Bioactive molecular sheets from self-assembly of polymerizable peptides†

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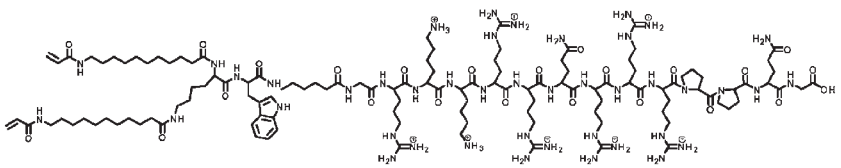
We have demonstrated that polymerizable peptides self-assemble into a unique sheet-like 2D structure in bulk solution that can be covalently fixed to produce 2D molecular objects which were shown to be efficient at delivering cargos into living cells and are nearly nontoxic in contrast to non-polymerized nanostructures.

Self-assembly of biomolecular building blocks plays an important role in the discovery of new biomaterials and scaffolds, with a wide range of applications in nanotechnology and medical technologies.[1–4] Especially, coating nanostructures with peptides endows them with unique opportunities to explore the vast biological events that peptides mediate.[5–11] In recent years, a few research groups have reported a 2D sheet structure formed by peptide self-assembly.[12] Because the peptide 2D sheet structure has some implications for biology and for biomaterial research, such as slow-diffusion drug delivery systems and artificial skin, this research field possesses great potential. Recently, we reported biomolecular building blocks consisting of the Tat cell penetrating peptide (Tat CPP) and a hydrophobic lipid dendrimer.[13] These peptide building blocks self-assembled into well-defined nanostructures while showing highly different cytotoxicity profiles according to the nano-aggregate stability, which, as a rule of thumb, is proportional to the critical micelle concentration (CMC). Unstable aggregates might disassemble into their individual amphiphilic components during the cell entry process, which can perturb and/or disrupt the amphiphilic cell membrane. Hence, one can envision that the covalent capture of the individual components could be one of the strategies to overcome the intrinsic instability of some useful nanostructures.[14]

In this communication, we present the unique self-organization of polymerizable peptide-based amphiphilic building blocks into a sheet-like 2D structure in bulk solution and the covalent capture of the preformed bioactive nanostructure (Fig. 1). Here, we synthesized peptide-based amphiphilic supramolecular building blocks consisting of a cell-penetrating peptide and a dibranched alkyl chain. As hydrophilic blocks, a Tat cell-penetrating peptide (Tat CPP) and oligo-arginine (R9) were employed in **1** and **2**, respectively. Both Tat CPP and R9 blocks were designed to have a similar volume fraction. Regarding the hydrophobic block, polymerizable acryl amide groups were incorporated at the distal part of hydrophobic alkyl chains to covalently capture the preformed nanostructures after polymerization.

The aggregation behavior of the peptide building blocks in aqueous solution was first investigated by using CMC measurements following encapsulation of the fluorescent dye Nile Red. The calculated CMC for **1** was approximately 150 μM, while that of **2** was much higher than 300 μM. As most cell-based delivery experiments are performed at much lower concentrations (at best 10–20 μM), the CMCs of both building blocks are too high to use in bio-applications. Moreover, if the nanostructures are used at concentrations higher than their CMCs to maintain the aggregated state, there arises a cytotoxicity issue.[13,15] Therefore, one way to overcome this issue is to cross-link self-assembled building blocks within the nanostructure, making aggregates as molecular objects.[16]

Transmission electron microscopy (TEM) investigations of the nanostructures revealed that **1** self-assembles into 2D sheets (Fig. 1a), while **2** self-assembles into spherical aggregates (Fig. S5†). Atomic force microscopy (AFM) images of **1** showed that the thickness of the sheets is ~7 nm, indicative of an interdigitated bilayer (Fig. 1b and c). According to classical theory of amphiphiles, the morphology of amphiphiles is dictated by the relative volume fraction of hydrophilic and hydrophobic blocks. Although both building blocks were designed to have a similar volume fraction, it is



