DISCUSSIONS

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General discussion

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Professor Alexander opened the discussion of the paper by Zhanglin Lin: Are there changes in motility of the bacteria following changes in gene expression?

Professor Lin answered: This is something we did not look into. A very interesting question.

Professor van Hest enquired: Is it possible to control the type of aggregates formed in the bacteria, for example could vesicular structures be formed?

Professor Lin responded: This is a very intriguing question. We have only concerned with ourselves with aggregates purely of proteins/peptides. To have vesicular structures, one would have to involve other biopolymers such as lipids.

Dr Saiani commented: Using bacteria to produce protein, how reliable is the protein sequence obtained? Is the cost of the approach used strongly affected by the length of the peptide tag used?

Professor Lin noted: The sequence is very reliable and the cost is generally not affected by the length.

Professor Hamley asked: Why do you need such a specific peptide sequence in order to drive self-assembly? There has been work by others using amyloid peptides for example to drive the aggregation of large globular proteins (you cite some of this work in your paper) – these give non-specific assembly, perhaps your sequences are more specific?

Professor Lin replied: The amyloid peptide that has been reported to perform such a function is A β 42, a peptide with 42 amino acid residues. Our work is significant in two ways:

(1) Scientifically, we found there are a number of short peptides (beyond the amyloid peptide) that can cause proteins to form aggregates *in vivo*, in particular the amphiphatic peptides that are commonly found in proteins. This might be relevant to protein aggregation-related diseases.

(2) Technically, the short peptides found in our work are much safer to use in applications such as in peptide tags for protein expression and purification. Also, it is much more economical as these peptide tags account for a much smaller percentage in fusion with a target protein.

Dr Saiani remarked: Do the ion pair modifications change the conformation adopted by the peptides?

Professor Lin responded: Theoretically the modifications should slightly change the 18A peptide structure, but then it should remain in a helical conformation.

Dr Saiani asked: Is the efficiency of the protein recovery process affected by the conformation adopted by the peptide tag *e.g.* β -sheet or otherwise.

Professor Lin answered: Indeed, it seems at this point that the β -sheet conformation works better.

Dr Kros asked: If Lys is changed to Glu, do you lose the snorkel effect?

Professor Lin replied: Indeed yes. This is exactly what we set out to prove, and we saw reduced association of the fusion protein with the cell membrane.

Dr Saiani enquired: Can you use the bacterial approach to synthesise other species, such as polymers?

Professor Lin answered: Except for a limited number of cases such as poly-(3-hydroxybutyrate), generally bacterial cells cannot do that.

Dr Saiani asked: How long are the bacteria kept alive for? What is the correlation between length of experiment and amount of protein produced?

Professor Lin answered: Bacteria normally are kept alive for 1–2 days, but often the protein production reaches the peak within one day.

Dr Gouveia commented: How much easier it is to solubilize proteins using this system compared to those produced in usual inclusion bodies?

Professor Lin responded: There is no need to solubilize. The proteins in the aggregates are in the native state. If we cleave off the attached self-assembling peptides (say *via* the self-cleaving activity of an intein inserted in between), the proteins are released in the soluble fraction.

Dr Gouveia noted: The folding of a recombinant peptide or protein produced by bacterial systems after its dissociation from inclusion bodies seldom corresponds to the native one, which usually impairs its function. Does the use of these self-assembling peptides allow recombinant proteins with native-like conformations to be retrieved?

Professor Lin replied: It is somewhat the opposite: these self-assembling peptides allow the recombinant proteins to fold properly upon translation, and by the self-assembling action of the peptides, the properly-folded proteins are driven into active aggregates which retain the native state. At least, it seems this way given the fluorescent activity of GFP, and the activity results from other model enzymes.

Professor van Hest asked: How does your system compare to the yeast expression system with an excretion signal?

Professor Lin responded: It is quite different. We have protein aggregates formed inside *E. coli*, which is not amenable for secretion. Generally, the expression level for *E. coli* is much higher than that for yeast, whether aggregates are formed or not.

Dr Gouveia asked: Could these peptides be applied to other protein/peptide production systems (*e.g.* yeast, insect, or mammalian cells)?

Professor Lin replied: In theory, they could. We have not tried this yet.

Dr Saiani asked: How much protein gets trapped in the aggregates after cleavage and centrifugation?

Professor Lin responded: It varies. For lipase A (21 KD), about 32% gets trapped, for amadoriase II (49 KD), about 38%, for beta xylosidase (61 KD), about 86%.

Dr Saiani wondered: Which approach or technique could you use to improve the protein extraction from the aggregates?

Professor Lin answered: It appears that the protein size matters – the larger the target protein is, the more it gets trapped. Therefore, one way to improve the efficiency of release is to make the aggregates loose upon recovery *via* centrifugation.

