxicity of our nanoparticles using secondary and cancer cell lines (e.g. 3T3, HeLa, *etc.*). However, in the eyes of a biologist, such toxicity tests should be conducted using primary cell lines, such as HUVEC, since secondary and cancer cell lines are not sensitive towards a toxic environment unless the toxicity dose becomes very high.

Mauro Prato added in response to Lanry L. Yung's remark: As a biologist, I definitely agree on that point. Nevertheless, I would like to stress that it is crucial

to keep in mind that the physiology (and phenotype) of cancer cells is far different to that of normal healthy cells. If the new nanomolecule is aimed at targeting cancer cells, a comparison with normal tissues should always be deemed necessary. Of course, if the target is normal cells (such as in wound healing, for instance), no further comparisons are needed. Please also note that the main disadvantage of primary cultures lays on the high donor's variability. Therefore, depending on the context, using immortalised cell lines might also do the job. For instance, if I am studying normal (noncancer) skin cells for helping wound healing, HaCaT cell line might be sufficient instead of using highly variable primary keratinocytes.

Maya Thanou agreed: The point and suggestion is correct. HUVEC cells mimic the vein endothelium and indeed such tests would provide an indication of the potential toxicity of nanomaterials to the blood vessels. There is a list of suitable assays for new nanomaterials[1] by the Nanotechnology Characterisation Lab (NCL) that suggests proper immunological and toxicological evaluation of novel nanomaterials. The only primary cell line assay found in that list is the cell line of primary hepatocytes (for identification of reactive oxygen species using novel nanomaterials). We have performed MTT, LDH and glutathione assays using cancer cell lines to identify the toxicity of previously widely studied QDs, with the addition of surface modification with biphosphonates.

1 Assay Cascade Protocols, Nanotechnology Characterisation Laboratory, http://ncl.cancer.gov/working_assay-cascade.asp

Paresh Ray remarked: Why do the fluorescence spectra show a blue shift of about 100 nm after coating?

Siti Fatimah Abdul Ghani replied: We have observed this effect with methylene diphosphonate capping (coating). We are not aware of any similar blue shift upon capping with (other) water-soluble molecules. This may require thorough investigation with structurally different capping agents.

Kristian Göeken asked: In the paper, you state that QDs coated with ethylene diphosphonate (EDP) are found in the cytosol, while methylene diphosphonate (MDP) QDs are located around the nucleus. Do you have an explanation for the possible mechanism behind this "targeting"?

Siti Fatimah Abdul Ghani responded: We would not like to call it targeting. It is an observation that shows that the structure of a small molecule used as capping agent on the quantum dots may have an effect on its cellular distribution.

Peter Harvey opened the discussion of the paper by Matthew Todd : When observing the signal corresponding to the nanoparticle in the dye-sensitised example, is the lifetime multi-component? If so, do the components correspond to lifetimes associated with both the dye and the dot?

Matthew Todd responded: The data related to this question were presented at the meeting, but not in the relevant discussion paper, so we would prefer to

address this question after the relevant data have been through formal peer review.

Peter Harvey asked: Does the normalisation of the spectra take into account the fact that the increase in the QD emission intensity could be due to a contribution from a "shoulder" of the dye emission?

Matthew Todd replied: Yes. The spectra were deconvoluted such that contributions from the dye were removed.†

Zoe Pikramenou queried: Can you exclude photo-induced electron transfer taking place between the QD and the organic dye?

Matthew Todd answered: We can exclude electron transfer from dot to dye, since the 4-aminonaphthalimide is linked to the QD *via* an ethylene bridge. The imide functional group, being electron rich, is a very poor electron acceptor and thus not prone to PET. Studies on the direction of PET in structurally very similar molecules have been reported by A. P. de Silva and give proof of a photo-induced electron transfer happening only in one direction, which is from the dimethylamine to the 4-aminonaphthtalimide core.[1] The reversed case of electron transfer from the dye to the QD can reasonably be excluded, since it lacks appropriate amines to trigger the electron transfer.†

1 A. P. de Silva *et al.*, *Angewandte Chemie*, 1995, **34**, 1728–1731.

Zoe Pikramenou asked: How does the pH dependent fluorescence response of the dye on its own compare to the dye on the QD?

