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DISCUSSIONS
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General discussion

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Professor Alexander opened the discussion of the paper by Samuel Stupp: Have you considered the effects of pK_a/pH variation with the surface *vs.* buried carboxyls?

Professor Stupp replied: Generally we expect differences in pK_a for carboxyl groups present on the surface *vs.* the interior of the supramolecular nanofiber. In our earlier papers there is evidence for this effect. The strong driving force to form hydrogen bonds and side chain interactions in the so called beta domains of peptide amphiphiles responsible for fibril self-assembly should suppress the ionization of carboxyls closer to the hydrophobic core.

Professor Lecommandoux asked: How can you explain the difference in targeting properties between fibres and spheres?

Professor Stupp responded: We explain it by the large difference in surface area between filamentous and spherical nanostructures with the same characteristic diameter. Fibrils would have much higher surface area displaying the peptide sequence that we use to target the therapy relative to nanospheres. An additional effect which needs to be verified is the fact that fibrils might have longer circulation times in the bloodstream compared to spheres. There is evidence in the literature that would support this possibility.

Professor Hamley commented: I am wondering about your comments on the repair of blood vessel walls using peptide amphiphile fibrils; you mentioned that you believed that this was due to absorption of fibrils, as the same effect is not seen with spherical micelles. However, have you considered the nature of the equilibrium between fibrils and oligomers and/or monomers? Is it possible that in fact the latter species are adsorbed from fibril "depots"? Fragmentation of fibrils is a distinct possibility under flow.

Professor Stupp answered: Fragmentation of fibrils under flow is certainly a possibility and at the present time we do not have experimental access to this information. In my view even if flow-induced fragmentation of fibrils or enzymatic breakdown of fibril monomers occurs *in vivo*, I speculate that variations in shape must be preserved among aggregates of the peptide amphiphile monomers deriving from spheres *vs.* fibrils. These variations in shape must reflect their configurations in aqueous media prior to injection into the bloodstream. Those

derived from fibrils are likely to have larger surface areas containing the binding motif to collagen in the injured vessel when compared to those derived from spheres of the same diameter. Thus the difference between fibrils and spheres in their capacity to target would still be preserved in the fragmented supramolecular aggregates. The difference between both in terms of binding and therapeutic effect is very clear from the *in vivo* experiments.

Professor van Hest asked: Could you modify the persistence length of the virus-like particles, by changing PEG length or cationic charge of the leucine zipper-based oligomers that interact with dsDNA?

Professor Stupp responded: Based on our first results the persistence length of the virus-like particle could be changed through differences in the length of PEG segments. The most important phenomenon observed so far is the inability to precisely template the virus-like particle when PEG segments are insufficiently long. We never varied the cationic charge density and control of the persistence length through this approach is possible. However, I believe the critical molecular feature that controls templating is the steric effect of the PEG segments working synergistically with electrostatic binding to dsDNA.

Dr Singh asked: Was there any toxicity involved in the delivery systems presented, for example the NO delivery system?

Professor Stupp replied: We did not observe any obvious sign of toxicity in the *in vivo* model utilized to test the NO delivery. By this I refer to signs of strong inflammatory or immune response. We did follow the presence of the therapeutic system in different organs. In this regard it was important to observe that the nanostructures do not remain in the lung. Instead they accumulate in the liver and then are cleared through the kidneys.

Professor Guler addressed the general audience and Professor Stupp: The dynamics of the monomers in the self-assembled system is an interesting question. Is it possible to have monomers coming in and going our after the assembly process?

Dr Paternostre answered: I am working on reversible self-assembled systems that posses a critical assembly concentration. Through dilution, the assembly disappears by the depletion of the self-assembled system in favor of the non-assembled one. In this case, I think that yes there could be monomers coming in and out of the assemblies.

Dr Korolkov responded: I guess that would be largely down to the thermodynamics of this process. If that is an equilibrium process than such a possibility should exist. That could be true for weakly assembled systems where the molecules are held together *via* weak, non-specific interactions. Once we are dealing with strong electrostatic or multiply hydrogen-bonded assemblies that would be less possible. Another point is how quickly such exchange could happen. I guess that could be kinetically limited as the activation barrier and steric hindrance for a single molecule to leave the assembled structure could be quite high.

Professor Stupp added: I believe there is some dynamic exchange of monomers in and out of the fibrils after the assembly process. My suggestion is based on earlier experiments carried out in our laboratory using neutron scattering. However, results from these experiments indicate that the process is rather slow, and therefore not extremely significant compared to the more rapid biodegradation of peptide amphiphile molecules in physiological media.

Professor Lin enquired: Any comments on the possible strategies for disassembling amyloids?

Professor Stupp answered: This is a fascinating question that will require a deeper knowledge than presently available about the internal structure of the amyloids. This may require targeting specific sites in the supramolecular structure with ions or small molecules. An interesting example will be described by Professor Safinya later where cytoskeleton fibers are disassembled by specific ions that disrupt specific sites of electrostatic interaction between positively and negatively charged amino acids.

Professor Kinbara asked: What is the mechanism of the formation of the hierarchical structures by bundling of nanofibers? Is it likely that the nanofibers are formed first and then they assemble together to form the bundle structure?

Professor Stupp responded: In the formation of hierarchical structures, we expect that fibers are formed first and then create bundled structures as they adsorb to viscous liquid surfaces containing oppositely charged groups. This is the case in the formation of hierarchical structures between positively charged supramolecular nanofibers and the negatively charged biopolymer hyaluronic acid. This interaction creates the diffusion barrier which is critical in the formation of the hierarchical structures.

Professor Cavaco-Paulo opened the discussion of the paper by Cyrus Safinya: Did you check the stability of your structures in physiological buffers (like 9 g L^{-1} of NaCl) ?