Professor Hamley remarked: Why did you use the L₆KD sequence? Is there something specific about it?

Professor Lin replied: We have screened quite a number of peptides for the ability to drive proteins into active aggregates in *E. coli*. L₆KD is one of the peptides that we found to perform such a function. At this point, we do not have a specific rule as to which peptide may work.

Dr Saiani commented: What are the conditions, pH and salt content, in the bacteria?

Professor Lin replied: As far as *E. coli* is concerned, the intracellular pH is around neutral. The salt content is a more complex issue, but the concentration is in the submolar range.

Dr Bittner opened the discussion of the paper by Saul Tendler:[†] Do you have additional proof that your structures are tubes and not solid fibres? AFM alone may not suffice (see Bittner *et al.*, *Adv. Mater.*, 2008, **20**, 2332–2336).

[†] Prof. Tendler's paper was presented by Dr Korolkov, The University of Nottingham, Nottingham, UK.

Dr Korolkov answered: Our study of the first stages of dipeptide assembly have indicated a tubular structure. Whether it remains so during the whole assembly is under investigation.

Dr Saiani asked: What are the globular structures present on the tubes in the pictures?

Dr Korolkov replied: These are structural elements whose structure needs to be assessed with other techniques, perhaps UHV STM or TEM. Ambient AFM on its own cannot resolve their inner arrangement.

Dr Adams enquired: Do you know for definite that the diphenylalanine is molecularly dissolved in the HFIP?

Dr Korolkov answered: Di-PhePhe solution in HFIP at given concentrations appeared as a clear transparent liquid with no evidence of colloidal systems. It did not scatter light when a laser was passed through it nor did it show any precipitation over a long period of time. So, yes, we believe it to be a true solution as there is no experimental evidence indicating otherwise.

Dr Adams asked: What time scales are involved when changing from one structure to another?

Dr Korolkov responded: I believe you are referring to the transition of dendritic structures into needle-like crystals upon the exposure to 100% ambient humidity. The exact time scale is not known to us. But we understand that it happens very rapidly, probably within seconds or even less. That is based on our observation of the process with a conventional CMOS-camera built into the optical microscope.

Dr Adams remarked: Have you looked at the crystal structure after exposure to humidity? Does it agree with other published structures?

Dr Korolkov replied: No we have not. Morphologically, as seen by AFM or optical microscopy, they appear to look very similar to di-PhePhe monohydrate crystals as supplied by Sigma-Aldrich.

Dr Saiani remarked: What are the characteristics of the mica surface used? Freshly cleaved mica is charged; do the surface characteristics affect the structure formed? Have you tried to change the surface characteristics?

Dr Korolkov answered: We used a freshly cleaved mica surface. We have not modified it further. We have not established if surface charge has any impact on the formation of structures.

Dr Saiani asked: Why do mica surfaces trigger the formation of these structures?

Dr Korolkov replied: We believe two factors play an important role. Namely, the atomic flatness of mica and strong substance–substrate interactions.

Dr Nieuwland wondered: Is there a way to stabilise the structures and use them in an application?

Dr Korolkov replied: We believe that this could be done *via* electron induced polymerisation, although the possible electron induced damage may affect the fine structural arrangement.

Professor Hamley asked: Is there a monolayer of dipeptide coating the whole mica, meaning the dendritic structure is unaffected by the mica substrate?

Dr Korolkov replied: It is a monolayer of dipeptide particles assembled in a random fashion. They do cover areas of mica in between the dendritic structures, although such a monolayer is absent in close proximity (100–200nm away) to the dendritic structures. Our understanding is that such particles further assemble into tubular-like structures which further interact with each other. We did observe such tubular assembly directly on mica surface without the formation of randomly assembled particles monolayer first. We argue that mica provides an atomically flat and strongly binding support for tubes' assembly.

Dr Saiani enquired: How can the amount of water present be evaluated? What is the role and effect of water?

Dr Korolkov responded: We have not evaluated quantitatively the effect of water on the dendritic structure formation, although, as we have observed, water does transform them into needle-like crystals. The effect needs further investigation.

Dr Saiani commented: We need tools to evaluate the role of water in the self-assembly of peptides.

Dr Singh noted: Trifluoroacetic acid is a common contamination source in commercial peptides.

Dr Korolkov answered: We have not observed that contaminant when the sample was analysed using high resolution MS.

Dr Squires asked: Can you take a small amount of diphenylalanine from your dendritic structure and use this as a seed to grow crystals, to determine whether the packing of diphenylalanine molecules within the dendritic structure is the same as that in previously observed crystals?

Dr Korolkov responded: We have not performed such experiments. But I believe it should be possible to initiate crystal growth in such a way.