Matthew Todd replied: The pH response of the dye in conjugation with the QD is very similar to its response as "free" dye in its monomer form (in the range of pH 7.5 to 9.6), which we can conclude by comparison with the reference compound 2. Studies on compound 2 have been published in our related paper.[1] What differs is the change in intensity, which is large for compound 2 (7-fold), but small for the dye–QD conjugate (2-fold). This loss in intensity could be due to the energy transfer to the QD.†

1 S. Ast *et al.*, *Phys. Chem. Chem. Phys.*, 2014, **16**, 25255–25257.

Sara Carreira asked: If your pH responsive dye works best at pH 7–10, would you expect it to be sensitive enough to work in the intracellular space, where the pH can be much more acidic? If not, could you use a dye that has a better response to acidic pH?

Matthew Todd answered: Yes, this prototype has a pH range that makes it useful for measurements of physiological conditions, but, if we were to want to image more acidic environments, we would need to adapt the system. Note that

† Both Matthew Todd and Sandra Ast collaborated on the response after the meeting.

the dots themselves were not particularly stable at acidic pH, so that would be a component of the system that would need to be modified, as well as the appended dye.

Thomas Carter added: Extracellular physiological pH varies within a very sensitive range, and tumours may affect local extracellular pH, making the pH slightly lower than this. Would the pH-responsive nanoparticles be sensitive enough within the appropriate pH range to provide diagnostic information, or is the response range something which can be modified?

Matthew Todd responded: For use in realistic sensing environments (*i.e.* for diagnostics), we would need to show that the particle is bright enough (*e.g.* for cell culture experiments) and is stable at relevant physiological conditions – note that we observed significant instability of the QD itself at low pH values *in vitro*. While we certainly see clear responsiveness in model cases, it is likely we would want to try to increase the responsiveness for what will presumably be "noisier" real-world applications. These are the general areas it would be interesting to target next.

Dejian Zhou asked: Have you tried the QDs without the dye – are they still pH sensitive?

Matthew Todd replied: Yes, that has been tested and is reported in the paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00110A) for the case of QD 570. The relevant data are shown in Fig. 7 (acidic sweep) and Fig. 8a and b (alkaline sweep). The QD signal remains stable from 7.5–9.6, so that the pH-induced changes in the QD emission of the conjugate can only be attributed to energy transfer from the pH-responsive dye.†

Paresh Ray asked: What is the mechanism of transfer – is it fluorescence resonance energy transfer (FRET)?

Matthew Todd replied: We assume so, based on voluminous literature of related systems, but we have not explicitly shown the transfer to be FRET here. Recent work by others has shown the distance dependence of dot to dye transfer follows the expected FRET model.[1]

1 G. Beane, K. Boldt, N. Kirkwood and P. Mulvaney, *J. Phys Chem. C*, 2014, **118**, 18079–18086.

Peter Dobson asked: Have you got information about all the HOMO and LUMO levels – how do these line up with the QD levels?

Should this information be reported more widely in any discussion involving energy transfer between light, quantum dots and dyes?

Matthew Todd responded: This is an interesting question. While we do this for small molecule sensing applications of related molecules,[1] we have not analysed the HOMO and LUMO levels for a dot. I agree that there is an underlying assumption in the literature here that we could explicitly address in the future. We did match the energy levels according to their photophysical data: the basic

requirement for FRET to occur is obviously a match between the absorption of the QD (at the first exciton peak) with the emission of the dye.†

1 S. Ast, P. J. Rutledge and M. H. Todd, *Eur. J. Inorg. Chem.*, 2012, 5611–5615.

Dejian Zhou asked: Have you checked the hydrodynamic sizes of the QDs after the addition of the dye molecules?

