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DISCUSSIONS

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General discussion

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Dr Singh opened the discussion of the paper by Sebastien Lecommandoux: Why did you use an excess of copper sulfate? Is it correct to say that copper sulfate is a catalyst if you are using four times excess of copper sulfate in comparison to alkyne?

Professor Lecommandoux answered: The click chemistry presented in this work has indeed been optimized previously with glycopolypeptides that include hyaluronan oligosaccharides. Hyaluronan is negatively charged and a lot of copper is required to overcome the possible chelation with carboxylic acids from the glycan. Whereas this coupling method is useful to avoid CuBr and its O_2 sensitivity[1–3], and is a method that was used previously in the team, we did not re-optimize the reaction for dextran coupling. If correctly balanced in stoichiometry compared to sodium ascorbate, less copper will certainly work.[4] It is important to note that in all cases, copper can be easily eliminated by dialysis. Indeed, from elemental analysis, we have observed that only ppm levels of the metal can be detected after purification in the copolymer material.

1 K. K. Upadhyay, J. F. Le Meins, A. Misra, P. Voisin, V. Bouchaud, E. Ibarboure, C. Schatz and S. Lecommandoux, *Biomacromolecules*, 2009, **10**, 2802–2808.
2 K. K. Upadhyay, A. N. Bhatt, E. Castro, A. K. Mishra, K. Chuttani, B. S. Dwarakanath, C. Schatz, J. F. Le Meins, A. Misra and S. Lecommandoux, *Macromol. Biosci.*, 2010, **10**, 503–512.
3 C. Bonduelle, J. Huang, E. Ibarboure, A. Heise and S. Lecommandoux, *Chem. Commun.*, 2012, **48**, 8353–8355.
4 M. Meldal, *Macromol. Rapid Commun.*, 2008, **29**, 1016–1051.

Professor Cui asked: In all your TEM images for the disk morphology, there is consistently a white dot in the disk center. Is this an imaging artifact or something real? It is very interesting.

Professor Lecommandoux replied: For TEM imaging, we currently use staining with uranyl acetate and one can hypothesize that this white dot comes from a staining effect. CRYO-TEM did not evidence such a white dot in the disk center but the overall contrast that this imaging technique uses is low compared to uranyl acetate stained TEM. Interestingly, AFM topography also evidenced a small dot at the center of the nano-object (see Fig. 1), the same dot that is perhaps at the origin of the white dots observed by uranyl acetate stained TEM. One can hypothesize that this is an effect of solvent evaporation during drying, but more experiments would be needed to fully elucidate the underlying mechanism.

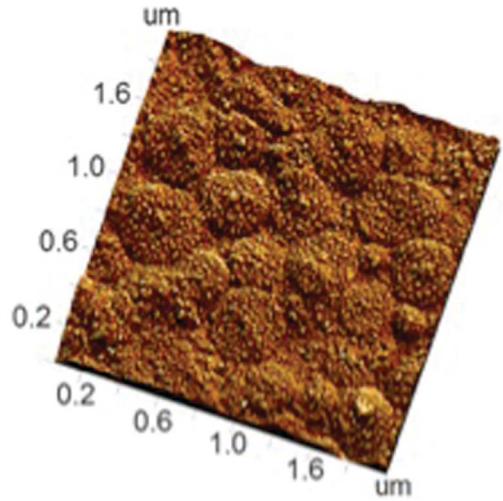

Fig. 1 AFM topography image of disk sample.

Professor Cui asked: Regarding the formation mechanisms for the disk morphology, do you think they are stabilized by spontaneous curvature or by crystallization of the PBLG block? Also, in the morphological transition from spheres to disks, did you observe any cylindrical or elongated objects inbetween?

Professor Lecommandoux replied: I guess the liquid crystalline behavior of PBLG is a strong driving force for self-assembly. As a result, rod–coil systems generally stabilize easily flat interfaces and their phase diagram in the bulk has a very broad lamellar domain. In solution, rod–coils have also been shown to stabilize vesicular morphology over a broad range of compositions, in good agreement with the bulk phase diagram and experimental observations. The fact that we did not observed cylindrical micelles between spheres and disks in our system is indeed quite intriguing. Such a sequence of morphologies with decreasing curvature is indeed expected at the thermodynamic equilibrium, which is not the case here, as we used a solvent-displacement method for sample preparation. This might also add complexity into the system as the kinetics and dynamics of exchange are also significant in the resulting structure.

Professor Mezzenga said: Presumably there is a loss in liquid crystalline interactions at the edges of the disk micelles. What are your thoughts about the stabilisation and structure at edge?

Professor Lecommandoux answered: Indeed, a disk-like shape is locally flat, but needs to curve at the edges in order to minimize the interactions of the hydrophobic segments with water. Actually, one can imagine two possible models. The first one is based on the "hockey-puck" mechanism. In that case, the long and/or sterically hindered hydrophilic blocks are covering the edges, preventing the growth of the crystalline or liquid-crystalline order, and in the mean time protecting the rigid hydrophobic blocks from water. A second possible

model would be related to an increased curvature at the edges. In that case, a loss of the liquid crystalline interactions would be necessary. If we consider that liquid crystal order is the driving force for self-assembly, this second hypothesis is not expected. However, this can happen due to the polydispersity of the block copolymers. As a result, one can only at this stage hypothesize about the different models, but further experiments are needed to clearly understand such unusual morphology.

Professor Alexander said: The amount of homopolymer used to stabilise your micelles was up to 30 mole percent, is that a limiting concentration? How sensitive is the structure to the nanoprecipitation technique?

Professor Lecommandoux responded: In this particular case, we did not try to go to higher concentrations. Based on previous observations, we can probably increase the concentration to about 50 mole percent, as the branched polysaccharide block efficiently stabilizes the interface. In addition, the structure was not very sensitive to nanoprecipitation in the present case. Indeed, looking at compound 3′, no significant difference in the resulting morphology from direct dissolution or nanoprecipitation could be detected (see Fig. 5 and 6 in the paper).

Dr Paternostre remarked: From TEM and AFM your objects appear as disks. As some interaction/drying of the object on the surface (microscopy grid or mica) could induce this specific shape, could you do freeze fracture studies to observe your sample?

Professor Lecommandoux replied: Freeze fracture is certainly an interesting technique to complete the full set of characterizations presented in our manuscript. Whereas surface influence may play a significant role when TEM or AFM is used, we believe that our cryo-TEM imaging is an additional proof of the existence of the disk-like morphology that certainly minimizes surface interaction.