Professor van Hest opened the discussion of the paper by Mustafa Guler: Do you see raft or domain formation? Is efficient uptake an indication of domain formation?

Professor Guler answered: How the peptides are localized on the liposome is not well-known. The enhanced uptake of the liposomes upon peptide integration is an indirect sign of peptide integration.

Dr Singh asked: Have you done any temperature dependent studies on the delivery of anticancer drugs?

Professor Guler replied: We have not tested our formulations at varying temperatures.

Professor Lecommandoux enquired: Can you quantify or at least estimate how much peptide you need at the surface of the liposomes to have good uptake?

Professor Guler responded: Over the range of peptide concentrations we have used in this study, an increased amount of peptide can enhance the cargo uptake results.

Professor Cui commented: Is there any particular reason for you to use the C12 (lauric acid) and not C16 (palmitic acid) hydrocarbon as the tail in your molecular design? Also, is the purpose of using proline in your PA to disrupt its assembly into nanofibers?

Professor Guler answered: Lauric acid was used as a hydrophobic segment to integrate peptide into the liposomal system. Other lipids should work as well.

Dr Kros asked: What is total lipid concentration used in this study? How does this system compare to cationic liposomes?

Professor Guler replied: We do not know.

Dr King commented: Do you know the mechanism by which your system can transfer cargo across a cell membrane?

Professor Guler answered: We have not tested the uptake mechanism. It is possible that it is through electrostatic interactions between the cellular membrane and the peptides.

Dr Garanger asked: How does the amphiphilic peptide alone (without liposomes) self-assemble? How do the self-assemblies formed from the peptide alone (used as the control in your biological experiments) compare to peptide-functionalized liposomes in terms of size?

Professor Guler responded: The peptide alone was not used as a carrier system. Liposomes and peptides were mixed during the liposome formation. We have not studied structures formed by peptides.

Mrs Kessler asked: What is the loading capacity of the hydrophobic and hydrophilic compound in comparison to the amount you need for a therapy?

Professor Guler noted: It is in the range of typical liposomal systems.

Dr Paternostre commented: Did you measured the solubility of your amphiphilic peptide and its critical aggregation concentration? Upon dilution there is risk that the peptide is extracted from the lipid bilayer of the liposome through a dynamic equilibrium process.

Professor Guler responded: We have not.

Professor Hamley asked: Can you please comment on the mode of interaction of your cell penetrating peptide amphiphile with the lipid membranes? Can you eliminate the possibility of peptide amphiphile molecules interacting at the surface of the liposomes rather than embedding within the membrane?

Professor Guler said: Unfortunately, we do not have direct evidence to support organization of peptides within the lipid membrane. We expect that the lipid segment could be localized in the liposomal membrane and that the arginine residues are interacting with the surface of the membrane.

Professor Alexander noted: There was a notable step up at higher concentration. Why?

Professor Guler replied: We do not know.

Dr Nieuwland commented: In the introduction of the paper you mention that a non-covalent approach is preferred over a covalent approach because more activity is retained. How does the activity of your systems compare to systems with covalent linkage (for example chemical ligation?).

Professor Guler answered: We have compared our peptide functionalization results against bare liposomes. We have not studied a covalent functionalization.

Dr Kros asked: At the highest concentration used, are you sure liposomes are still being formed? Is this proven by, for example, TEM?

Professor Guler replied: We imaged the liposome samples with TEM. No change in size was observed.

Professor Lin opened the discussion of the paper by Honggang Cui: Will the nanoparticle structures be disrupted in the stomach environment (pH around 2)?

Professor Cui answered: These drug nanoparticles are expected to be administered though intravenous injection. I have done some testing at low pH to check their stability and found the nanotubes could be stable at low pH for several hours. But eventually, all these nanoparticles are designed to break down, releasing the free CPT.

Professor Lin asked: How would you use the nanotubes for drug delivery?

Professor Cui replied: There are two issues we need to address before we evaluate these CPT nanotubes in an animal setting. The first issue is the control of the nanotube length and length polydispersity. Then, we need to study how to change the surface chemistry without losing the nanotube morphology. After these, we will plan to test these nanotubes in appropriate models through an intravenous injection pathway.

Dr Adams noted: The TEM images of folic acid show large objects. Doesn't the CD data suffer from scattering issues?

Professor Cui responded: Yes. This is a good point. The CD spectra are affected by the presence of larger objects that scatter very strongly. The overall intensity of absorption would likely be attenuated. However, given that the CD measures the absorption difference of left and right handed circularly-polarized light, it is unlikely the observed supramolecular chirality (intensity sign, peak position) is induced by the large object scattering, although their intensity is more or less affected. We did not attempt to extract too much information from the CD spectra.