Matthew Todd answered: The dots are covered in a polymer shell, to aid water solubility. The dye attachment occurs beneath that. We do not expect the hydrodynamic radius of the dye to change appreciably after the addition of the dye, particularly as the attachment method is a substitution for a surface-bound molecule of similar size.

Katherine Brown remarked: Does your QD–dye have enhanced photophysical properties appropriate for studying dynamic biological processes, considering the limitations of organic dyes (bleaching) and QDs (blinking)?

Matthew Todd responded: We have not seen either bleaching or blinking in these systems to date. One of the reasons for our wanting to look at the dot, rather than the dye, in a sensing environment is because the dot ought to be bright, and dots have been used for this reason in biomedical imaging applications. Whether our conjugate behaves well in a real biological setting (*i.e.* displays the same mode of energy transfer and is clearly detectable in cell culture) is a subject of future work.

Matthew Todd opened a general discussion of Hedi Mattoussi's, Siti Fatimah Abdul Ghani's and his own papers: One of the features of Hedi's work[1] is the highlighting of lifetime as a key determinant of whether it is sensible to expect a dye to be able to excite a dot. The lifetime of a dye is typically much shorter than that of a dot, so it makes little sense to expect energy transfer towards the dot when it is likely that the dot is in an excited state. We wondered whether in our case such transfer might be possible, because there are so many dyes per dot (around 100), meaning there is a statistical chance that the dot could be receptive to transfer from some dyes (essentially the "long tail" of the decay profile).

1 A. R. Clapp, I. L. Medintz, B. R. Fisher, G. P. Anderson and H. Mattoussi, *J. Am. Chem. Soc.*, 2005, **127**, 1242–1250.

Hedi Mattoussi responded: The data by Todd and co-workers confirms that FRET between organic dyes and QDs is much higher and better controlled when the QDs are used as energy donors. This produces a FRET quenching that is commensurate with the number of dyes interacting with the same QDs. The reverse configuration, energy transfer from a dye to a QD, is very weak even when a large number of dyes are used. This is caused by the fact that direct excitation of the QDs is highly efficient; these systems have very large extinction coefficients.

Sandhya Moise asked: How stable are the surface coatings on the quantum dots and cobalt particles in biological systems? How much knowledge is there about *in vivo* retention times and modes of excretion, and are the surface coatings

stable enough to protect the cores sufficiently throughout the time scales and conditions to make them safe for *in vivo* applications?

Oliver Reiser replied: We do not have any *in vivo* data yet. From the chemical data we have, the carbon coated particles are extremely robust over a high temperature (>100 °C) and pH range (pH 4–10). For biological applications, we will use the analogous iron particles, which have the same properties, but the metal core obviously less toxic. The reason we have conducted the study shown here with cobalt particles is that they were available first through our collaborators (W. Stark, ETH Zurich and Turbobeeds Inc.). The iron particles are a more recent development by them.

Hedi Mattoussi responded: Knowledge about the *in vivo* retention of nanoparticles is still rather scattered, though several studies have recently looked into these issues. The stability of the surface coating on nanoparticles (such as QDs, metallic and magnetic nanoparticles) in biological media strongly depends on the strength of the ligand interactions with the metal-rich surface of these nanoparticles. These interactions are driven by metal-coordination. That is why ligands presenting multiple strongly coordinating groups (anchors) can bind more tightly onto the nanoparticles. For example, we have shown that iron oxide nanoparticles coated with multi-dopamine polymer ligands exhibit long-term colloidal stability (for more than 1 year) using *in vitro* test conditions, including acidic and basic pH, 1 M NaCl and in RPMI growth media.

1 W. Wang, X. Ji, H. B. Na, M. Safi, A. Smith, G. Palui, J. M. Perez and H. Mattoussi, *Langmuir*, 2014, **30**, 6197–6208.

