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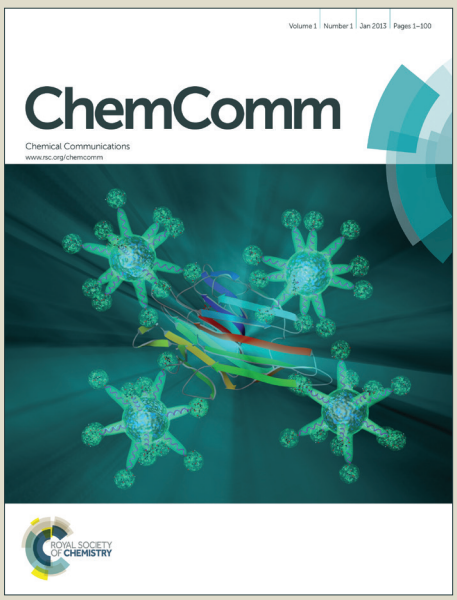

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Supramolecular Polymeric Peptide Amphiphile Vesicles For The Encapsulation of Basic Fibroblast Growth Factor†

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The synthesis of a supramolecular double hydrophilic peptide-conjugated polymer held together by cucurbit[8]uril (CB[8]) ternary complexation and its subsequent temperature triggered self-assembly into vesicles is described. Basic fibroblast growth factor can be easily loaded into the vesicles under benign conditions and their bioactivities can be preserved without the need for excipients such as heparin.

Peptide-amphiphiles are attractive candidate biomaterials for bio-nanotechnology and tissue engineering.[1] Typical covalent peptide-amphiphiles have limited ability to respond to external triggers.[2] Temperature sensitive capsules which can release drugs upon the application of a temperature cue can be fabricated by chemically conjugating the temperature sensitive poly(N-isopropylacrylamide) (PNIPAAm) to other hydrophilic block copolymers.[3] However, it remains challenging for non-chemists to prepare such polymers by typical chemical synthetic methods. Therefore, the supramolecular approach is highly attractive as a non-chemical facile preparation method for the fabrication of drug delivery carriers for hydrophobic drugs and proteins.

Cucurbit[8]uril (CB[8]) presents an attractive choice for building stable, modular supramolecular structures in an aqueous environment. The CB[8] ternary complex system offers strong binding of two complementary motifs (binding constants K_{eq} = 10^{12} M^{-2}) in water and can be used to prepare a variety of stable structures *via* a non-covalent route.[4,5] The ternary complexation ability of CB[8] has been exploited to simultaneously bind two polymer chains bearing complementary guests.[6] This concept was expanded upon in the construction of temperature induced supramolecular micelles through a dynamic CB[8] "handcuff" linking two stimuli-responsive hydrophilic blocks.[7] Hierarchical self-assembled vesicles in the aqueous environment can be prepared from either functionalised CB[6] or CB[8] ternary complexes.[8–10] Herein we report a supramolecular double hydrophilic peptide-conjugated polymer held together by CB[8] ternary complexation, which self-assembles into vesicles at physiological temperature and can be used to encapsulate and release the biologically active basic fibroblast growth factor (bFGF).

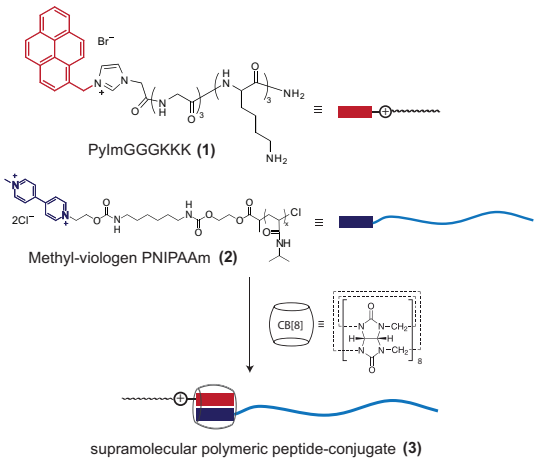

Fig. 1 Schematic representation of the chemical structures of pyrene-imidazolium labeled peptide and viologen functionalised PNIPAAm and the formation of ternary complex with CB[8].

A pyrene bearing an imidazolium group was conjugated to a short peptide (Figure 1).[11] Pyrene-functionalised peptide **1** is conjugated to methylviologen-terminated PNIPAAm **2** by CB[8] ternary complexation leading to the formation of a peptide-polymer conjugate **3**, as shown in Figure 1. The formation of the pyrene-viologen-CB[8] ternary complex causes quenching of the fluorescence as shown in Figure 3 (a) and Figure S1. Once the supramolecular peptide-polymer conjugates are formed, they readily undergo subsequent self assembly into vesicle **4** (Figure 2) by heating the solution from room temperature to 37 °C. When the temperature is raised to 37 °C, the PNIPAAm segment becomes hydrophobic and makes supramolecular conjugate **3** amphiphilic. As a result, double layer vesicles were observed in water (0.05 mM) as illustrated in Figure 2.