Professor Kinbara commented: Both filamentous nanostructures and micron-sized platelets are formed from the same compound in different solvent compositions. If the composition of the solvent is changed after the formation of these assemblies, do these structures revert to the other?

Professor Cui replied: The transition from filamentous nanostructures to micron-sized platelets will take place with the addition of water into methanol, however, this transition is not reversible. Once we obtain these single-crystal-like platelets, they do not change to the nanofiber structures even when the solvent composition was reversed back to containing a higher percentage of methanol. In the presence of water, the platelets are very stable. One way to access the nanofiber morphology from these platelets might be to lyophilize the solution and then resolubilize the resulting powder in pure methanol.

Professor Cavaco-Paulo asked: What happens when the self-assembled structures are put in water with a physiological buffer? What happens to the structures in blood?

Professor Cui answered: Folic acid in PBS buffer assembles into nanofibers, similar to those formed in pure methanol. For the CPT drug amphiphiles, they could form either nanofibers or nanotubes depending on the treatments. We have not studied the stability and morphological changes of the produced nanostructures in blood, and expect to conduct these experiments soon.

Professor Lecommandoux commented: Using drugs as building blocks for self-assembly is a nice concept. In this area, the group of Patrick Couvreur (Chatenay-Malabry, France) especially demonstrated the benefit of the concept of "squale-nization" for the delivery of gemcitabine, the self-assembled nanoparticles thus formed being able to be transported by a particular mechanism. In your case, and

in a more general perspective, to what extent you can anticipate the bioavailability of the drug after conjugation and self-assembly?

Professor Cui responded: Thanks for pointing out the work by Patrick Couvreur. The “squalenization” is an elegant demonstration of the concept of creating drug assemblies with a high and controlled drug loading. This is indeed an emerging and promising area that may have a great impact on cancer nanotherapeutics, although there are still many experiments to be done to push this conceptual work to the clinical stages.

Regarding the bioavailability, I cannot give you a straight answer because we have not done any *in vivo* experiments to evaluate it. In pharmacology, bioavailability is usually used to evaluate a formulation that is not given through intravenous (i.v.) injection. By definition, the bioavailability of a drug given through intravenous injection is considered to be 100%. Since both our drug amphiphiles and the “squalenization” formulation are expected to be delivered through i.v. injection, the bioavailability of the drug amphiphile should be 100%. I think what you really refer to is the percentage of the “free drug” available to the tumors. It will be very difficult to evaluate this because not only do the supramolecular drugs need to be delivered to the target sites and dissociate into monomeric units, but also the free drugs (CPT in this case) must be released from the conjugated form. In our *in vitro* experiments, we have already found out that the retarded release of CPT, as a result of forming supramolecular nanostructures, has a great impact on the efficacy of the drug amphiphiles in several cancer cell lines.

Dr King enquired: Do you have any thoughts on how you might be able to target the delivery of your system to specific places in the body, or to specific cell types?

Professor Cui answered: This is exactly what we expect to do with our system eventually. In addition to the passive targeting strategy of taking advantage of the EPR effects, we plan to incorporate cell-specific or organ-specific ligands into our system for active targeting. This is not trivial and there are lots of experimental parameters that need to be worked out.

Dr Squires asked: If you wanted to add functional groups to give your drug assemblies specific targeting (eg RGD motif), where might you add these to your structures?

Professor Cui replied: Synthetically speaking, this can be done rather readily. If the specific targeting ligands are peptides, for example, the RGD motif to which you referred, this incorporation process could be just part of the peptide synthesis. If the targeting ligands are non-peptidic small molecules, this can be done using lysine as a branching point.

Dr Squires enquired: Have you done X-ray diffraction to provide evidence for the tetramer stacking shown in Fig. 4?

Professor Cui responded: No, we have not done X-ray diffraction experiments to provide direct evidence for the tetramer stacking among folic acids. Folic acid is known to form a tetramerized disk through Hoogsteen hydrogen bonding, and this has been studied by others using X-ray diffraction techniques. On the basis of the previous reports, we assume this would be the case in our system.

Dr Squires asked: What is the evidence so far for the molecular arrangement shown in the nanofiber structure?

Professor Cui replied: For the folic acid nanofibers formed in methanol, we measured the diameters of these nanofibers as observed using TEM imaging and found the measured value is in general agreement with the expected size of the tetramerized folic acid disk. We therefore propose that the observed folic acid fibers are formed by stacking the folic acid disks in one dimension.

Professor Stupp commented: One would expect that targeting efficiency would be affected by the shape of the supramolecular assemblies. The shape is likely to change with the nature of the drug. Could you comment on the strategies that might be used for maintaining shape invariance over widely varying chemical structures among drugs?