Dr Nieuwland said: With the addition of extra "trunks" to the systems, a mixed system (micelles and disks) rather than a gradual transition from the micelles to the disks is observed. How could that be explained from thermodynamics?

Professor Lecommandoux responded: I addressed this in my reply to Professor Cui's question about formation mechanisms.

Dr Kros said: What do you know about the thickness of the bilayer? Are they actual bilayers?

Professor Lecommandoux replied: This is a very good point that would help with the understanding of the underlying mechanism of the formation of disks. Actually, we do not know the actual thickness but we are aiming to perform SAXS and SANS to determine the thickness and internal structure of these self-assembled nanostructures.

Dr Gouveia commented: Regarding the bio-functionalization of the developed glycopeptides, did you consider introducing glycan moieties containing sialic

acid for finer control of the interactions between the self-assembled structures and cells/tissues/*in vivo* environment?

Professor Lecommandoux replied: Among possible oligosaccharides that can be used to interact with biological systems, we have already developed glycopolypeptides incorporating hyaluronan that target CD44 receptors.[1–3] We are currently trying to develop biomimetic systems incorporating other oligosaccharides for lectin binding but few oligosaccharide fractions having bioactivity are commercially available. So far, no oligosaccharides made of sialic acid are available on the market and to introduce such bio-functionalization, it is certainly possible to use an alternative approach that incorporates sialic acid monosaccharides along a polypeptide backbone.[4] This fully synthetic approach is much more versatile and is currently used in our team to prepare glycopolypeptides that integrate unnatural carbohydrates known to inhibit glycosidases.

1 K. K. Upadhyay, J. F. Le Meins, A. Misra, P. Voisin, V. Bouchaud, E. Ibarboure, C. Schatz and S. Lecommandoux, *Biomacromolecules*, 2009, **10**, 2802–2808.
2 K. K. Upadhyay, A. N. Bhatt, E. Castro, A. K. Mishra, K. Chuttani, B. S. Dwarakanath, C. Schatz, J. F. Le Meins, A. Misra and S. Lecommandoux, *Macromol. Biosci.*, 2010, **10**, 503–512.
3 C. Bonduelle, J. Huang, E. Ibarboure, A. Heise and S. Lecommandoux, *Chem. Commun.*, 2012, **48**, 8353–8355.
4 J. Huang, C. Bonduelle, J. Thevenot, S. Lecommandoux and A. Heise, *J. Am. Chem. Soc.*, 2012, **134**, 119–122.

Professor Stupp asked: What would make these disk-like structures (with symmetric or asymmetric surfaces) unique in terms of bioactivity?

Professor Lecommandoux responded: Glycopolypeptide materials hold significant promise in biomedical applications as they display biologically relevant carbohydrates at the surface of well-defined nanoparticles.[1] The non-linear glycopolypeptides presented here have the ability to spontaneously self-assemble into spherical micelles or in disk-like structures in water, a property that is not observed with their linear counterparts.[2,3] One can hypothesize that these disk-like structures may bring some advantages in terms of bioactivity and we are currently developing drug delivery systems to be tested *in vitro* and *in vivo*.

1 C. Bonduelle and S. Lecommandoux, *Biomacromolecules*, 2013, **14**, 2973–2983.
2 C. Bonduelle, J. Huang, E. Ibarboure, A. Heise and S. Lecommandoux, *Chem. Commun.*, 2012, **48**, 8353–8355.
3 J. Huang, C. Bonduelle, J. Thevenot, S. Lecommandoux and A. Heise, *J. Am. Chem. Soc.*, 2012, **134**, 119–122.

Professor Stupp remarked: Is there a possibility of controlling the polydispersity of the polysaccharide given that its volume fraction should affect the type of supramolecular packing observed? Can you comment further on the polydispersity of both components?

Professor Lecommandoux replied: As already highlighted in the literature, the best way to prepare monodisperse polypeptides is the use of protein engineering. Whereas this approach allows the preparation of natural proteins, by using such approach, the introduction of unnatural aminoacids is now well documented and

is certainly an alternative way to prepare PBLG or poly(propargylglycine) blocks that would allow absolute control over molecular weight. It is certainly one of the aspects of our study that would help to well understand the influence of the polypeptide polydispersity on the self-assembly properties. Concerning the oligosaccharide segment, polydispersity is generally controlled little because these materials are generally extracted from biomass and (bio)chemically treated. We are currently working on fully synthetic systems that can be branched *via* radical polymerization to further understand this influence.

Professor Stupp asked: Is there any evidence of crystallinity or other intermediate types of order within the disk-like aggregates? Does this factor determine if the surfaces of the aggregates are symmetric or not? (This may depend on the rod length).

Professor Lecommandoux responded: This is a very good point, as the driving force for self-assembly is certainly related to the liquid-crystal order from the rod segments. The understanding of the local structure of the aggregates would certainly give relevant insight to their mechanism of formation. We intend to perform SAXS and SANS to elucidate the internal aggregate structure.

Dr Adams asked: Regarding $R_G/R_H = 1$, could you comment on whether you think that people may have assumed they had vesicles previously in the literature when actually they had disks?

Professor Lecommandoux responded: By using the following equations to determine R_G and R_H for a cylinder, one can estimate the values expected for the system.

$$R_H = \frac{3R_{disk}}{2}\left\{\left[1+\alpha^2\right]^{1/2} + \frac{1}{\alpha}\ln\left[\alpha + \left[1+\alpha^2\right]^{1/2}\right] - \alpha\right\}^{-1}$$

where α is the thickness of the cylinder and R_{disk} the radius.

$$R_G{}^2 = \frac{R^2}{2} + \frac{h^2}{12}$$

where R is the radius and h the length of the cylinder. If the thickness of the disk is at least 10 times lower than its diameter, it provides an R_G/R_H value between 0.9 and 0.95, depending on the thickness. This value is close to the expected one for a hollow structure and it is reasonable to complete a form factor determination by other characterizations (such as imaging) to verify the formation of vesicle morphology.

Dr Adams commented: In the past when we tried to make vesicles and the TEM was ambiguous, we assumed we had vesicles because $R_G/R_H = 1$. Perhaps we were incorrect in light of your data.

Professor Lecommandoux answered: Indeed, this point has been somehow neglected in the literature. A ratio of $R_G/R_H = 1$ is one way to discriminate clearly between spherical micelles and vesicle morphology, but actually not between vesicles and disks. Nevertheless, imaging (TEM or AFM) is also often presented alone to justify the presence of vesicles (or hollow structures), which is not

sufficient as many preparation artefacts and surface effects can occur during sample preparation. Examples of disk structure in the literature are not abundant, however. I believe this combination of both scattering and imaging together can give a precise idea of the actual morphology.