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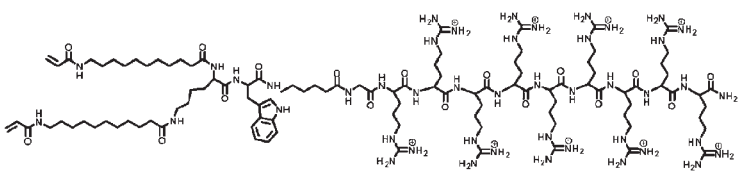

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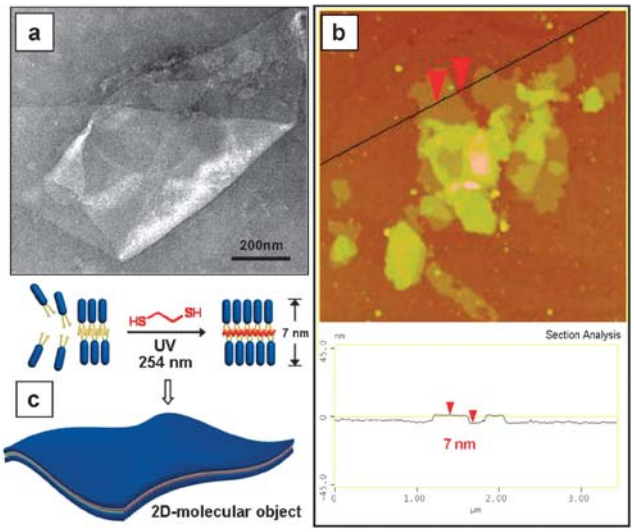

Fig. 1 (a) TEM image of self-assembled sheets of **1**. (b) AFM image of self-assembled sheets of **1** with height information. (c) Schematic representation of the confined polymerization of 2D peptide sheets.

likely that their self-assembly behaviors are quite different from those of conventional non-ionic amphiphiles as they are composed of highly charged residues.[17] The high charge density in **2** due to closely located cationic arginine residues, might induce repulsive forces among oligo-arginine blocks, thereby increasing the curvature of the nanostructures.

We next asked whether the nanostructures can be covalently captured by polymerization. For polymerization, 1,2-ethanedithiol (EDT) was used as a cross-linker. The building blocks were mixed with EDT at a concentration above their CMC, and photo-polymerization was performed under UV light for 2 h (254 nm). The ^{1}H-NMR spectrum of **1** after polymerization revealed complete disappearance of the resonances corresponding to acrylic double bonds, indicating that all of the acryl groups within the nanostructures of **1** have been successfully converted into thioether linkages upon polymerization (Fig. 2a and Fig. S4†). Remarkably, the 2D sheet morphology of **1** was preserved even after the polymerization (Fig. 2b). The macroscopic 2D sheets of **1** could also be observed by fluorescence microscopy following encapsulation of fluorescent dye in aqueous solution (Fig. 2c). AFM investigation of the polymerized nanostructure revealed plate-like nanosheets with a uniform thickness of $\sim$7 nm (Fig. 2d). Similarly, the nanostructure of **2** was cross-linked successfully and the morphology was maintained after polymerization (see the ESI†). These results indicate that this type of cross-linking strategy offer an opportunity to covalently capture amphiphilic peptide-based nanostructures, while preserving the morphologies. To corroborate the above described results in solution-based measurements, we performed dynamic light scattering (DLS) experiments. As shown in Fig. 2e, no changes in the autocorrelation function were observed after the polymerization of the nanostructure of **1** in concentrations quite below its CMC. This result reconfirms that the nanostructure of **1** was successfully converted into the polymeric objects.