Professor Cui answered: You are right that the targeting efficiency of supramolecular assemblies will most likely be affected in several ways if their shapes are changed. This could be good or bad, depending on how it is altered, and also on the purpose of the intended use. In the design of a self-assembling drug amphiphile, it is almost certain their self-assembly behavior is heavily dependent upon the chemical structure of the conjugated drug. The question here is to what extent such a difference will be sufficient to lead to a change in shape or morphology. We recently have found that for drug amphiphiles containing one drug and one short peptide capable of forming intermolecular hydrogen bonding, the resulting morphology is often one dimensional and dominated by the peptide segment. This observation is consistent with your work on the peptide amphiphiles – the nanofiber morphology is very tolerant to the length of the alkyl tail. However, if two or more drugs are conjugated, we found that the drug structures have a huge impact on the shape of the assembly.

Professor Stupp asked: In addition to the loading issue, what are the other critical features that self-assembling drugs would improve for therapies?

Professor Cui replied: Our recent studies show that the release rate of the drug from the self-assembled nanostructures can be regulated by the packing order of drug molecules within the assemblies in addition to the design of the linker chemistry. Another benefit is the protection of the hydrolysable or degradable drugs from being accessed during circulation.

Professor Stupp enquired: Could the self-assembling drug platform be improved through the use of cross-linking using hydrolysable bonds? This would presumably help preserve the supramolecular shape, which is an important feature for targeting.

Professor Cui responded: Yes, we are currently working on a few cross-linking strategies to stabilize the drug assemblies. We are particularly interested in enzymatic degradable linkers such as MMPs. The cross-linking chemistry, as well as the triggered release of the drug molecules by enzymatic cleavage of the cross-linker, is not trivial and we are seeking several strategies to resolve the stability issues. Stability is a major concern when using small molecular building units to create nanocarriers for the delivery of drugs and imaging agents. We always expect the resulting supramolecular assemblies are stable during the circulation so that we could have good control over the carrier's pharmacokinetic properties. At the same, when the particles reach the targeted site, we hope these nanocarriers can break down to effectively release its cargo because only monomeric, unassembled drug molecules are actually functional and biologically relevant. The use of hydrolysable or enzymatically degradable cross-linkers presents a potential solution to solve this issue.

Dr Squires asked: As the carrier is the drug itself, how can you control the loading? If you change the loading don't you also change the self-assembly?

Professor Cui said: The drug loading is defined by the molecular design, precisely the fraction of the drug within the drug amphiphile. For a given drug amphiphile, regardless of the nanostructures it could potentially form, the drug loading is always the same. It is highly likely that when you change the molecular design, the self-assembly behaviors will change accordingly. I think that this is truly the beauty of the drug amphiphile design. You have control not only over the drug loading, but also on the assembled nanostructures. The nanostructure characteristics (size, shape and surface chemistry) are a very important design consideration for the development of nanomedicines targeted for cancer chemotherapy.

Professor Guler enquired: Do peptide conjugated drugs behave similar to the drugs themselves in terms efficacy?

Professor Cui replied: In terms of the *in vitro* efficacy, none of the self-assembling Camptothecin (CPT) drug amphiphiles is better than the free drug. In our paper published early this year in *J. Am. Chem. Soc.* (2013, **135**(8), 2907–2910), we did a thorough study to evaluate CPT drug amphiphiles' efficacy against a number of cancer cell lines. The free drug CPT is always the most effective one with the lowest IC₅₀ value. This is not surprising actually, given that CPT itself can effectively penetrate the cell membrane through diffusion and is directly bioavailable. Our recent results suggest that the retarded release of CPT from the self-assembled supramolecular nanostructures accounts for one of a few factors that contribute to the reduced efficacy. The true advantage of using nanocarrier-based systems is to improve the drug's targeting efficiency to tumors, which can only be evaluated in animal models. We expect that these drug amphiphile nanostructures will perform much better than the free drug in animal experiments.

Professor Guler commented: Peptide conjugated drugs should be tested in terms of their efficacy and side effects. These cannot be compared to the initial drug.

Dr Singh opened the discussion of the paper by Joerg Kressler: Why does the branched polymer adsorb faster than the linear one? What is the role of branching?

Professor Kressler replied: The hyperbranched Ch-PEG-*hb*PG adsorbs quickly and strongly, the linear Ch-PEG-*l*PG more slowly. The reason for this difference is thought to be the polymers' architecture and the resulting spatial molecular conformations in the aqueous environment. For Ch-PEG-*hb*PG, the cholesterol unit is exposed and free for direct insertion into the phospholipid membrane and the kinetics of the interaction depends mainly on the diffusion of the polymer from the aqueous bulk phase. The coiled conformation of the linear copolymer, PEG-*l*PG, shields the hydrophobic moiety, hindering the hydrophobic interaction with the phospholipid membrane.