Dr Bittner said: In the cryo-TEM images, nearly all of the disk-shaped vesicles appear round. Why are none oriented with the thin side facing up (towards the beam)?

Professor Lecommandoux replied: For cryo-TEM grid preparation, to get vitreous ice, an excess of the solution is absorbed forming a thin film before freezing. A ice thickness of about 100–300 nm provides a good contrast (with thicker samples electrons have multiple scattering events and eventually cannot cross the specimens).[1] In vitreous ice, the specimens, small protein objects whose sizes are smaller than the ice thickness usually adopt a random orientation. With larger specimens such as disk-shaped vesicles, specimen orientation is more restrained and some orientations are even forbidden due to surface tension at the air–water interface. Nevertheless, they do not all have the same orientation with their thin side facing up. Indeed in Fig. 2B, the disk-vesicle marked with a white arrow has a round shape while the one marked with a black arrow has an ovoid shape. In cryo-TEM, specimen images correspond to their true projections; the shape of the disk-vesicle is directly related to its orientation. At 60° tilt (Fig. 2C), it is obvious that their shapes are different. This is simply because their orientations with respect to the electron beam are different. One vesicle is lying flat (white arrow) while the other is slightly tilted (black arrow).

1 J. Dubochet, M. Adrian, J. J. Chang, J. C. Homo, J. Lepault, M. A. McDowall and P. Schultz, *Q. Rev. Biophys.*, 1988, **21**, 129–228.

Dr Paternostre remarked: If a phase separation is at the origin of the disk-like shape, why could you not see the bulky head of the glycolipids on the edge of the disks?

Professor Lecommandoux answered: As shown in our manuscript, by taking the molecular modeling of **1′** to **4′** as an example, the head of our tree-like structure is within the 1–2 nm range. It is very hard to visualize such small details

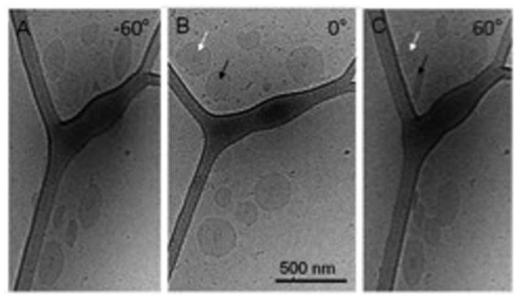

Fig. 2 Cryo-TEM imaging of sample 3′ + 30 wt% PBLG (scale bar indicated in the images). The sample grid was tilted from −60° (image A) to +60° (image C) to reveal the disk-shape structure of the sample.

by using TEM or AFM. On the other hand, one can hypothesize that disk-like morphologies could be stabilized without glycopolypeptides on the edge of the disks if they are stabilized *via*, for example, a "hockey-puck" mechanism.[1]

1 T. W. Schleuss, R. Abbel, M. Gross, D. Schollmeyer, H. Frey, M. Maskos, R. Berger and A. F. M. Kilbinger, *Angew. Chem. Int. Ed.*, 2006, **45**, 2969–2975.

Dr Lobaskin commented: You have discussed different polydispersity types being important, the backbone length and the arm length. Can you control polydispersity in the number of arms? For example, can you separate the molecules with different numbers of arms by sorting and then prepare well-defined compositions, *e.g.* a monodisperse or a bidisperse one to control the shape of the aggregates?

Professor Lecommandoux answered: Purification of amphiphilic structures is not an easy task. To the best of our knowledge, oligosaccharide polydispersity is not well controlled because they are materials generally extracted from biomass. The control of the polypeptide polydispersity may be achieved *via* protein engineering but this does not resolve the polydispersity that comes from the glycan. We are currently working on fully synthetic systems that can be branched *via* radical polymerization to further understand this influence.

Professor van Hest noted: You could use protein engineering to get absolute control over the number of arms attached to PBLG.

Professor Lecommandoux responded: As already highlighted in the literature, the best way to prepare monodisperse polypeptides is the use of protein engineering.[1] Whereas this approach allows the preparation of natural proteins, by using such an approach, the introduction of unnatural aminoacids is well documented[1] and is certainly an alternative way to prepare polypropargylglycine blocks that would allow absolute control over the number of arms attached. It is certainly one of the aspects of our study that would help to well understand the influence of the polypeptide polydispersity on the self-assembly properties.

1 S. M. Yu, V. Conticello, G. Zhang, C. Kayser, M. J. Fournier, T. L. Mason and D. A. Tirell, *Nature*, 1997, **389**, 187–190.

Professor Stupp commented: In the late 90s we reported on the self-assembly of rod–coil molecules in which the rod segment was perfectly monodisperse. In these systems the critical parameter that led to disk-like aggregates was a rod volume fraction with respect to the coil that was lower than 0.36. At higher rod volume fractions the aggregates had more one-dimensional character. This parameter could be investigated systematically in these branched rod systems as it may yield different types of aggregates. Do you have any estimate of what rod volume fraction range was covered in this work? It should be possible to estimate it for the co-assembled systems of rods and branched rods.

Professor Lecommandoux answered: Indeed, some nice contributions come from the 90s, especially from your group. They mostly concern systems in the bulk however, for which one can establish comparisons. Nevertheless, the behavior in solution is a bit more complex. In addition, as estimated in the ESI for our paper,

the rod volume fractions with respect to the coil that were covered in this work were 0.11 to 0.27 for copolymers **1′** to **4′**. No disk-like aggregates were observed after the self-assembly of these nanomaterials. We further probed the influence of this rod volume fraction by incorporating PBLG homopolymers and we obtained disk-like aggregates for a rod-volume fraction of 0.43, a value that was slightly higher than the given 0.36. We are currently doing a comprehensive study on these systems to fully understand the influence of this ratio on the self-assembly behavior. As you mentioned, these branched rod systems may yield different types of self-assembled structures, even unpredicted or unexpected ones.

Professor Stupp opened the discussion of the paper by Raffaele Mezzenga: What determines the width of the ribbons and why are there both periodic and non-periodic twisted ribbons?

Professor Mezzenga replied: Twisted ribbons are always periodic by definition (the entire angular twist expressed as $2p$ corresponds to a period). The factor which determines the width of the ribbons is the number of protofilaments which aggregate laterally to form it. In general, the longer the incubation time, the wider the ribbons will be.