Professor Safinya answered: The buffer that we used for these experiments consisted of PEM50, which contains 50 mM Pipes buffer, 1 mM Mg^{2+}, 1 mM EGTA, and 0.1 mM GTP. This buffer corresponds to an equivalent ionic strength of about 120 mM for a 1 : 1 electrolyte. However, in previous work (M. C. Choi *et al.*, *Biophys. J.*, 2009, **97**, 519–527) we have seen that our similarly prepared taxol-stabilized microtubule preparations are stable at least up to 300 mM added KCl (in addition to the buffer). Thus, the cationic divalent depolymerization effects we are reporting in the paper, which occur at much lower ionic strengths, are due to other effects.

Professor Cui questioned: Have you looked at how long it takes for taxol-stabilized microtubules to depolymerize in the presence of these multivalent ions? Given that Manning condensation may happen relatively quicker, would you expect to see some transient bundling upon the addition of multivalent counterions, and then the bundled microtubules starting to depolymerize gradually?

Professor Safinya replied: We have not looked at the time dependence of depolymerization of taxol-stabilized microtubules for the ions presented (Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}) in the current Faraday Discussion paper. From the experiments we know that a possible transient bundling would have to be occurring on the time scale of hours (if it happens at all). This is a very interesting question, which is worthy of further study. In a separate study using a larger multivalent ion we do indeed find a transient bundle state before depolymerization (M. A. Ojeda-Lopez, D. J. Needleman, C. Song, A. Ginsburg, Y. Li, H. P. Miller, L. Wilson, U. Raviv, M. C. Choi, C. R. Safinya, *Nature Materials*, in press).

Professor Cui said: Taxol is a very potent anticancer drug. In *in vitro* assays, the IC50 values of taxol against most cancer cell lines are of the range of several nanomolar. However, in your experiments, the taxol concentration seems to be much higher. Is that biologically relevant? Why is such a high taxol concentration is required?

Professor Safinya answered: The taxol concentrations used in our study are in the micromolar range, much larger than what you say is needed as an anti-cancer drug. Our study could have been performed at a much lower taxol concentration by probably another factor of ten. However, if one goes too low in the taxol/tubulin molar ratio then microtubules will not be stabilized in their GDP-tubulin state (the state where taxol is efficient in maintaining the microtubule structure and preventing depolymerization under most conditions). The main effect of reducing the taxol/tubulin molar ratio is to reduce the concentration at which the divalent ions depolymerize microtubules.

Dr Bittner enquired: Does the Manning condensation depend also on the freely mobile anions?

Professor Safinya answered: There is an effect of the freely mobile anions (*i.e.* due to their electrostatic interactions with all other ions including the counter-ions) but in most cases the effect may not be very large. Manning condensation (where a fraction of counterions condense on the charged biopolymer rod) occurs when the distance between unit charges on the backbone of the charged polymer rod (*i.e.* the bare length) is less than the Bjerrum length of 7.1 Å (in water with a dielectric of 80). The Bjerrum length corresponds to the distance at which the repulsive electrostatic energy between two unit charges, with the same sign, is equal to the thermal energy. Thus, the condition of condensation corresponds to the regime where the repulsive Coulomb energy between the charges on the rigid rod is reduced by more than thermal energy every time a counterion condenses on the rigid rod backbone.

Dr Bittner asked: Would van der Waals forces not become important at distances as close as those between the tubes? In which way are they included in the Manning theory?

Professor Safinya replied: Van der Waals (vdW) interactions are not included in Manning theory or theories that consider attractions between two similarly charged rods resulting from the correlation between rods due to the presence of

the cloud of fluctuating counterions surrounding the rods (*i.e.* the Manning layer of ions). The separate contribution of the vdW interactions is usually left out because it is typically smaller than the attractions arising from the non-uniform distribution of counterions condensed around the rods.

Professor Stupp wondered: Are there any details known about the state of hydration at the critical ion pair that is targeted by the cations that leads to the disassembly of the microtubules?

Professor Safinya replied: This is a very relevant question. To my knowledge there is no direct information in the case of microtubules regarding the hydration level of the ion pair (Glu53 in the M-loop of one beta subunit and Arg282 in the H1-S2 loop of the neighboring beta-tubulin subunit). What is generally believed is that positively charged amino acids (*e.g.* Arg) are less hydrated compared to negative amino acids (*e.g.* Glu). Thus, the oppositely charged amino acids are not ideally matched in their affinity for water and so the ion pair bond may be more susceptible to disruption by more hydrated divalent cations that our paper shows disassemble microtubules.

Professor van Hest opened the discussion of the paper by Jeroen Cornelissen: Is the reduction in size of the CCMV particles upon irradiation a result of denaturation or a change in assembly number?

Professor Cornelissen responded: No, denaturation is unlikely as SDS-PAGE analysis of the particles after irradition indicates intact proteins. The exact assembly number is difficult to determine.

Professor Alexander asked: Can you comment on the balance between stabilisation and the valence of the assembly?

Professor Cornelissen replied: The more interactions, the more stable would be the intuitive answer. Sodium (1+) does not stabilize, but Mg^{2+} or Ca^{2+} does. Higher valencies have not (yet) been tried.

Professor Alexander queried: Could you co-add lysine to help stabilise the structure?

Professor Cornelissen responded: Good suggestion, we have not tried that. Any cross linking might add to the stabilization.

Professor Cavaco-Paulo asked: Do molecules leak out?

Professor Cornelissen replied: Yes, if the particles are loaded with small molecules.

Professor Cavaco-Paulo further asked: Is the protein degradable?

Professor Cornelissen responded: Yes, it is! It looses its N-terminus quite quickly upon standing when disassembled. The virus, on the other hand, is very

stable. Empty capsids are also stable, but some degradation will occur over the course of weeks.

Professor Cavaco-Paulo asked: What is the fate of the capsules inside cells?