We next investigated the effect of nanostructure polymerization on cell viability. The result showed that unpolymerized

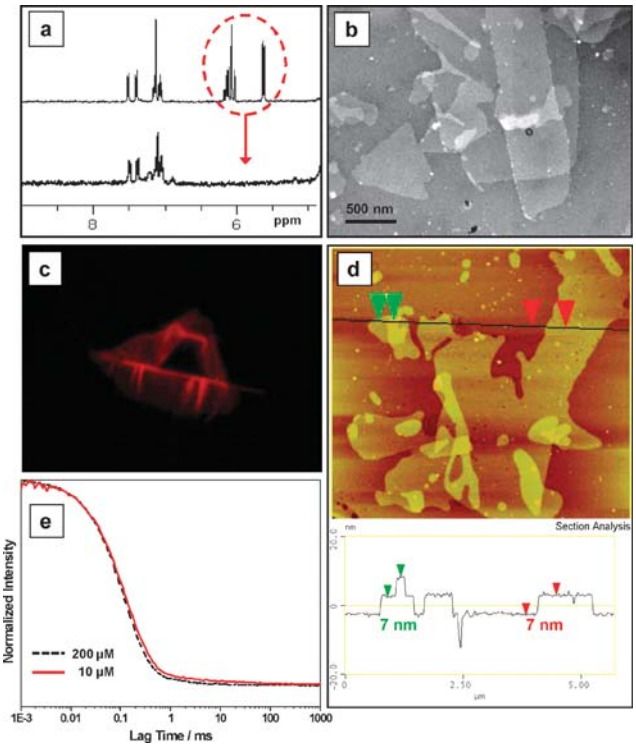

Fig. 2 (a) ^{1}H-NMR spectra of **1** (upper) and polymerized **1** (lower) in D_2O. The ^{1}H-NMR spectrum of polymerized **1** reveals complete disappearance of the acrylic double bond at around 6 ppm. (b) TEM image of polymerized objects of **1** with negative staining. (c) Fluorescence microscopy image of polymerized sheets of **1** encapsulating Nile Red in aqueous solution. (d) AFM image of polymerized sheets of **1** with height information. (e) Autocorrelation functions of an aqueous solution of polymerized objects of **1** with different concentrations.

1 was found to be highly cytotoxic, whereas the polymerized nanostructure was nearly nontoxic within the concentration range tested (Fig. 3a). These results can be explained in that unpolymerized block peptides exist as isolated molecules during interaction with the plasma membrane due to their weak association strength, thereby disrupting cell membranes similarly to conventional surfactants.[13,15] This finding clearly demonstrates that polymerization-mediated covalent capture

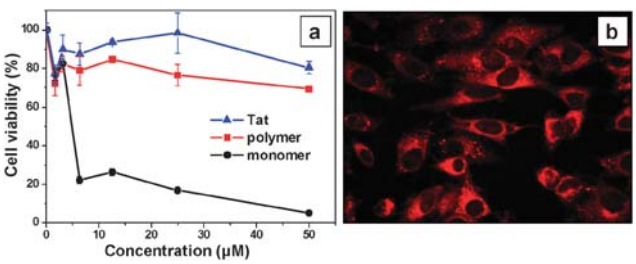

Fig. 3 (a) Cytotoxicity in Hela cells by MTT assay. Mean $\pm$ s.d. (n = 3). (b) Confocal laser scanning microscopy (CLSM) image (400×) of intracellular delivery of Nile Red by polymerized sheets of **1**. Concentration of polymerized sheets of **1** was 10 μM and the amount of the encapsulated Nile Red was 5 mol% relative to that of **1**. The cells were treated for 3 h.

of nanostructures can be developed as a general means to lowering the cytotoxicity of amphiphilic nanostructures.