Professor Stupp asked: Do you have any insight on the molecular scale of the transformation from twisted ribbons to helical ribbons and then rigid tubes?

Professor Mezzenga replied: We do know that upon progressive lateral growth (that is, with increasing numbers of protofilaments forming the fibrils), the twisted ribbons turn into helical ribbons, where the transition occurs at a critical width to thickness ratio. Beyond this critical ratio, energy is associated with bending rather than with torsion. The helical ribbons can then close into nanotubes by simply reducing the line tension associated with their edges. Thus, the usual polymorphic evolution with time is twisted ribbons → helical ribbons → nanotubes (see ref. 1 and 2).

1 J. Adamcik *et al.*, *Angew. Chem. Int. Ed.*, 2011, **50**, 5495.
2 C. Lara *et al.*, *Nanoscale*, 2013, **5**, 7197.

Professor Lecommandoux commented: In some other contributions dealing with "classical" amphiphilic molecules, the nanotube structure seems to be reached over time. In your system, what happens if you let the system equilibrate with time? Would you expect a ribbon–nanotube transition?

Professor Mezzenga responded: We do know that the transition from twisted ribbon to helical ribbon is promoted by an increase in later ribbon growth. The transition from helical ribbon to nanotube, on the other hand, is promoted by the closure of edges and the reduction of line tension. Therefore, *a priori*, whether the nanotube is the lowest thermodynamic energy state would depend primarily on the structural features of the fibrils.

Dr Squires asked: Are some of the different protein fragments shown on the gel after acid hydrolysis preferentially incorporated into the fibres over others?

Professor Mezzenga responded: To fully answer this interesting question, one would be required to do MALDI-TOF on the fibrils, with the exact identification of the fragment primary structure. This analysis has been carried out in the literature on other globular systems (β-lactoglobulin, lysozyme), but to our knowledge, not yet on bovine serum albumin. Extrapolating from the available literature, one can expect that different peptide fragments contribute to the formation of fibrils.

Dr Adams asked: Is there a chance you have low molecular weight peptides not visible in gels acting as surfactants and affecting the self-assembly?

Professor Mezzenga answered: We have no evidence for such a mechanism, although this remains possible.

Professor Hamley said: I was wondering how the fibril morphology depends on the preparation conditions, in particular the pH value, and how it's controlled? Also whether ultracentrifugation has any effect on fibril morphology?

Professor Mezzenga replied: We do know that pH is crucial to induce hydrolysis, an important step in the fibrillation of these proteins. In this specific case, the pH changed during fibrillation from 2 to 2.8, and we let it evolve naturally. Ultracentrifugation has no effect on the fibril morphology.

Professor Hamley said: You made a very interesting remark about Young's modulus. Is it possible to extract it from persistence length measurements, and have you done this for BSA? If so, how does it compare to other amyloid fibril systems?

Professor Mezzenga answered: The persistence length can be routinely converted into Young's moduli by simply considering that this equals the product of the Young's modulus and the area moment of inertia, over kT. This requires, however, accurate measurements of the cross-section area moment of inertia, that is, the height and width. Our preliminary data seem to indicate that the Young's moduli in BSA can differ substantially from data reported in other amyloid systems. However, in contrast to our previous studies (see ref. 1), in the present case the width does not come out straightforwardly from an integer number of constituent protofilaments, and therefore, the estimation of Young's moduli in BSA could suffer from weak assumptions of the cross-section area moment of inertia. We are committed to elucidate in detail this issue in future work.

1 J. Adamcik *et al.*, *Appl. Phys. Lett.*, 2011, **98**, 193701.

Professor Hamley commented: Do BSA fibrils behave differently compared to other natural protein amyloid fibrils, *e.g.* with different molecular weights such as lysozyme or β-lactoglobulin which you have studied in previous work?

Professor Mezzenga answered: In contrast to other smaller globular proteins (lysozyme, β-lactoglobulin), in the present case we have to work at much higher concentrations and we do have much longer lag times. This may be due to the

unfolding and hydrolysis of a much larger protein, with substantial reduction of the fibrillation kinetics.

Dr Kasotakis asked: How do you determine if the ribbons have a left or right-handed twist by AFM?

Professor Mezzenga responded: AFM is one of the most, if not the most, used technique to determine the handedness of chiral macromolecules. Basically left or right handed ribbons can be identified straightforwardly by measuring the sign of the twist angle with respect to the fibril axis at the height maxima along the fibril contour length.

Dr Kros remarked: Do you have information on the secondary structure as a function of time?

Professor Mezzenga responded: Yes, we do have CD measurements *versus* time showing an increase in the β-sheet content during fibrillation. These data will be published in a separate manuscript.

Dr Kros remarked: Are there preferential sites for hydrolysis?

Professor Mezzenga responded: In other systems, fibrillation can be promoted by selective cleavage and hydrolysis of specific sites *via* enzymatic reactions. This can be the case also in the present system, although hydrolysis here was not carried out in a selective way, but rather the combined use of low pH and high temperature was employed as a unique hydrolysis source.

Dr Kros asked: If you raise the pH do you stop further fibre formation?

Professor Mezzenga answered: We cannot exclude fibrillation under milder conditions. Nonetheless, for the mechanisms described here, hydrolysis, promoted by low pH, is crucial to achieve the observed morphologies.

Miss Zhou asked: Do you have any methods to determine if the nanotube structures are hollow?

Professor Mezzenga answered: Definitely, yes. In our earlier works, we validated a hollow nanotube structure using small angle X-ray scattering and fitting of the form factor with hollow tubes. In the present case this additional method has not yet been applied because several morphologies coexist, which can smear the scattering profile. Nonetheless, the assembly mechanisms show strong analogies with our earlier observations on heptapeptide fibrillation[1] or lysozyme nanotubes.[2]

1 J. Adamcik *et al.*, *Angew. Chem. Int. Ed.*, 2011, **50**, 5495.
2 C. Lara *et al.*, *Nanoscale*, 2013, **5**, 7197.

Dr Squires opened the discussion of the paper by Maite Paternostre: We have carried out polarized Raman microscopy on oriented samples to determine the

orientations of specific groups; see for example ref. 1 and 2. Have you considered carrying this out on your sample, to provide further evidence to support your structural model?

1 I. W. Hamley, V. Castelletto, C. M. Moulton, J. Rodríguez-Pérez, A. M. Squires, T. Eralp, G. Held, M. R. Hicks and A. Rodger, *J. Phys. Chem. B*, 2010, **114**(24), 8244–8254.
2 J. C. Rodríguez-Pérez, I. W. Hamley and A. M. Squires, *Phys. Chem. Chem. Phys.*, 2013, **15**, 13940–13950.