Professor Cornelissen answered: We don't know.

Dr Nieuwland commented: In figure 5 (p7) of the paper, DLS data are shown for the vesicles in presence and absence of Mg^{2+} ions. Even before irradiation, a size difference seems to be present. My question is therefore: how far is the structure of the CCMV virus changed by the presence of Mg^{2+} ions (without irradition)?

Professor Cornelissen responded: We do not observe this difference in the TEM, so it might be an effect of surface charge. On the other hand the Mg makes the virus-like particle more robust and in that way reduces the dynamics. This is a similar effect as observed when the virus capsid is empty compared to a capsid loaded with a polyelectrolyte. In the latter case the size and dynamics are also slightly different as we have shown by a combination of DLS and SANS. This is published in *Soft Matter*.[1]

1 M. Comellas-Aragonès, F. D. Sikkema, G. Delaittre, A. E. Terry, S. M. King, D. Visser, R. K. Heenan, R. J. M. Nolte, J. J. L. M. Cornelissen and M. C. Feiters, *Soft Matter*, 2011, **7**, 11380–11391.

Dr Lobaskin asked: What are the molecular weight and size limits for the permeability of the capsules?

Professor Cornelissen answered: The pore sizes are ~2 nm, we have seen molecules with an estimated size in that range (*e.g.* phthalocyanines) enter the capsule. Charged, flexible polymers of high molecular weight slowly diffuse in, but colloidal particles of >7 nm do not (smaller colloidal particles have not been tested)

Mr Hernandez-Garcia enquired: Have you used Circular Dichroism spectroscopy to probe if the viral coat protein is completely folded when self-assembled into the viral particle?

Professor Cornelissen responded: CD spectroscopy has been used to monitor the dis-assembly/assembly pathway, but since it only provides information on the local folding and hardly on the capsid structure we have not carried out more detailed studies.

Professor Kinbara asked: Is the shrinkage of SIP-CCMV reversible? Namely, does the shrunken particle swell again by addition of Mg^{2+}?

Professor Cornelissen answered: No they don't! It's likely that it is a kinetically trapped structure.

Professor Stupp opened the discussion of the paper by Carlos Alemán: Could you elaborate further on all the different algorithms that you use to carry out

simulations? In particular can you clarify the level of atomistic detail that is included in the simulations?

Professor Alemán answered: We used molecular dynamics algorithms with an explicit description of the whole chemical system. In other words: all atoms of the conjugate assembly (including both the cyclic peptides and the polymer blocks) were explicitly included; solvent molecules and counter-anions to neutralize the peptide charge were also explicitly described (one-by-one); and the mica surface was also explicitly included. The latter is perhaps quite interesting since many times inorganic and metallic substrates/surfaces have been described at the physical level only using a simple hard-potential. In this case, we included a chemical description, the mica substrate being constructed with explicit consideration of all atoms using the corresponding crystallographic structure.

Dr Nieuwland remarked: The authors take the ring systems as explored by Biesalski and Ghadiri as the starting point for polymerization on self-assembled peptide systems. However, the paper of Couet and Biesalski in *Macromolecules* (J. Couet and M. Biesalski, *Macromolecules* 2006, **39**, 7258–7268) shows that these systems fall apart when a polymer is attached to them. Is something similar observed in the simulations, and if not, why not?

Professor Alemán responded: As you rightly stated the peptide nanotube is the same in Couet and Biesalski (*Macromolecules*, 2006, **39**, 7256-7268) as in the paper by Ten Cate and coworkers (*Macromolecules*, 2006, **39**, 7831–7838). However, the polymers are completely different not only from a chemical point of view but also from a physical point of view. More specifically, the polymer of Ten Cate *et al.* is low molecular weight and monodisperse (*i.e.* it was synthesized with chemical precision) while that of Couet and Biesalski is high molecular weight and polydisperse. Furthermore, the former authors used a polyacrylate while the latter prepared a polyacrylamide. We did not observe any disruption in the case of the studied system (that of Ten Cate and co-workers). However, if simulations with the system of Couet and Biesalski are done they should lead to a full agreement with experimental results. Unfortunately, it is not easy to predict *a priori* if such disruption is mainly provoked by amide groups (competing interactions), molecular weight, polydispersity, or a combination of factors. One of current utilities of computer simulations is that they provide understanding of these features with reasonable/high accuracy.

Professor van Hest asked: What will be the effect on peptide assembly when the polybutylacrylate length is increased?

Professor Alemán replied: The studied systems are clearly dominated by the interaction between polymer blocks and the mica surface, which indeed is accurately described at the atomic level, rather than by the intermolecular interactions between peptide units. This effect is not expected to change with increasing length. However, what I want to note is the importance of the procedure used in the model for the deposition of the assembly on the surface, which should mimic as closely as possible the experimental procedure. Thus, the deposition procedure clearly affects the structure of the system. In this specific

system polymer blocks can organize as extended chains on the mica surface or can be wrapping the peptide assembly, depending on how the deposition and desolvation processes are carried out.

Professor Mezzenga enquired: Have you tried to change the solvent quality in your simulations? Does this affect the breaking of the fibrils?

Professor Alemán responded: Simulations in solution have been carried out using two different solvents chloroform and *N*,*N*-dimethylformamide, which were those used for the experimental measurements. These solvents present very different properties (*e.g.* the dielectric constant is 4.7 and 37, respectively) and abilities to interact with the nanotube. The parallel assembly is unstable in solution because of unsuitable intermolecular interactions between the side groups of the amino acids contained in adjacent peptide cycles. This behavior is independent of the solvent, as observed experimentally. So, the only possible assembly in solution is the anti-parallel one because avoids unfavorable interactions between adjacent cyclopeptides.

Professor Mezzenga asked: Is the breaking of the fibrils caused by the side chains' loss of entropy or by some other effect?