Peter Dobson said: When dealing with complex systems such as QDs with a core and a shell and ligands attached to the surface, few researchers are reporting on the energy levels of the valence and conduction bands and HOMO/LUMO levels relative to the surroundings. This situation is not helpful, because to ascertain the mechanisms of detection or the mechanisms of destroying cells or pathogens it is necessary to know these facts.

Hedi Mattoussi added: There actually have been studies where groups looked into characterizing the energy levels of semiconductor QDs and their dependence on the nanocrystal size. Nonetheless, often only the lowest level in conduction and the highest level in the valence band are reported for simplicity. The ligand shell has little to no contribution to these levels for common alkyl type molecules, though recently groups have probed the effects of using benzenethiol ligands on the energy levels of PbS QDs. The shell can, however, strongly affect the rate of electron–hole radiative recombination, which manifests itself in the measured photoluminescence quantum yields of the nanocrystals.

The issue of QD interactions with cells is not necessarily associated with the energy levels *per se*. Often this problem is discussed in terms of generation of reactive oxygen species in the medium following UV excitation, or possible leaching of the metal ions into the surrounding medium. The latter can be controlled by the nature of the ligand used (stronger binding ligands provide better shielding).

Liane Rossi addressed Hedi Mattoussi and Matthew Todd :The purification of nanoparticles after ligand-exchange can be challenging. Can you please discuss the importance of removing the excess ligands and how to achieve this?

Hedi Mattoussi replied: Removing excess ligands from the nanoparticle dispersions is crucially important for using these systems to develop applications, such as sensor design and cell and tissue imaging. Excess free ligands can interfere with simple procedures, such as coupling to target biomolecules. As an example, we address ligand exchange as applied to hydrophobic AuNPs. The nanoparticles are purified in two steps. First, we apply two rounds of precipitation by adding hexane to the dispersion after ligand exchange; this primarily removes oleylamine (the native ligand). Second, the nanoparticles are dispersed in deionised (DI) water, sonicated for 2–3 min. and filtered through a 0.45 μm disposable syringe filter; this step is carried out without loss of materials. Removal of excess free hydrophilic ligands is achieved by applying 3 to 4 rounds of concentration/dilution using a centrifugal filtration device (Millipore, M_w cutoff = 50 kDa). To prepare the samples used to collect the NMR spectra, the solvent is switched from hydrogenated DI water to deuterated water by applying two rounds of concentration/dilution using 2 mL D_2O each. We also characterize the filtrate collected in the bottom of the device using ^{1}H-NMR spectroscopy. We have found no sign of free ligands in the filtrates.

Matthew Todd responded: We were unable to isolate the conjugate in a stable form due to non-specific interactions with the carboxylic acid groups. Instead, as described in the paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00110A), we studied the ligand uptake by the dot and worked with a concentration at which we were certain that all of the dye was conjugated to the QD.

Peter Dobson opened the discussion of the paper by Gil Lee‡: I would just like to check that your image is really convincing visual proof that the nanorods and the target molecule are sticking on the cell surface?

Anna Lesniak replied: Movies showing the active targeting of MCF-7 cells by HRG-functionalized rods and their subsequent mechanical stimulation can be found in the supplementary material of our most recent publication.[1]

1 D. Kilinc, A. Lesniak, S. A. Rashdan, D. Gandhi, A. Blasiak, P. C. Fannin, A. von Kriegshelm, W. Kolch and G. U. Lee, Mechanochemical stimulation of MCF7 cells with rod-shaped Fe–Au Janus particles induces cell death through paradoxical hyperactivation of ERK, *Adv. Healthc. Mater.*, DOI: 10.1002/adhm.201400391

Peter Dobson asked: If you remove the nanorods using your magnetic tweezers and try again, do the nanorods attach in the same places?

Anna Lesniak replied: When higher forces were applied, nanorods resulted in membrane tether formation, possibly through the disruption of the bonds

‡ Gil Lee's paper was presented by Anna Lesniak, *Bionanotechnology Group, Conway Institute and School of Chemistry, UCD, Dublin, Ireland*.

between the ErbB3 receptors and the underlying cytoskeleton. We have not tried to target stretched cells one more time.