Dr Paternostre answered: I tried to do polarized IR spectroscopy but not polarized Raman microscopy. This is a good idea, but we have to find an efficient way to align the peptide fibers and for the moment we don't have one because we would like to work on hydrated systems and not on dried ones.

Professor Mezzenga commented: The co-existance of isotropic and nematic phases is expected, as this is a first order transition. Do you know anything about your fibre lengths/aspect ratios?

Dr Paternostre replied: We did not do these experiments. The fibres are very long (a few hundreds of micrometers sometimes) and it is therefore very difficult to measure the size from electron micrographs.

Professor Mezzenga said: Although the I–N coexistence is to be expected, there are other amyloid systems where this coexistence is not observed. Why is this system special in this respect?

Dr Paternostre answered: In contrast to most amyloid systems, ours is reversible and the peptide is highly soluble. This allows us to work at equilibrium and a step by step concentration increase allows us to describe the phase diagram and indeed to characterize both the isotropic and nematic phase. I think that the solubility of the peptide and the reversibility of the self-assemblies are the reasons why we could study this I–N coexistence.

Dr Bittner commented: Have you found any shifts in the IR spectra? Of course this depends also on the standard spectra used to assign the IR peaks. Is it possible to assign α-helix or β-sheet structures (see W. Nuansing *et al.*, especially the ESI, in this volume (DOI: 10.1039/c3fd00069a)), or is the peptide too small?

Dr Paternostre responded: We based our spectral interpretation on ref. 46 in our paper that deals with the IR spectra of the peptides. From this interpretation we did not find any α-helical structure within our assembly. To obtain the data for Table 1 we decomposed the spectra into individual components as Nuansing *et al.* did. However, one strict rule that should be applied to perform such decomposition is that all the individual spectra should have similar widths, which is obviously not the case in the reference herein.

Professor Hamley noted: This is very interesting work. I was wondering about the bioactivity, since you mentioned amyloid formation within secretory granules. Do amyloid fibrils form simply because the concentration is so high within these compartments, or is there a functional role for the fibril structure itself?

Dr Paternostre replied: This is a very interesting question but I am afraid I cannot answer. From what I know, the observation of amyloid assemblies of hormone within the secretory granules has been done in dendritic cells. The concentration of hormones in these granules is higher than in the Golgi where the peptide is synthesized. There should be a mechanism that allows this increase of intravesicular concentration of the hormone. The function proposed for the hormone amyloids is a storage function as self-assembly allows an increase in the hormone concentration in the vesicles without increasing the osmotic pressure. When released out of the cell, the amyloid is dissolved in the external medium and the monomer can circulate.

Professor van Hest asked: Do you think the amyloid droplets are the most biologically relevant structures?

Dr Paternostre responded: I don't know, it just is an hypothesis. I find very interesting that these droplets are formed only in a very narrow range of concentrations and I propose that this peculiar property could regulate the concentration of hormone within the granules, but I have no proof of it.

Dr Singh opened the discussion of the paper by Kostas Kostarelos†: What is the mechanism of internalization of PNFs? Do they go by endocytosis or cytoplasm directly?

Dr Mazza answered: Peptide nanofibres have been reported to enter the cells through endocytosis[1] and internalization may occur for the monomeric peptide amphiphiles through energy-dependent endocytosis and/or through caveolae-independent and clathrin-independent pathways.[2]

Peptide nanofibres might also pierce the lipid cell membrane in the same manner as another class of fibrillar nanostructures, carbon nanotubes, to access the intracellular environment[3,4] due to their similarity in shape.

1 E. Beniash, J. D. Hartgerink, H. Storrie, J. C. Stendahl and S. I. Stupp, Self-assembling peptide amphiphile nanofiber matrices for cell entrapment, *Acta Biomaterials*, 2005, **1**, 387–397.
2 D. Missirlis, H. Khant and M. Tirrell, Mechanisms of Peptide Amphiphile Internalization by SJSA-1 Cells in Vitro, *Biochemistry*, 2009, **48**, 3304–3314.
3 D. Pantarotto, R. Singh, D. McCarthy, M. Erhardt, J. P. Briand, M. Prato, K. Kostarelos and A. Bianco, Functionalized carbon nanotubes for plasmid DNA gene delivery, *Angew. Chem. Int. Ed.*, 2004, **43**, 5242–5246.
4 L. Lacerda, J. Russier, G. Pastorin, M. A. Herrero, E. Venturelli, H. Dumortier, K. T. Al-Jamal, M. Prato, K. Kostarelos and A. Bianco, Translocation mechanisms of chemically functionalised carbon nanotubes across plasma membranes, *Biomaterials*, 2012, **33**, 3334.

Professor Alexander said: Would you expect to see initial dilution (burst release or degradation over time)? Could you follow release after intercranial injection?

Dr Mazza answered: We think that it is possible that degradation will take place. While enzymes will degrade the PNFs, the cells of the immune system (*e.g.* microglia) will also be recruited to clear out the PNFs from the site of injection.

† Dr Kostarelos' paper was presented by Dr Mazza, *University College London, UK.*

This will cause a dilution of the signal generated by the fluorescence labelled PNFs injected intracranially in a specific brain area (as it can be seen in Fig. 5B of our paper) and therefore of the overall concentration of the system.

Dr Paternostre asked: Do you have an idea of the stability of the lipido-peptide fibers upon dilution?

Dr Mazza answered: We did not try dilution of the PNFs presented in this paper, because on other PNFs based on a different amino acid sequence dilution was not sufficient to cause disassembly of the nanostructures. Conversely to other self-assembled structures based on amphiphiles (*i.e.* micelles, liposomes, *etc.*), the driving force determining the self-assembly is not only the hydrophilic/hydrophobic balance, but also H-bonds that contribute to stabilize the supramolecular assembly.

Dr Singh asked: Have you examined the toxicity of the PNFs?

Professor van Hest also asked: Did you look into the cytotoxicity of the lysine functional peptide fibres?

Dr Mazza replied: The cytotoxicity was not under investigation in the present work. However we are assessing the cytotoxicity of these nanostructures in an ongoing project and so far we have not observed relevant signs of stress/toxicity for neuronal cells treated with the nanofibres. However, as one may expect, this will also be dose related.