Professor Alemán replied: There is no entropic effect at this point. Simply, in the parallel arrangement the Lys side groups of neighboring peptides confront one another, producing electrostatic repulsions. In order to alleviate such unfavorable interactions, the Lys side chains adopt different conformations, provoking in some case the rupture of backbone–backbone intermolecular hydrogen bonds (*i.e.* breaking the nanotube). In contrast, the anti-parallel assembly favors the formation very stable cooperative assemblies because of the attractive interactions between the Gln and Lys side chains of neighboring peptides. Thus, these residues are arranged alternately facilitating the formation of side chain–side chain electrostatic interactions.

It is relatively easy to deduce the anti-parallel assembly from the experimental results. However, modeling helps to explain the physical association to the stability and instability of the anti-parallel and parallel assemblies, respectively.

Professor Mezzenga asked further: Does the stability of the system change with the molecular weight of the side chain?

Professor Alemán responded: Actually we did not study assemblies with polymer blocks higher than those used for the experiments (simulations were carried out using the experimental molecular weights – *i.e.* the blocks were monodisperse). What can be anticipated is that a small increment of the molecular weight will not affect the stability. However, a drastic increase may induce a change in the balance of the interactions, which in turn may affect the stability of the assemblies.

Dr Surin asked: How did you take into account the ions naturally present at the surface of mica ? What is the effect of those ions on the supramolecular

organization of the polymer chains on the surface ? Is there any effect of the symmetry of mica on the assembly ?

Professor Alemán answered: We used a mica K1.0[Si3Al1O8][Al2O2(OH)2] (dioctahedral phyllosilicate) surface that included all atoms explicitly (an all-atom model). More specifically, the mica super-cell was constructed to adapt the unit cell of mica to the dimensions of the simulation box. An accurate force-field was specifically developed for mica by Heinz *et al.* (*Chem. Mater*, 2005, **17**, 5658–5669).

The effect of the ions on the assembly of the cyclopeptides is relatively small. For example, the circularity of the cyclopeptide was not altered while the distance between adjacent cycles decreased 0.15 Å only. In contrast, mica has a drastic effect on the polymer blocks' organization. Indeed, the stability of the whole system is dominated by mica–polymer block interactions rather by mica–cyclopeptide or cyclopeptide–cyclopeptide interactions.

Regarding the effect of the symmetry of mica on the assembly, honestly we do not know since all models were built using the same surface. I should say that this is a very interesting point widely studied for the adsorption of ions onto metallic surfaces but scarcely studied for the adsorption of organic macromolecules/assemblies onto inorganic surfaces. In spite of such scarcity, a few results (for example for adsorption of collagen onto hydroxyapatite) suggest that the surface plane plays, in some cases, an important role. However, this importance is lower than in metals where the disposition of active adsorption sites provokes important geometric restrictions. In any case, this is a topic that I would like to investigate in the near future.

Dr Kros wondered: Did you consider using hybrid models to study your system?

Professor Alemán replied: Not for this specific system since our objectives were fully reached using atomistic classical simulations. However, in previous works we used different kinds of hybrid models. For example, quantum mechanics/molecular mechanics (QM/MM) models were used to study charge transfer processes in tubular nanostructures constructed using protein building blocks (F. Rodríguez-Ropero, D. Zanuy, X. Assfeld and C. Alemán, *Biomacromolecules*, 2009, **10**, 2338) and helical peptides tethered to a gold surface (D. E. López-Pérez, G. Revilla-López, D. Jacquemin, D. Zanuy, B. Palys, S. Sek, and C. Alemán, *Phys. Chem. Chem. Phys.*, 2012, **14**, 10332); and coarse grain/MM (GC/MM) models were used to study large nanotubular assemblies (D. Curcó, R. Nussinov and C. Alemán, *J. Phys. Chem. B*, 2007, **111**, 10538 and D. Curcó, R. Nussinov and C. Alemán, *J. Phys. Chem. B*, 2007, **111**, 14006).

Professor Cavaco-Paulo asked: Can you model peptides for transdermal delivery?

Professor Alemán responded: I am assuming this is using atomistic modeling, isn't it? I cannot say if we can without knowing about the focus of the modeling: peptide structure, mechanism of delivery, role of water, interaction with a receptor after delivery, *etc.*?

Dr Kros opened the discussion of the paper by Elisabeth Garanger: How can the particles escape from the endosome? Can you comment on the stability of the polymer block in the endosome?

Dr Garanger answered: Tat-*b*-PTMC nanoparticles contain *a priori* no "signal" encoded in the Tat segment to drive their escape from endosomes as intact particles. However, it is very likely that in the "harsh" conditions of endosomes (acidic pH, high enzymatic activity), the PTMC block will be digested and thus the nanoparticles will fall apart. Or they will be excreted in endosomal vesicles as efficiently as they entered the cytoplasm, but we need to perform additional experiments to understand the fate of the Tat-*b*-PTMC nanoparticles.

Professor Hamley asked: I am wondering if you could explain more about the choice of PMTC as the polymer; more particularly what is its glass transition temperature, and does this affect micelle dynamics, or might you have "frozen" micelles?

Dr Garanger answered: Poly(trimethylene carbonate) (PTMC) was chosen as a biocompatible, biodegradable, low T_g polymer less prone to hydrolytic degradation as compared to polyesters such as PLA.[1] Our group has long-term experience using PTMC as a hydrophobic block in amphiphilic block copolymers forming well-defined nanoparticles by spontaneous self-assembly.[2,3] The glass transition (T_g) of PTMC segments in the Tat-*b*-PTMC chimera studied here is around -20°C (-19°C for Tat-*b*-PTMC66 and -24°C for Tat-*b*-PTMC30).[4] The micelles are thus formed, conserved and used under conditions allowing chain mobility, which is in favor of dynamic micelles and not "frozen" micelles. Regarding the semi-crystalline character of PTMC however, the debate is open. As described in the literature, homoPTMC segments used in our study are semi-crystalline with a T_m of around 30–40°C. However, we did not observe any exothermic crystallization peak (or endothermic melting transition) in the DSC thermograms of Tat-*b*-PTMC chimeras either in bulk or self-assembled into micelles in solution. It is, however, tricky to decide unambiguously on its amorphous or semi-crystalline character since the crystallization kinetics are very slow. We also tried AFM imaging to determine the status of the softness or rigidity of the micelle cores with no clear answer so far.