As the nanostructure of **1** is decorated with CPPs, it might be used as an intracellular drug carrier following encapsulation of hydrophobic drugs. It would be quite interesting to investigate how CPP-decorated 2D macroscopic sheets interact with the cells. For this, a model hydrophobic drug, Nile Red, was encapsulated in the polymerized nanostructure of **1**, and the Nile Red-encapsulated nanostructure was treated in mammalian cells. The result showed that the nanostructure of **1** was quite efficient in delivering encapsulated cargos into the cells. Especially, the successful intracellular delivery of the nanostructures of **1** demonstrates that macroscopic 2D sheets interact with the outer cell membrane strongly and deliver the guest molecules efficiently into the cells (Fig. 3b). It is not currently clear whether the 2D sheet itself does internalize into the inside of the cells or, due to its very large size, the sheet just releases the payloads while it is still bound to the cell surface, which will be the subject of further in depth study. The macroscopic 2D sheets, contrary to conventional spherically or cylindrically-shaped nanostructures, should offer a unique opportunity for developing nanocarriers with unexplored and unexpected functions.

In conclusion, we have demonstrated that polymerizable Tat peptides self-assemble into a unique sheet-like 2D structure that can be covalently fixed to produce molecular sheets, which would open new possibilities for fabricating morphologically diverse and controlled bioactive nanostructures. Subsequently, covalent capture of such controlled bioactive nanostructures by the polymerization strategy should enable efficient intracellular delivery of cargo molecules in concentrations quite below their CMCs.

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Notes and references

1 V. Percec, A. E. Dulcey, V. S. K. Balagurusamy, Y. Miura, J. Smidrkal, M. Peterca, S. Nummelin, U. Edlund, S. D. Hudson, P. A. Heiney, H. Duan, S. N. Magonov and S. A. Vinogradov, *Nature*, 2004, **430**, 764.
2 H. G. Börner and H. Schlaad, *Soft Matter*, 2007, **3**, 394.
3 C. A. Mirkin, R. L. Letsinger, R. C. Mucic and J. J. Storhoff, *Nature*, 1996, **382**, 607.
4 K. Lu, L. Guo, A. K. Mehta, W. S. Childers, S. N. Dublin, S. Skanthakumar, V. P. Conticello, P. Thiyagarajan, R. P. Apkarian and D. G. Lynn, *Chem. Commun*., 2007, 2729.
5 S. Zhang, *Nat. Biotechnol*., 2003, **21**, 1171.
6 R. Langer and D. A. Tirrell, *Nature*, 2004, **428**, 487.
7 G. A. Silva, C. Czeisler, K. L. Niece, E. Beniash, D. A. Harrington, J. A. Kessler and S. I. Stupp, *Science*, 2004, **303**, 1352.
8 M. R. Ghadiri, J. R. Granja, R. A. Milligan, D. E. McRee and N. Khazano-vich, *Nature*, 1993, **366**, 324.
9 E. P. Holowka, V. Z. Sun, D. T. Kamei and T. J. Deming, *Nat. Mater*., 2007, **6**, 52.
10 Y.-b. Lim, S. Park, E. Lee, H. Jeong, J.-H. Ryu, M. S. Lee and M. Lee, *Biomacromolecules*, 2007, **8**, 1404.
11 Y.-R. Yoon, Y.-b. Lim, E. Lee and M. Lee, *Chem. Commun*., 2008, 1892.
12 (*a*) S. Zhang, T. Holmes, C. Lockshin and A. Rich, *Proc. Natl. Acad. Sci. U. S. A*., 1993, **90**, 3334; (*b*) T. A. Martinek, A. Hetényi, L. Fülöp, I. M. Mándity, G. K. Tóth, I. DéKány and F. Fülöp, *Angew. Chem., Int. Ed*., 2006, **45**, 2396.
13 Y.-b. Lim, E. Lee and M. Lee, *Angew. Chem., Int. Ed*., 2007, **46**, 9011.
14 M. A. Gauthier and H.-A. Klok, *Chem. Commun*., 2008, 2591.
15 L. M. Pakstis, B. Ozbas, K. D. Hales, A. P. Nowak, T. J. Deming and D. Pochan, *Biomacromolecules*, 2004, **5**, 312.
16 L. Y. Jin, J. Bae, J.-H. Ryu and M. Lee, *Angew. Chem., Int. Ed*., 2006, **45**, 650.
17 J. N. Israelachivili, *Intermolecular and Surface Forces*, Academic Press, New York, 1985.