Professor Cui commented: Enzymatic degradation is currently a very hot and important topic in biomaterials. In the context of peptide assemblies rich in β-sheets, one would think these assembled structures should be resistant to enzymatic degradation because the cleavage site might not be embedded inside, not directly accessible to the targeted enzymes. In your case, you proposed two degradation mechanisms to break down the PA nanofibers: one from the end, the other from the middle. Do you think that the enzyme you used can actually dive into the fibers and cut them into pieces? There is likely to be a third mechanism in which the enzyme cuts the unassembled peptide amphiphiles in solution, and the cleavage event would shift the balance between PA nanofibers and unassembled PA molecules that eventually lead to nanofiber breakdown.

Dr Mazza replied: We actually include this possibility in mechanism II that we have proposed in this work. The enzyme (*e.g.* carboxypeptidase) starts to degrade the K amino acids simultaneously at different sites along the same nanofiber. In this case the equilibrium from an ordered structure will shift toward a more unstable structure, where the molecular mobility of the single peptide amphiphile is increased. For example in Fig. 3A in our paper, at 24 h it can be observed that the peptide nanofibers show a less ordered structure and the cylindrical morphology is lost. In a less ordered/packed structure more sites for degradation (that were more sterically hindered in the ordered/packed nanofiber) will become more readily available to the action of the degrading enzyme. As a result PNFs can be broken down into smaller pieces and be degraded.

Professor Stupp commented: One of the interesting features of peptide amphiphile nanofibers with cylindrical shapes is their internal hydration, which allows proteins to penetrate into fibers; this has been observed by placing enzyme substrates in parts of the peptide sequence buried within the fibers and observing catalytic activity.

Professor Stupp noted: The toxicity to cells from nanofibers containing lysine residues can be diminished by enhancing intermolecular interactions among the peptides.

Professor van Hest commented: Nanomaterials with cationic charges prove to be often less toxic than their individual building blocks.

Professor Guler commented: The effect of amine groups on toxicity may change due to the supramolecular self-assembled system. The amine groups on peptide nanofibers could be easily neutralized by water compared to quaternary amines and linear peptides.

Professor Guler asked: Can enzymes degrade the peptides from anywhere in the peptide nanofiber system?

Dr Mazza responded: Depending on the amino acid sequence of a peptide amphiphile, sites for enzymatic degradation can be sterically hindered as well as "hidden" within the core of the supramolecular assembly that makes a peptide nanofiber.

Dr Nieuwland commented: The paper from Professor Stupp (DOI: 10.1039/c3fd00120b) and this paper both show that enzymatic degradation of peptide amphiphile fibers is possible. I would like to comment that this is highly dependent on the peptide amphiphile systems. A few of the peptide amphiphile fibres with GANPNAAG as a peptide (from the lab. of Jan van Hest) do not show enzymatic degradation.

Dr Saiani commented: Wouldn't you expect an immune response to the introduction of these fibres before enzyme interaction can take place?

Dr Mazza responded: It is possible that an immune response takes place, for example by activation of resident microglia, as indeed we are introducing an exogenous material by intracranial injection directly within the brain parenchyma. However, we did not investigate at this stage the response of the immune system.

Dr Saiani commented: Were the fibers injected as a solution? If so, did you also try to inject the fibers as a gelled network?

Dr Mazza answered: The nanofibers were injected as a solution, but we did not try to inject a gelled network.

Dr Paternostre commented: There is an example of a gel (peptide + water) that is commercialized by IPSEN as a sustained release formulation for therapy against acromegaly: Somatuline Autogel®.

Professor van Hest opened the discussion of the paper by Stephen Boothroyd: Did you look into the effect of counter-ions and ionic strength on gel formation and mechanical properties?

Dr Boothroyd responded: We have tested the effect of ionic strength on the gel properties. It was found that this increased the strength of the gels significantly. It can be hypothesised that this acts in a similar way to moving the gel pH towards its isoelectric point. Salts can screen the charge on the peptide, lowering electrostatic repulsion and hence the energetic barrier to assembly, increasing fibre association. The effect of charged guest molecules on hydrogel formation has also been studied. It was found that oppositely charged molecules compared to that of the peptide increased gel strength, while similarly charged molecules decreased gel strength. For further information I refer the reader to ref. 11 in our paper.

Dr Paternostre addressed Dr Boothroyd and Dr Saiani: You use ATR-FTIR to characterize the secondary structure of the peptide within the assembly. 80% of the amide I spectra is due to TFA absorption. How can you draw conclusions about the secondary structure of the peptide as the TFA signal is hiding a large part of the amide I spectra?

Dr Boothroyd replied: While the TFA does absorb within the amide I region the extent to which this occurs is lower than you suggest. Of course this is dependent on the amount of TFA in the system. In addition we find that the TFA band at 1672 cm^{-1} is very well defined. Other bands that absorb in the region can be accounted for by the peptide secondary structure, and side chains of the amino acids. Band deconvolution of the sample was performed as described in the materials and methods section and the bands then assigned appropriately to the relevant secondary structures, amino acid side chains, and single TFA peak. From this it was possible to estimate the amount of β-sheet in the structure. It is clear from the spectra that a sharp peak is present at wavenumbers of 1624–1618 cm^{-1}, depending on the sample pH, highlighting the presence of β-sheets. In addition a clear shoulder to the TFA peak at 1694 cm^{-1} was confirmed by the band deconvolution indicating an antiparallel structure. Weak bands between the TFA (1672 cm^{-1}) and β-sheet peak (1624–1618 cm^{-1}) indicate some disordered/α-helix structure, but the low absorption in this region (1640–1650 cm^{-1}) suggests qualitatively that β-sheet is the predominant structure, and this was confirmed by the band fitting. In addition we have done many tests with TFA solutions of different concentrations to subtract this proportionally from the peptide spectra. An ion exchange, removing TFA from the sample, was also conducted. These extra tests all suggested that our original band assignments and secondary structure assessment were correct, and the TFA was not hiding the presence of further structures.

Dr Paternostre addressed Dr Boothroyd and Dr Saiani: Could you also show amide II spectra? That could help for the interpretation.

Dr Boothroyd replied: While the amide II spectra can be instructive about the structure of the peptide, we find it to be broader and less well defined than the bands we see in the amide I region. Hence for this reason we concentrated our analysis on the amide I region.

Dr Adams said: Could you comment on the scaling values at different pH values?