1 Z. Zhang, R. Kuijer, S. K. Bulstra, D. W. Grijpma, J. Feijen, *Biomaterials*, 2006, **27**, 1741–1748.
2 C. Sanson, J.-F. Le Meins, C. Schatz, A. Soum, S. Lecommandoux, *Soft Matter*, 2010, **6**, 1722–1730.
3 C. Sanson, C. Schatz, J.-F. Le Meins, A. Brulet, A. Soum, S. Lecommandoux, *Langmuir*, 2010, **26**, 2751–2760.
4 C. Drappier *et al.*, *Polym. Chem.*, 2013, **4**(6) 2011–2018.

Professor Hamley commented: Following on from my previous question, have you considered any molecular labelling technique to examine possible micelle exchange dynamics?

Dr Garanger answered: I cannot think of an "easy" molecular labelling technique to examine possible micelle exchange dynamics. Conjugating a dye or a

quencher at the chain end of the PTMC block, we could possibly measure the percentage of fluorescence of each micelle population individually and over time when mixed. But in our experience, self-assembly of the Tat-*b*-PTMC chimeras proved very sensitive to any changes of the chemical structures. This is still something that we will consider in the future.

Dr Paternostre asked: Did you measure the critical micellar concentrations of your compounds? What was the rationale behind using a cell penetrating peptide in your TAT-PTMS chimiras?

Dr Garanger responded: Our initial goal was to use a biofunctional peptide as the hydrophilic block as an alternative to a polypeptide segment traditionally used in our group in the design of amphiphilic polypeptide-based block copolymers. As ring-opening polymerizations of α-amino acids, N-carboxyanhydrides afford an intrinsic limited level of control in terms of macromolecular composition, and the use of a peptide obtained by solid-phase peptide synthesis would provide a bioactive hydrophilic block perfectly defined in terms of primary sequence and length. Tat47-57 was chosen as a model peptide with well-established bioactivity, namely cell-penetrating properties, so that the resulting self-assembled structures would present cell internalization properties that can relatively easily be studied *in vitro* using model cell lines and well-established protocols. Since we ultimately aim at correlating macromolecular structures with resulting self-assembled morphologies and their biological activity, it is crucial to access polymer materials with the highest possible precision. This also explains why we used Tat peptide as the full hydrophilic block instead of a polyethylene glycol-Tat conjugate.

Professor Alexander enquired: Is the clustering related to Tat percentage; are the particles changed in the serum, and how does this affect uptake?

Dr Garanger responded: Tat-*b*-PTMC nanoparticles were found to be stable for a couple of hours when diluted in serum but start to aggregate after 5 h only. At that time however, all of them have already been internalized into the cells since the internalization process occurs within less than 30 min. Of course, serum proteins can adsorb at the micelle surface and have an effect on the internalization pathway and procedure. An analysis of the protein corona potentially formed has not been performed so far.

Professor Cui said: The peptide-polymer molecular chimeras you described are very similar to the "crew-cut" polymeric micelles developed by the Eisenberg lab in the 1990s. In the "crew-cut" system, where typically a very long polystyrene block is conjugated to a very short polyacrylic acid block, the Eisenberg lab were able to access a wide range of micellar shapes from spheres, to cylinders and vesicles. In your current peptide-polymer system, you mentioned that due to the strong electrostatic interactions among the Tat segment, only spherical objects can be observed. I am wondering if this might be due to the fact that in your system the polymer block is too short to have access to cylindrical or vesicular morphologies?

Dr Garanger replied: We initially chose the different PTMC block lengths to obtain hydrophilic weight fractions in the chimeras from 20 to 40% in an attempt to access different morphologies.[1] However, we have observed the formation of spherical micelles solely for all chimeras and we assume now that this is indeed due to the relatively short polymer segments. Also considering the highly charged Tat segment, we may need to synthesize much longer PTMCs (typically DP 100–200) to possibly start observing other morphologies with lower curvatures such as worm-like micelles and vesicles.

1 D. E. Discher and A. Eisenberg, *Science*, 2002, **297**, 967–973.

Professor Cui remarked: In your system, self-assembly was promoted by directly dissolving the polymers into water. This suggests that the resulting solution could contain a mixture of both the unassembled molecules (unimer) and micelles. In your cell experiments to evaluate the effect of micellar size on the cellular uptake, how do you know it is the micelles not the unassembled molecules that actually get into cells?

Dr Garanger replied: Considering amphiphilic molecules, an equilibrium between unimers and micelles is indeed expected, especially for concentrations close to the CMC. For amphiphilic copolymers, the CMC is very low and the association is generally described using a model of closed association. In these conditions, the presence of unimers is not expected. Together with the different techniques that we have now used to characterize or study our micelles (AFM, TEM, DLS, SANS, SAXS and different biophysical studies), this leaves no doubt about the self-assemblies and the absence of remaining unassembled unimers.

Miss Nogueira asked: Why do you use PEGylated nanoparticles as the negative control in the cellular uptake studies?