Professor van Hest asked: How generally applicable is the stabilisation of the polymersome structure using BSA? Could you take any type of biotemplated protein?

Professor Kressler said: As observed using CLSM, the protein coating (BSA-Streptavidin) triggers the formation of polymersomes by allowing the adsorption of an ordered polymer layer on its surface. However, our results indicate that the protein layer does not have a stabilizing role once the polymersomes have been formed.

Although we only tested the BSA-Streptavidin system, it is possible that other protein coatings could also trigger the formation of polymersomes, but certainly not just any protein would do it. For example, a specific interaction between the PGM block of PGM-PPO-PGM and streptavidin was found previously using molecular dynamic simulations (J. Kressler *et al.*, *ACS Macro Lett.*, 2012, **1**, 1016–1019).

Dr Kros enquired: Do the polymersomes detach from the surface?

Professor Kressler replied: Yes, they do. As shown in Figure 2a-A of the paper, an overview of the surface after polymersome formation shows some round spots where only the BSA layer is left behind (blue spots) after the polymersomes detached from the surface.

Professor Alexander asked: What is the driving force behind crossing the membrane?

Professor Kressler answered: The driving force for the membrane crossing ability of the polymer is not fully understood. However it is known that some amphiphilic polymers are able to diffuse across biological membranes, but endocytosis is also a possibility. The actual mechanism will only be known after further experiments.

Dr Singh wondered: Does hydrogen bonding in the hyperbranched polymer play any role for increased transfection?

Professor Kressler replied: We have not yet had any indications in that direction. Both hyperbranched Ch-PEG-*hb*PG and linear Ch-PEG-*l*PG are able to cross the membrane and to form plenty of hydrogen bonds, therefore the difference could be more related to the polymer conformation.

Dr Kros asked: Did you observe flip-flopping, as the molecules are not very hydrophilic?

Dr Squires added the question: Are the polymer molecules (and fluorescent probes) distributed symmetrically in both sides of the bilayer? Is there any evidence for this?

Professor Kressler responded: Since we observed membrane-crossing of the polymers, flip-flopping is a possibility. However, we could not observe it because a discrimination between the two leaflets of the membranes is below the resolution limit of the CLSM. Experiments allowing for such a discrimination, such as FRET with GUVs filled with fluorescent solutions, have not been performed yet. On the other hand, the mechanism of membrane crossing could also be endocytosis.

Dr Kros asked: What happens if the salt concentration is increased?

Professor Kressler answered: All experiments with polymersomes and GUVs were performed in pure water, thus the influences of salts were not investigated at all. For the cells experiments a standard DMEM cell culture medium was used.

Dr Squires enquired: In the cell penetration experiments, do the polymers permeate as individual molecules or are they self-assembled? Do the fluorescence signals offer any clues?

Professor Kressler replied: In the experiments with murine embryonic stem cells the concentration of the Ch-PEG-*hb*PG polymer was 2 μ M, which is below its CMC. Therefore, it can be assumed that only single chains crossed the cell membrane. We have not got any hints from the CLSM observations, such as self-quenching phenomena, for self-assembly.

Dr Squires opened the discussion of the paper by Olga Vinogradova:[‡] Where you had a heterogeneous charge distribution, down to what range of length scales for the spacing of negative and positive regions is your model applicable to?

Dr Lobaskin said: The problem has at least two important length scales: the Debye length and the scale for the charge inhomogeneities. The characteristic distance at which the heterogeneity can be detected is the longest spatial period of the charge distribution. In the limit of small patches, the periodicity can be felt

[‡] Professor Vinogradova's paper was presented by Dr Lobaskin, University College Dublin, Dublin, Ireland.

down to the molecular length scales. I can cite the interactions between polyelectrolyte multilayer capsules, where the patterns are formed by individual molecules, or experiments by Stellacci *et al.*,¹ where the molecular size surface stripes were shown to affect the nanoparticle–membrane interaction as compared to the homogeneously charged particles.

- 1 A. Verma, O. Uzun, Y. Hu, H.-S. Han, N. Watson, S. Chen, D. J. Irvine, and F. Stellacci, Surface-structure-regulated cell-membrane penetration by monolayer-protected nanoparticles, *Nature Materials*, 2008, 7, 588–595.

Professor Mezzenga commented: Irregularity of charges on surfaces can lead to a reduction in long-range repulsion, an effect described by some authors as patchy hydrophobic interactions. Can you account for this effect in your approach and how do you do this?