Dr Boothroyd replied: The scaling values at different pH show the important influence of charge on the self-assembly of the peptide into fibres and the lateral association of these fibres with one another. It also shows that while several models for the scaling behaviour have been outlined in the literature it is important to understand the nature of the network, and also its evolution with concentration in a system that assembles hierarchically. At pH 2.8 we see a scaling value of 2.1 which matches well with that predicted (2.2) by Mackintosh and colleagues (ref. 19 in our paper) for entangled solutions where the network mesh size was much larger than the fibril persistence length. Heavily cross-linked gels where the mesh size is equivalent to the fibrillar segment length were predicted to have a scaling value of 2.5. At pH 4 we see a value much higher than this, suggesting that the change in mechanical properties cannot be solely attributed to the formation of a more densely cross-linked network. Indeed, we find the presence of thicker fibres formed from associated fibres. We see that these form as a function of concentration, as shown from TEM at 5 and 40 mg mL^{-1}. We believe that not only do these fibres form very stable junction points, but that they will also be much stiffer than the single fibrils. For this reason increasing the amounts of these thick fibres lead to a greater increase in the strength of the gel with concentration, and a larger scaling value. At pH 10 we see that the gel strength is much higher at lower concentrations compared to at pH 2.8 and 4, yet the scaling value is lower than those observed at pH 2.8 and 4. This can be attributed again to the difference in network formation at pH 10. At this pH fibres are seen to be more twisted, and have a lower persistence length, as indicated by SAXS results. This leads to an inability to form the laterally associated fibres observed at pH 4 (and to a lesser extent at pH 2.8) and a much lower scaling behaviour. Despite this the fibres are clearly able to entangle and entwine around each other. This can lead to a more heterogeneous network of entanglements, and strong gels.

Dr Adams asked: Do you ever see changes in gradient?

Dr Boothroyd answered: For these experiments we conducted tests within the gelation range of the samples. It can be expected that as we cross over the gelation concentration boundary there will be a significant change in the properties and strength of the gels. A test on a sample at pH 4 suggests a lower gradient across the gelation transition in the range 10–20 mg mL^{-1}. Further tests were not conducted on samples at pH 2.8 and 10, but it can be anticipated that at pH 10, which has a lower scaling exponent but forms strong gels at low concentration, as we head below the gelation concentration there will be a significant drop in the sample moduli.

Professor Cui asked: What are the observed structures at pH 6 in your TEM image (Fig. 5 in your paper)? Are those membrane structures?

Dr Boothroyd answered: At pH 6 and 7 we see the formation of highly aggregated peptide material, because of the lack of net charge on the peptide. This is reflected in the TEM image at pH 6, where large aggregates of peptide are found. At high magnification aggregates appear to show highly aligned filamentous material, although due to the high level of staining it is difficult to determine structure with any great clarity. In other areas it is possible to see aggregated peptide fibres, as in the image at pH 7, where the individual fibres resemble those found at lower and higher pHs, where such aggregation is not observed.

Professor Hamley remarked: Could I ask whether it is the width of the fibril itself that controls the modulus, as you propose, or whether there is an indirect effect of width on persistence length, with the latter influencing the modulus? If determined, how do you obtain the persistence length? Also, are different levels of fibril entanglements observed at different pH values, and if so does this affect the modulus?

Dr Boothroyd answered: It can be seen that thickening of the fibres by lateral association of single fibres will make the thicker fibre stiffer, increasing its modulus. This may then have an effect on the persistence length, the stiffer the fibres are the larger the persistence length is likely to be, and this is also indicated by the relationship $l_p \sim \kappa/kT$, where κ is the bending modulus of the fibre, and l_p the persistence length (ref. 19 in our paper). So the modulus of the fibres and the persistence length are directly related. While the persistence length has not been directly measured SAXS results indicate that fibres at pH 2.8 and 4 are similar, while those at pH 10 have a lower persistence length. This is also apparent in the TEM images, where at pH 10 the fibres appear to have a lower persistence length, and greater twist. In addition this appears to lead to a greater number of entanglements between fibres at pH 10, and stronger gels at lower concentrations.

Dr Kros asked: Did you consider including amino acids in the sequence in your system?

Dr Boothroyd replied: Our peptides are based on an alternating structure of hydrophobic and hydrophilic amino acids, which drives the assembly. This peptide sequence is FEFEFKFK, where F is the hydrophobic amino acid phenylalanine, E is the negatively charged glutamic acid, and K is the positively charged lysine. Using two E and K residues each gives charge neutrality at pH 7, while changing the number of these in the sequence helps to tune the assembly at different pH values. The use of a different hydrophobic amino acid in place of F will also lead to changes in the self-assembly and gelation behaviour of the peptide.

Professor van Hest opened the discussion of the paper by Alexander Bittner: Can you comment on the mechanical properties of the electrospun peptide fibres?

Dr Bittner answered: We have not tested the fibres quantitatively. We have some simple indications: the fibres can be manipulated and transferred, *e.g.* with a spatula, and they are in some cases quite brittle, compared to polymers such as

polyethyleneglycol. AFM in noncontact mode is straightforward. Some microscopy techniques (confocal Raman and SEM) allow us to detect 3D structures, *i.e.* some stretches of the fibre extend at least tens of micrometres normal to the substrate. When we spin large amounts, this effect is macroscopic, hence we produce a very porous solid.

Professor Alexander asked: Did you always use one pure enantiomer? Did you try mixed enantiomers and if so, did you observe different properties?

Dr Bittner replied: We only used natural amino acids (L). Here we found no crystalline fibres, so we would not expect to observe differences for enantiomers.

Enantiomers should have an influence in the case of crystalline fibres (known for Phe–Phe, see ref. 1). For example, mixing L-Phe–L-Phe with D-Phe–D-Phe and electrospinning the mixture should result in two crystal morphologies, reminiscent of Pasteur's classical work. Self-assembly of this mixture from the gas phase indeed causes chiral recognition of very small aggregates on a surface.[2]

1 W. Nuansing, E. Georgilis, T. V. A. G. de Oliveira, G. Charalambidis, A. Eleta, A. G. Coutsolelos, A. Mitraki and A. M. Bittner, Electrospinning of tetraphenylporphyrin compounds into wires, *Particle and Particle Systems Characterization*, 2013, accepted.
2 M. Lingenfelder, G. Tomba, G. Costantini, L. Colombi Ciacchi, A. de Vita and K. Kern, Tracking the chiral recognition of adsorbed dipeptides at the single-molecule level, *Angew. Chem. Int. Ed.*, 2007, **46**, 4492.

Dr Nieuwland said: Spinning polymers requires interaction between the polymers. Can you elaborate on the interactions inside the peptide fibers and between the peptide fibers during the spinning process?