Dr Garanger replied: We wanted to address the point of the bioactivity of the Tat peptide when attached though its C-terminal end to a PTMC polymer block and when presented on the surface of Tat-*b*-PTMC core–shell nanoparticles. We thus needed control nanoparticles devoid of Tat segments but almost identical in all other characteristics. Nanoparticles obtained from the amphiphilic block copolymer PEG-*b*-PTMC happened to have these features: same hydrophobic PTMC block, same morphology obtained by spontaneous self-assembly, similar size, identical amount of labeling, but with a stealth PEG segment (with no cell transduction properties described so far!).

Professor van Hest said: What is the effect of PEGylation on the availability of the Tat peptides? Would it be interesting to couple the Tat peptide to the PEG chain end?

Dr Garanger replied: The poly(ethylene glycol) segment of the PEG-*b*-PTMC block copolymer used to access nanoparticles with lower Tat content was chosen to have a molecular weight similar to the one of the peptide (~1700 g mol^{-1}) to obtain amphiphiles (Tat-*b*-PTMC and PEG-*b*-PTMC) of similar hydrophilic weight fractions. A 2,000 MW PEG was thus used. However the total length of this PEG

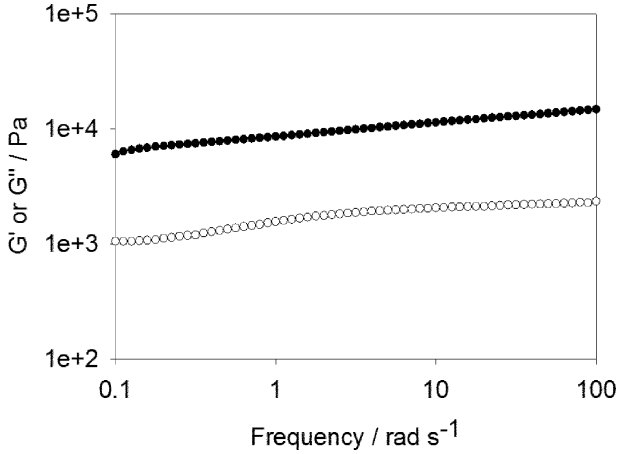

Fig. 1 Example frequency sweep data for a gel prepared at 20 °C.

block, consisting of around 45 (-*O*-CH_2-CH_2) repeats, is much longer than the length of the peptide roughly made of 13 (-NH-CH(R)-CO-) repeats (assuming the PEG and Tat are in fully extended conformations). We can then suspect a potential shielding of the bioactive peptide by the hydrophilic polymer resulting in an apparent decreased bioactivity of the resulting nanoparticles and explaining why, in our *in vitro* experiments, the higher the Tat density, the more efficiently the particles bind to biological membranes. We are currently performing additional experiments to address this point. The second part of your question has already been addressed in my response to Dr Paternostre.

Miss Makwana remarked: Critical micelle concentration (CMC) studies allow you to determine the exact concentration at which self-assembly takes place. In order for characterization studies to show accurate results, you must work above the CMC. Not having performed this experiment, how confidently can you suggest that the features are you seeing in the AFM images are actually micelles?

Dr Paternostre added the question: Did you measure the critical assembly concentration of the TAT-PTMC chimeras?

Dr Garanger responded: CMCs of amphiphilic block copolymers are usually very low (as compared to the ones of low molecular weight amphiphiles such as surfactants). It was impossible to determine the exact CMC of our Tat-*b*-PTMC chimeras by dynamic light scattering, because the dilution we need to apply to reach the CMC is too large to measure a significant count rate before and immediately after the CMC. Of course we could have performed surface tension measurements but we did not! Also, regarding the application we target, as well as the effective amount of materials we are able to produce, we have not studied the phase diagram over the whole concentration scale. We, however, have now a sufficient amount of data obtained using different techniques (DLS, SLS, SANS, SAXS) to feel confident about the morphologies we observed on AFM (or TEM) images.

Dr Nieuwland opened the discussion of the paper by Dave J. Adams: The absence of an influence of kinetics on the final structure of the dipeptide hydrogels is explained by the authors by assuming a two-step process - first the formation of fibrils, then gel formation. Could a highly dynamic system in a steady state be an alternative explanation?

Dr Adams answered: This is an interesting point. In theory, you are right that this could also be caused by a highly dynamic system. However, we have no evidence for this. Our gelators are very hydrophobic below their pK_a. As such, I would expect that the solubility is very low and so it is unlikely to be in a highly dynamic state. We have tried to probe the concentration of gelator that is 'dissolved' or mobile in the gel state in a number of ways, including NMR. This shows an absence of detectable gelator, implying that the equilibrium is very far over to the gel fibre state. However, you are correct that this is a possible alternative explanation that we cannot fully rule out at present.

Dr Bittner asked: Can you measure G′ and G″ as a function of frequency, and if yes, what are the results and interpretations?

Dr Adams replied: Yes, we can do so and indeed routinely do so. For example, here is an example frequency sweep for a gel prepared at 20 °C (Fig. 1). This is typical of our gels (and those of similar gels in the literature), where G′ and G″ are only weakly dependent on frequency.

Dr Bittner commented: Drying (in vacuum for SEM) might influence the fibre morphology. Environmental SEM can help to characterise the fibre during drying (or during rehydration).

Dr Adams replied: I would say that I suspect that any drying has a strong potential for influencing fibre morphology. We have certainly seen that drying can induce crystallisation (K. A. Houton, K. L. Morris, L. Chen, M. Schmidtmann, J. T. A. Jones, L. C. Serpell, G. O. Lloyd and D. J. Adams, *Langmuir*, 2012, **28**, 9797–9806). We try to avoid this wherever possible. Also, we tend to observe discrepancies between fibre widths as measured by EM and those measured by SANS (*e.g.* L. Chen, J. Raeburn, S. Sutton, D. G. Spiller, J. Williams, J. S. Sharp, P. C. Griffiths, R. K. Heenan, S. M. King, A. Paul, S. Furzeland, D. Atkins and D. J. Adams, *Soft Matter*, 2011, **7**, 9721–9727), even when using cryo-TEM, where drying should not be an issue. I think that this is an important point that is often overlooked in this area. In general, we rely heavily now on confocal microscopy, since this can be used to probe the gel state when wet and with minimal sample preparation. This does not allow imaging of individual fibres in detail, but our data suggests that the mechanical properties of the gels is more related to the microstructure as opposed to the nanostructure.