Dr Lobaskin replied: This was exactly the main point of this work. We aimed to assess the contribution of the charge patches on the wall to the long-range repulsion. We introduce the patchiness through the boundary conditions at the surface and calculate the corrections to the disjoining pressure coming from the average surface charge. In all cases, the patchiness reduces the interaction as compared to the homogeneous case, which could indeed provide an explanation for the phenomena you mention. In what regards to the hydrophobic patches, the problem would be more challenging, as there is currently no simple general model for hydrophobic interactions.

Professor Hamley asked: Using Fourier expansion, how can you account for irregular charge patches?

Dr Lobaskin answered: The Fourier expansion of the surface charge distribution works equally well for arbitrarily-shaped patches or irregular patterns. The difference with the case of regular charge stripes considered in this work is that the expansion for irregular patterns would contain further terms with larger wave vectors, which would reflect all the important length scales of the charge distribution. I should note, however, that the decay length for the potential away from the surface and for the terms in the disjoining pressure depends on the wavelength of the charge pattern. It is longer for the larger patches and shorter for the smaller ones. Therefore, the biggest contribution to the pressure always comes from the structures with the largest period or longer characteristic wavelength.

Dr Squires wondered: Can active transport be incorporated into the model?

Dr Lobaskin replied: Yes. In our theory we calculate the induced potential on the membrane due to the presence of the charged surface nearby. The corresponding potential can be used to calculate the additional force acting on the molecules travelling across the membrane.

Dr Squires enquired: What experimental data would you like to have available to test your model?

Dr Lobaskin responded: Ideally, we would like to see the surface force measurements (AFM or SFA) combined with the independent measurements of the electrokinetic potential for the same surfaces. In this case, we would be able to evaluate the main parameters and test the model in full.

Dr Squires asked: Is the experimental data on stability quantitative enough to tell the difference between this model and a more basic model?

Dr Lobaskin replied: As the disjoining pressure between the membrane and the charged surface behaves similar to the pressure between two charged surfaces, it would be impossible to tell the case with a semipermeable wall from a hard particle in a single experiment observing just the stability of the dispersion. We can only extract some effective surface charge on the membrane from the measured interaction potential. However, if an independent experiment with capsule electrophoresis is done, then the comparison of the zeta potential and effective surface potential can help us to evaluate the role of the membrane permeability.

In regards to probing the charge distribution on the solid surface and the presence of charge inhomogeneities, they can be detected by probing the distances comparable to the typical period of the charge distribution. At these separations, the force will deviate (decrease) from the exponential one that is predicted for the homogeneous surface. The distance, at which the deviations will start to appear, would indicate the largest length scale of the charge inhomogeneity.

Mrs Kessler opened a general discussion of Professor Guler's, Professor Cui's, Professor Kressler's and Professor Vinogradova's papers: There are two complete different approaches described in Professor Guler's and Professor Cui's papers to encapsulate cancer drugs. What are the advantages of these two different approaches? If you think of potential applications, for which applications would which approach be the best?

If you consider sensitive hydrophobic compounds like vitamins, does one of these approaches lead to a protection of the encapsulated material from environmental stress, like for example UV-light?

Professor Cui said: One advantage of using drugs to build their own nanostructures is that the drug loading can be well controlled. Most carrier-based systems possess a very low drug loading capacity (less than 5%), whereas in the drug amphiphile system the drug loading is precisely defined by the molecular design. In my opinion, the creation of drug nanostructures could potentially improve, or partially solve the drug loading issue in a carrier-based system, but it does not directly address the grand challenge in cancer chemotherapy – that of specific targeting to the tumor sites. It should be clear that high drug loading is not always desirable, particularly for drugs with very high potency. Nevertheless, the drug amphiphile system at least provides one additional option allowing for quantitative control of drug loading. Of course there are many experiments to be done in order to translate this concept into a platform technology. Speaking of the delivery of sensitive hydrophobic compound like vitamins, it is certain that encapsulation of the vitamin into the hydrophobic cores of polymeric micelles,

liposomes, or peptide amphiphile nanofibers would keep the drug away from water. It is a feasible and sound approach to use nano-carriers for drug protection. However, the most justifiable reason to use a nanoscale architecture as carriers to deliver anticancer drugs, in my opinion, is to modify the drug's pharmacokinetics profiles to achieve better tumor targeting, not to protect the drug from degradation although most carriers do protect drugs from hydrolysis and enzymatic degradation. For vitamins that are highly sensitive to water, you can simply increase the dose to generate the desired therapeutic effects. If vitamins do not go to the targeted organs, cells, or receptors, they less likely to lead to severe problems to the patients. As for anticancer drugs, simply increasing the dose is not an option due to the side effects; specific targeting to the tumor is the strategy, and the reason to use nanocarriers.

Professor Guler added: Our work aimed to contribute to liposomal drug delivery studies. Several drug delivery systems can be used as a carrier.