Dr Bittner responded: Interactions between fibres (microscale) are probably more important for applications. After discharging (instantaneous upon hitting the collector/substrate), stretches of fibre can cross easily (see *e.g.* Fig. 2 in our paper), so there is no repulsion. We observe such a repulsion during the process when the jet surface is highly charged.

Interactions on the nanoscale should guide the fibre formation in the liquid jet, during solvent evaporation. We expect that rheology studies of the educt solutions can give important hints. For now we restrict ourselves to vibrational spectroscopy of the product, which indeed shows intermolecular interactions (hydrogen bonds). See also ref. 1. Note that most fibres are not crystalline. To our knowledge there is no technique that is sufficiently fast to observe nanoscale interactions during the spinning process.

1 W. Nuansing, A. Rebollo, J. M. Mercero, J. Zuñiga and A. M. Bittner, Vibrational spectroscopy of self-assembling aromatic peptide derivates, *J. Raman Spectrosc.*, 2012, **43**, 1397.

Dr Kasotakis asked: Is it possible to control nanoscale patterning of the fibres with the electrospinning technique?

Dr Bittner answered: To the best of our knowledge, nanoscale patterns cannot be produced. It is already very difficult to produce fibre diameters below 20 nm.

There are some publications on "near-field" electrospinning, where the educt droplet is scanned or positioned in 2D over the collector. The emitted jet can then

be guided on the microscale. The jet should not exhibit true electrospinning with typical whipping because the latter cannot be controlled (it is an intrinsic instability). This means that diameters are normally rather large (microscale), and it is not always clear whether the jet solidifies.

Professor Hamley said: Have you tried electrospinning your peptides from substances in water? I am wondering specifically about Fmoc–FG–OH which has been shown to form fibrils by self-assembly in water, I think, by Saiani, Ulijn and coworkers.

Dr Bittner answered: We didn't try water as a solvent. First, its vapour pressure is rather low, so it is difficult to obtain dry and thin fibres. Second, the peptides have a very low solubility in water. For electrospinning, concentrations up to 50 wt% are required.

Self-assembly processes in water were found for this example in ref. 1. and 2. Such an assembly means that we have to work at exactly those conditions we have to avoid: Our droplet should be a solution, not a suspension of already formed fibres. In this case we would probably obtain solidified droplets, made up from straight short self-assembled fibres.

1 V. Jayawarna, M. Ali, T. A. Jowitt, A. F. Miller, A. Saiani, J. E. Gough and R. V. Ulijn, *Adv. Mater.*, 2006, **18**, 611.
2 D. M. Ryan, T. M. Doran, S. B. Anderson and B. L. Nilsson, *Langmuir*, 2011, **27**, 4029.

Dr Nieuwland commented: The question of why it is difficult to spin proteins was raised. We believe that has to do with the strong internal interactions in proteins that prevent them from forming the required entanglement (see ref. 1).

1 M. Nieuwland *et al.*, Food-grade electrospinning of proteins, *Innovative Food Sci. Emerging Technol.*, 2013, DOI: 10.1016/j.ifset.2013.09.004.

Dr Saiani asked: If the peptides within the fiber do not form any structured assembly what are the forces/interactions keeping the spun fibres together?

Dr Bittner replied: We expect the same as for crystalline aromatic peptides, *i.e.* mainly hydrogen bonds, but also π-stacking. The hydrogen bond network is at least analogous to that found in solids made from "amorphous peptide sequences".

Dr Saiani asked: If the spun fibres are an assembly of amorphous peptides can they be handled without falling apart?

Dr Bittner replied: Although the structure is amorphous, the intermolecular forces should be qualitatively the same as for a peptide crystal. We have no data on mechanical stability yet, but it appears the fibres are rather brittle. It is worth noting that the fibres can be electrospun on any desired substrate, so normally no transfer is required.

Professor van Hest asked: Do the protein fibres display hydrophobic patches? Do proteins found in the medium tend to adsorb onto the fibres?

Dr Bittner answered: We expect that hydrophobic patches are exposed, but we have not tested for them. Patches inside a single protein are probably exposed whenever the protein that forms the fibre is denatured. We have not investigated the fibres for adsorption of proteins.

Professor Stupp commented: The nature of surfaces in electrospun fibers is important for their application as scaffolds for cells. Will it be possible to control the chemistry of these surfaces in electrospun proteins and peptides, that is what parts of their structure are actually displayed to cells on the surfaces of the fibres?

Dr Bittner replied: The advantage of properly chosen peptides and proteins (over polymers) is that they already possess the desired chemical properties. While the conformation might change to various degrees, the chemical identity is preserved in electrospinning. It is possible, but not likely, that on the fibre surface the conformation is such that the biological function is lost.

On the application side, we have found that certain peptides do remain functional on electrospun fibres, and that they are suitable as scaffolds for cells. On the fundamental side, determination of surface biochemistry on fibres is a difficult task that probably can only be solved by combining various advanced methods.

Professor Lecommandoux asked: To what extent can you electrospin proteins into fibres without damaging them irreversibly?

Dr Bittner responded: Our CD results suggest that the solvent, rather than the electrospinning, causes denaturation. This is obvious here for the case of trifluoroacetic acid. More generally, one can probably in most cases find solvents that allow us to preserve at least the functionality. This has been shown for lipase.[1] Preserving the complete structure would first require a method that can prove this in the solid state.

1 J. Xie and Y.-L. Hsieh, Ultra-high surface fibrous membranes from electrospinning of natural proteins: casein and lipase enzyme, *J. Mater. Sci.*, 2003, **38**, 2125.

Professor Lecommandoux said: Did you observe any birefringence in the fibres you obtained by electrospinning that may indicate a strong orientation from the experimental conditions you have?

Dr Bittner answered: We have not checked for birefringence. We did test CD of the fibre mesh, but with unreliable results, probably due to scattering. In some rare cases the spun fibres are crystalline. We found this for Phe–Phe.[1] As for macroscopic orientation, there are various possibilities. The simplest is the presence of a sharp edge on the collector, which induces straightening and alignment.[2]

1 W. Nuansing, E. Georgilis, T. V. A. G. de Oliveira, G. Charalambidis, A. Eleta, A. G. Coutsolelos, A. Mitraki and A. M. Bittner, Electrospinning of tetraphenylporphyrin compounds into wires, *Particle and Particle Systems Characterization*, 2013, accepted.
2 G. Singh, A. M. Bittner, S. Loscher, N. Malinowski and K. Kern, Electrospinning of diphenylalanine nanotubes, *Adv. Mater.*, 2008, **20**, 2332.