Dr Korolkov asked: Could you please comment on the mechanism of viscoelasticity in your gels? Can you explain the formation of gel in the low molecular weight system?

Dr Adams replied: In general, at high pH, we have a system of dispersed gelator as some kind of micellar aggregate. For our systems, when we lower the pH, fibres

are formed. These then crosslink in some way, perhaps by the entanglement of fibres or perhaps by fibres' wrapping' around one another. These physical cross-links lead to the formation of the gel. The properties of the gels arise from the fibres themselves (persistence lengths and mechanical properties), the number and type of cross-links, and how the fibres and cross-links are arranged in space.

These gels differ from polymer gels in that they tend to break at low strain (so for example gentle agitation with a spatula will tend to break the network); they tend to be quite rigid and re-healing after agitation seems to require a specific microstructure (see for example L. Chen, J. Raeburn, S. Sutton, D.G. Spiller, J. Williams, J.S. Sharp, P.C. Griffiths, R.K. Heenan, S.M. King, A. Paul, S. Furzeland, D. Atkins and D.J. Adams, *Soft Matter*, 2011, **7**, 9721–9727 and work by Pochan). We have previously shown (D.J. Adams, L.M. Mullen, M. Berta L. Chen and W.J. Frith, *Soft Matter*, 2010, **6**, 1971–1980) that slow compression results in the z dimension being reduced, but not the x or y dimensions, which is very different to many polymer gels. When the force is removed, the gels do not recover their original shape.

Dr Korolkov said: What about comparing your systems with polymer gels? Do they return to their original state under pressure?

Dr Adams responded: No, these gels do not return to their original states when force is removed. These gels behave very differently to polymer gels.

Professor Alexander enquired: Can you use ammonia to change the pH in a controllable way – *i.e.* to go steadily back through the pH series to control/modify gel structure?

Dr Adams replied: Certainly ammonia has been used as a gaseous means of adjusting the pH in the past by Stupp *et al.* I would expect that we would be able to utilise this also adjust the pH, but we have not attempted to do so.

Professor van Hest asked: Would it be possible to change the concentration of CO_2 in order to get better control over the gelation process?

Dr Adams answered: Yes, this is indeed possible. We can change the thickness of the membranes by varying the time over which we expose the solution to CO_2 (at a fixed CO_2 pressure) or by varying the pressure of CO_2 we use. We will report on these finer details in the near future.

Professor van Hest wondered: Could you use the gel produced at the interface to direct the gelation process in bulk?

Dr Adams responded: This is an interesting question. We may be able to gel the bulk after membrane formation by adding an acidic trigger to the lower solution. We do not know yet whether this would direct the bulk gelation process in any way, but that's a very exciting idea.

Dr Saiani remarked: The mechanical properties of hydrogels depend on the intrinsic properties of the fibers that form the network as well as on the properties

of the network itself which are dependent on how the fibers associate/entangle. How does the topology of the network formed affect the mechanical properties of your hydrogels?

Dr Adams replied: The network strongly affects the mechanical properties of the gels. In fact, I would suggest that this is most important for the mechanical properties and that examining fibres using EM tells you little about the properties of the gels. We have previously shown that the microstructure of the networks determines whether a gel recovers after shear (L. Chen, J. Raeburn, S. Sutton, D.G. Spiller, J. Williams, J.S. Sharp, P.C. Griffiths, R.K. Heenan, S.M. King, A. Paul, S. Furzeland, D. Atkins and D.J. Adams, *Soft Matter*, 2011, **7**, 9721–9727) for example, with gels which contain spherulitic domains of fibres recovering well after shear, whilst those which have a more uniform fibre network do not. This seems to be a factor that is independent of the absolute G′ and G″. Similar results have been shown for an unrelated gelator by Pochan's group.

Professor Stupp commented: It is not clear how such small molecules as the dipeptides are arranged to form the much larger fibrous units observed in the hydrogels.

Dr Adams replied: This is true. A model has been suggested for one dipeptide gelator by Ulijn's group (A. M. Smith, R. J. Williams, C. Tang, P. Coppo, R. F. Collins, M. L. Turner, A. Saiani, R. V. Ulijn, *Adv. Mater.*, 2008, **20**, 37–41), but whether this model is appropriate for our naphthalene-based gelators is not clear. We have significant data for one example (L. Chen, K. Morris, A. Laybourn, D. Elias, M. R. Hicks, A. Rodger, L. Serpell and D. J. Adams, *Langmuir*, 2010, **26**, 5232–5242), where we were able for example to determine the angle of the naphthalene ring to some degree from the CD and LD data. However, we do not have a specific model as yet. In the literature, the crystal structures are often used to suggest a model. However, for our systems, we find that the data from the crystal structures does not agree with data collected *via* fibre diffraction in the gel state (see for example K. A. Houton, K. L. Morris, L. Chen, M. Schmidtmann, J. T. A. Jones, L. C. Serpell, G. O. Lloyd and D. J. Adams, *Langmuir*, 2012, **28**, 9797–9806). This seems to be true for all examples for which we have both crystal structures and fibre diffraction from the gel state (mostly unpublished). However, we are continuing to try and determine the packing from the fibre diffraction patterns.

Dr Squires asked: Have you tried diluting the material keeping the pH constant, to see whether the modulus decreases, as a method of differentiating between entanglement, or some chemical or physical cross-link, as the basis for the observed gelation?