Dr Squires wondered: What are the major scientific barriers at the moment to drug delivery in general?

Dr Bittner responded: Thinking about the future, I could imagine very small autonomous systems that *actively* move to the point of delivery and release their drug cargo. This is no doubt that there are many unsolved problems on a rather fundamental level (material, assembly, propulsion, fuel, immunogenicity, proper size), but they might be solved.

Professor Guler added: Liposomes are normally used for the systemic delivery of drugs. Sometimes, they might enhance solubility, stability and targeted delivery of drugs.

Professor Stupp addressed Professor Guler: Liposomes have presumably not been very successful due to poor stability *in vivo*. How does incorporation of the cell-penetrating peptide amphiphile in the liposome affects the stability issue? Also, how is the peptide amphiphile incorporated in the liposome; do you expect segregation of these molecules within the bilayer membrane?

Professor Guler answered: The addition of peptide molecules decreases the stability of the liposomes compared to the bare samples. The localization of the peptides on the liposome could not have been detected in this work.

Professor van Hest commented: Two main issues regarding the general acceptance of nanomedicine are reproducibility and scalability of nanocarrier systems.

Professor Cui responded: Precisely said. When filing for investigational new drug application for clinical trials in the United States, the FDA requires that the applicant must have the manufacturing information included in the application. This information is assessed to ensure that the company can adequately produce and supply consistent batches of the drug.

Dr Bittner noted: An interesting alternative to fibres are aggregates of proteins with a well-defined mass. They can be found in virus-based nanostructures, *e.g.* bacteriophages, plant viruses, or virus-like particles. Other examples are non-virus cages, such as apoferritin.

Professor Stupp said: One of the great challenges in the field is to understand the mechanisms and pathway dependence of the self-assembly of complex biomolecules such as peptides and their derivatives in order to achieve reproducibility of the supramolecular structure. This is particularly important in therapies for which the supramolecular construct is the therapy and not simply the peptide or some other biomolecule.

Dr Squires commented: Could it be argued that it is not worth worrying about targeting if the problem of reproducibility in self-assembling systems hasn't been resolved?

Dr Kros noted: Endosomal escape is another problem in drug delivery, which needs to be addressed in order to develop efficient delivery systems.

Dr Garanger stated: When developing a low molecular weight drug, one has NMR to exactly assess the chemical structure of the active substance and all impurities of the drug can be determined and quantified. It is much more difficult to characterize drug-loaded nanocarriers with the same level of precision, since routine characterization techniques only give a global picture of the population and not of each individual in that population. This is even more true when the complexity of the systems increases (ligand-decorated nanocarriers, multiple drug-loaded nanocarriers, *etc.*).

Professor Cui responded: This is exactly right. Accurate characterization of drug-loaded nanocarriers or drug-polymer conjugates presents a big challenge to the development of effective nanomedicines due to the inherent polydispersity of these systems.

Professor Stupp added: Yes I agree with this comment. However, what will transform the field of drug delivery will be our ability to target therapies to specific tissues and organs through the bloodstream. This requires ligand-decorated nanocarriers which are obviously more complex than simple drugs. The problem is addressed to some extent through the use of molecules to construct the nanocarriers which contain the drug as part of their covalent structure.

Professor Alexander commented: Knowledge of the self-assembly processes is important in the pharmaceutical industry, but a detailed mechanistic understanding across multiple length scales is missing.

Professor Lin responded: We have been screening for peptides that can promote the aggregation of proteins *in vivo*. Indeed, mechanistically, we still have yet to come up with a general rule as to what peptides can perform such a function, although we know that hydrophobicity and amphipathicity are important. A better mechanistic understanding would allow a rational design of such

peptides, and a better understanding of protein aggregation and the associated diseases *in vivo*.

Professor Cavaco-Paulo noted: The conditions of self-assembly are important. We need to get closer to reality and think about availability to cells.

Miss Makwana commented: Although the field of self-assembly has led to the development of many sophisticated architectures, for drug delivery applications there are a few developmental barriers which need to be addressed. *In vivo* issues such as immune recognition towards self-assembling systems can prove to be a limitation. For example consider opsonization, whereby the attachment of proteins to the self-assembled system can lead to enhanced immune recognition and clearance from within the body leading to decreased therapeutic efficacy. For this reason it is important that during development we routinely perform experiments that take this into account.

Professor Stupp said: In the context of systemic drug delivery applications of self-assembled systems, one must consider what aspects of the supramolecular structure, for example shape, are critical for effective targeting not only to tumors but also generally to specific organs and parts of the vasculature. It is also important to understand how supramolecular structures change once in the bloodstream. Furthermore, different aspects of supramolecular structure are possibly important in localized tissue delivery.