Maha Abdollah asked: Referring to Fig. 4 of your paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00115J), can you explain how you can differentiate between bound and unbound (passing) nanorods?

Anna Lesniak replied: The experiment was recorded at a rate of 1 frame per second and nanorods with a velocity lower than 15 μm s^{-1} were considered for cell targeting analysis. This time resolution is sufficient to differentiate between bound and passing rods. Corresponding movies are provided as supplementary material for our most recent publication.[1]

1 D. Kilinc, A. Lesniak, S. A. Rashdan, D. Gandhi, A. Blasiak, P. C. Fannin, A. von Kriegshelm, W. Kolch and G. U. Lee, Mechanochemical stimulation of MCF7 cells with rod-shaped Fe–Au Janus particles induces cell death through paradoxical hyperactivation of ERK, *Adv. Healthc. Mater.*, DOI: 10.1002/adhm.201400391

Maha Abdollah queried: When heregulin (HRG)-nanorods bind to the cells, do they stimulate any downstream signalling, or cause any cell death?

Anna Lesniak answered: They indeed activate ERK signalling pathway in active zones, but fail to induce a global cellular ERK activation. This was only possible with subsequent mechanical force application.

Paresh Ray asked: Since the size of a cell is usually 10 microns and the nanorod you have developed is more than one micron, why are the nanorods not visible in TEM images, like the cancer cells?

Anna Lesniak replied: Figure 4A in our paper (*Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00115J) is a phase contrast image of a cluster of cells being targeted by an individual rod. Once adhered, the cells we used in this study assume a diameter of 20–25 μm and, at the magnification we used, both the cells and the rods are visible.

Mauro Prato commented: Your study was performed by culturing cells in serum-free medium. Do you have any data showing binding to plasma proteins?

Anna Lesniak replied: Cells were starved for only 1 hour prior to targeting experiments, which is not enough to remove all the proteins from the cell membrane. Targeting experiments were also performed in complete cell culture media (10% fetal bovine serum (FBS) and media with increasing amounts of FBS up to 80%). Targeting rates decreased with increasing FBS concentration (data not shown).

Mauro Prato asked: You have stated that, before performing your experiments, cells were starved for 1 h in serum-free medium. What are the effects of starving your breast cancer cell lines on the phosphorylation status of ERK1/2?

Anna Lesniak responded: We did not directly compare ERK phosphorylation levels in starved *vs*. non-starved cells; however, in serum-starved cells we have only observed an increase in the ERK phosphorylation when MCF-7 cells were targeted with HRG-rods. Control cells (MDA) and control particles (STR-nanorods) were used to confirm this finding.

Kristian Göeken asked: ErbB receptors are known to cluster upon activation. Would it be feasible to use this technique to follow clustering kinetics? For instance by determining an increase in interaction strength over time, or by following the binding of rods to specific locations over time.

Anna Lesniak responded: Indeed, specific binding of HRG-conjugated Fe–Au rods to ErbB receptors resulted in the clustering of these receptors (Fig. 4 in ref. 1). We believe that it would be possible to determine the change in the interaction strength by applying a constant force after different periods of nanorod–cell interaction. Regarding your second suggestion, our method cannot determine where on the cell the rods will bind. However, one could study different regions of the cell to estimate local differences in the receptor presence or clustering.

1 D. Kilinc, A. Lesniak, S. A. Rashdan, D. Gandhi, A. Blasiak, P. C. Fannin, A. von Kriegshelm, W. Kolch and G. U. Lee, Mechanochemical stimulation of MCF7 cells with rod-shaped Fe–Au Janus particles induces cell death through paradoxical hyperactivation of ERK, *Adv. Healthc. Mater.*, DOI: 10.1002/adhm.201400391

Peter Dobson enquired: This allows you to study cells in tremendous detail – can you use magnetic tweezers to measure stiffness of cell membrane? Could you "shake cells to death"?