Dr Adams responded: Yes, we have done this in the past on gels that are closely related to those described here, with the pH changed using GdL. If we add water to the gel at the same pH as the gel, the gel does not swell or change form at all, implying a rigid network consisting of physical cross-links as opposed to entanglement. Of course, if we add high pH water, the gel network will slowly dissolve.

Dr Kasotakis remarked: Did you try chemical fixation of the samples before the visualization in TEM?

Dr Adams replied: No, we have not tried to do this. It is difficult to do for our systems. Fixing using a chemical reagent is difficult as it would require that the gelator has a specific functional group on it. Small changes in the chemical structure strongly affect the molecules' ability to form gels, so we have not invested time in trying to find a molecule that forms a gel that could be fixed prior to EM, although this is an interesting idea. Embedding in resin and sectioning might potentially work, although we have not tried this.

Dr Paternostre asked: Did you have any difficulties measuring the pH within a gel using a pH electrode, and what type of electrode did you use?

Dr Adams responded: No, we do not have issues. We use a pH probe that is designed for gel-like materials. Specifically, we use a FC200 pH probe (from HANNA instruments) with a (6 mm x 10 mm) conical tip for all the pH measurements.

Professor van Hest returned to the paper by Carlos Alemán: Can both types of assemblies (Elisabeth Garanger's micelles and Dave Adams' gels) be modeled following an atomistic approach?

Professor Alemán answered: Yes. Dr Garanger's system would probably require a mixed model combining an atomistic description with hard coarse-graining (that is nable to retain some chemical information). Regarding Dr Adams' system, simulations at an atomistic level should not be a problem, at least apparently. Indeed, many of the systems were within scales that could be rightly simulated at present time. For example, the organization of your own system in paper 20 (PA), is relatively similar to that of comb-like polypeptides, which we modeled a decade ago (D. Curco, D. Zanuy, C. Alemán, E. Rude and S. Muñoz-Guerra, *Biomacromolecules*, 2003, **4**, 87 and S. Muñoz-Guerra, F. López-Carrasquero, C. Alemán, M. Morillo, V. Castelletto, and I. Hamley, *Adv. Mat.*, 2002, **14**, 203-205). Indeed, at that time simulation of phase transitions associated to the heating of carbon tails was possible (D. Zanuy, A. M. Namba1, S. León, C. Alemán, S. Muñoz-Guerra, *Polymer*, 2001, **42**, 281 and D. Zanuy, C. Alemán, M. Laso and S. Muñoz-Guerra, *J. Comput. Chem.*, 2003, **24**, 770). The dimensions of your PA system are slightly larger than those of comb-like polypeptides but the speed and scalability of computer processes has increased enormously in the last ten years. In summary, the scale-length of many of the discussed systems is suitable for proper computer simulations.

Dr Garanger asked Professor Alemán: Would it be feasible (in a decent amount of time!) to use molecular modeling to predict how different macromolecular chains together interact and ultimately if their self-assembly would lead to homogeneous or heterogeneous nanoparticles (since this is a difficult point to address experimentally)?

Professor Alemán responded: Yes, interactions between macromolecules in aggregates/assemblies can be simulated within a reasonable time using reliable length scales. Therefore, a description of the homogeneity or heterogeneity for particles of nanometric dimensions (<100 nm) is then feasible and almost direct. For submicrometric particles (100–500 nm), heterogeneous or homogeneous distributions are usually described by extrapolating the results obtained at the scale of tenths of nm. This is usually done by applying appropriate algorithms when the analysis of the results is carried out. Indeed, your question is directly related with the fact that, at the present time, atomistic computer simulations are complementary to other nanometric techniques, providing qualitative or quantitative (depending on the case) information that cannot reached experimentally.

Dr Garanger further asked: If attempting to model our Tat-*b*-PTMC macromolecular chimeras, do we have to and is it possible to take into account all associated solvent (water) molecules, since we calculated from SANS experiments around 150 water molecules per chain in the self-assemblies (*i.e.* 4 layers of water)?

Professor Alemán replied: Indeed, it must be done in the way that you propose since water molecules are expected to play a crucial role in your system. Therefore, interactions between water molecules and the molecular chain (especially the Tat block) must be explicitly described in the system.

Dr Saiani commented: The role played by water in the self-assembly of peptides is still poorly understood. How much could simulation assist us in understanding the role played by water molecules?

Professor Alemán answered: The application of atomistic simulation methods for understanding of the thermodynamic and kinetic role of water in the self-assembly of highly-ordered peptides is actually feasible at the present time. This is because of a combination of different factors, such as the architecture of the computers, the efficiency of computer codes in terms of scalability, and mostly importantly the development of mixed methods that allow the creation of specific models for specific investigations. Simulations can provide microscopic insights into the indirect/direct effects of the water structure on the assembly, for example its role in the "lubrication" of the assembly, its role as a driving force of the hydrophobic collapse, participation of the peptide–water interactions in the whole assembly's stability, and many others.

Professor Hamley remarked: I would like to comment that the body of work done on water structure around antifreeze proteins, which has been studied in detail, may be relevant to understanding its role in peptide self-assembly, *e.g.* of amyloids.

Professor Alemán responded: Actually, I fully agree with you. This is an important point to consider.

Professor Stupp commented: The water structure around peptides, proteins, and other biopolymers is exceedingly complex, and therefore water cannot be

viewed as a solvent in self-assembling biomolecules. In the specific case of peptides discussed extensively at this conference, interactions with water are especially important and water needs to be regarded as part of their structure. The amount of water that is affected by the peptide will be particularly sensitive to the presence of charged amino acids and end groups and one may expect long range effects. The field needs to seriously consider integrating the techniques that are already known to characterize water structure and dynamics. One example is dynamic nuclear polarization enhanced NMR (DNP).