Anna Lesniak responded: Yes, since the applied force is known and the displacement (if any) in the cell membrane can be measured, the stiffness of the membrane can be estimated. No, in our hands, periodically stretching cells without any pharmaceutical intervention did not lead to cell damage. Please refer to our most recent paper[1] where we describe the local activation of the ERK pathway downstream of mechanical stimulation and how this could be used to induce cell death *in vitro*.

1 D. Kilinc, A. Lesniak, S. A. Rashdan, D. Gandhi, A. Blasiak, P. C. Fannin, A. von Kriegshelm, W. Kolch and G. U. Lee, Mechanochemical stimulation of MCF7 cells with rod-shaped Fe–Au Janus particles induces cell death through paradoxical hyperactivation of ERK, *Adv. Healthc. Mater.*, DOI: 10.1002/adhm.201400391

Amelie Heuer-Jungemann queried: Is cell death induced by overstretching of the cells, or by creating lesions in the cell membrane upon nanorod removal?

Anna Lesniak responded: Periodically stretching cells at low forces, which do not cause nanorod separation, activates cell signalling pathways that could be used for killing the cells, *e.g.* through combination with other treatments. Although we did not test this possibility directly, overstretching the cell membrane may cause mechanoporation, potentially leading to cell death.

Lanry L. Yung asked: In cases where a more gentle mechanical stretching was used, do the cells die from apoptosis pathway? Have any apoptosis pathway biomarkers been investigated?

Anna Lesniak responded: Although markers of apoptotic pathways were not directly assessed, we did not observe any noticeable cell damage following mechanical stretching at low forces (in the range of 10 pN). Our more recent results show that combining mechanical stimulation with B-Raf inhibitors, which activate the ERK pathway, results in cell death, likely through the hyperstimulation of the ERK.[1]

1 D. Kilinc, A. Lesniak, S. A. Rashdan, D. Gandhi, A. Blasiak, P. C. Fannin, A. von Kriegshelm, W. Kolch and G. U. Lee, Mechanochemical stimulation of MCF7 cells with rod-shaped Fe–Au Janus particles induces cell death through paradoxical hyperactivation of ERK, *Adv. Healthc. Mater.*, DOI: 10.1002/adhm.201400391

Dejian Zhou asked: Have you done any statistical analysis? How many nanorods are attached to each cell? This may allow you to identify the cell targeting specificity of the nanorod.

Anna Lesniak replied: For each condition, a number of independent experiments were performed and on average 45 particle–cell interactions were analyzed per experiment. We calculated the targeting rate by dividing the number of bound rods to the number of all passing rods (also normalized by the cell surface area). The targeting rates of MCF-7 cells by HRG-nanorods were indeed statistically significant compared to all other experimental groups (One-way ANOVA, followed by t-test, $p < 0.01$).

Lucio Litti enquired: In your opinion, on applying a magnetic field, could your nanorods self-assemble into an ordered surface, suitable for cell trapping and recovery after the removal of the magnetic field?

Anna Lesniak responded: We did not try to self-assemble rods into an ordered surface but similar rods, composed of Ni–Au, were previously used to capture different types of analytes.[1] Depending on the ligand or antibody functionalization at the tip of the rods it would be conceivable to trap specific cells in solution.

1 M. Platt, G. R. Willmott and G. U. Lee, Resistive pulse sensing of analyte-induced multicomponent rod aggregation using tunable pores, *Small*, 2012, **8**, 2436–2444.

Mauro Prato asked: The cell lines that you employed as a breast cancer *in vitro* model display prolonged ERK1/2 MAPK phosphorylation throughout the observational period. What is the phosphorylation status of other MAPKs, such as p38 and JNK?

Anna Lesniak responded: Only ERK1/2 phosphorylation was assessed in this study. We appreciate the suggestion of other pathways and will consider this for our future experiments.