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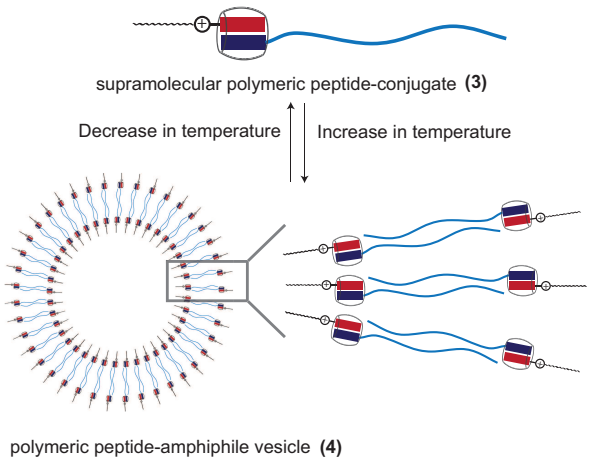

Fig. 2 Schematic representation of the temperature induced formation of a supramolecular polymeric peptide vesicle.

The difference observed in the solution properties of the mixture of **1** and **2** with and without CB[8] is remarkable as shown in Figure 3 (b). In the absence of CB[8], the aqueous mixture of **1** and **2** shows the usual phase transition typical of PNIPAAm both above and below its lower critical solution temperature (LCST). Upon addition of CB[8] to the mixture, the solution remains transparent, even when the temperature is raised above the LCST of PNIPAAm. In order to investigate the self-assembly behaviour of these supramolecular polymer peptide conjugates, dynamic light scattering (DLS) and transmission electron microscopy (TEM) studies were carried out. In the absence of CB[8], when the temperature is 25 °C, small particles were detected by DLS in the transparent solution. Above the LCST of 35 °C, large micrometer-sized particles are detected, resulting in a turbid solution as seen in the transmittance measurements shown in Figure 3 (b). When CB[8] is added, vesicles were observed above 35 °C. In Figure 3 (c), vesicles were observed after uranyl acetate staining with diameters of about 180 nm, which is in good agreement with the average diameter obtained from DLS, 200 ± 50 nm. The particle size observed by DLS at 25 °C is smaller than 10 nm, corresponding to unimers, while at temperatures above the LCST, the particle size increases to about 180 nm, indicating the self-assembly of compartmentalised superstructures, as shown in Figure 3 (d). These DLS measurements corroborate with the images obtained using TEM.

To investigate the self-assembly behavior of the supramolecular vesicles, the critical aggregation concentration (CAC) in water was determined by investigating the scattering intensity of light as a function of the polymer concentration.[7] The CAC values for the supramolecular vesicle were estimated to be 12 μM (Figure S2a). The CAC value of the supramolecular vesicle determined in a 1X phosphate buffer saline (PBS) solution containing 1% fetal bovine serum at 37 °C was about 6 μM (Figure S2b). After an additional 3 days of incubation, the CAC value remains largely unchanged suggesting that the system is stable under physiological conditions. Furthermore, the morphology of the vesicles are unchanged upon injection from a syringe, suggesting that these vesicles are useful as injectable vehicles of bio-relevant cargo.

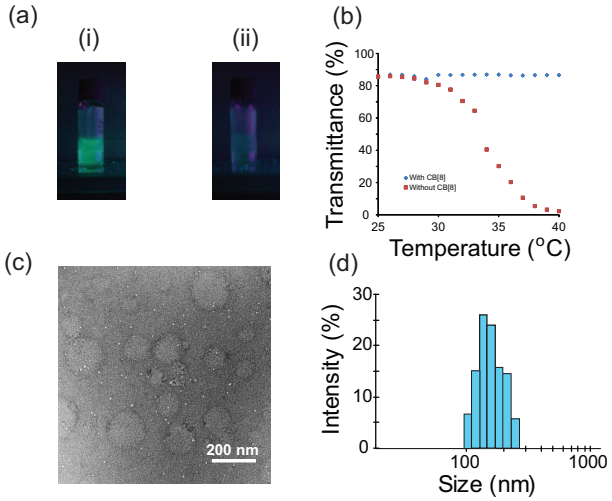

Fig. 3 (a) Photographs showing the effect of addition of CB[8] to a solution of **1** + **2** (i) without CB[8] and (ii) with CB[8]. The formation of supramolecular ternary complexes leads to quenching of the pyrene fluorescence (excitation at 303 nm). (b) Thermoresponsive behaviour of **1** + **2** with and without CB[8] (0.05 mM). (c) TEM micrographs of supramolecular micelles at 37 °C (solution concentration = 0.05 mM). (d) Particle size distributions of supramolecular vesicles at 37 °C.

Basic fibroblast growth factor is a mitogenic cytokine protein which regulates many aspects of cellular activity, such as cell migration and extracellular matrix metabolism. The proliferation of fibroblasts can be stimulated by bFGF leading to accelerated wound healing.[12] However, bFGF degrades rapidly when the external environment is above 40 °C or when the pH is less than 5 and heparin is required to stabilise the protein and to preserve its bioactivity.[13] The efficacy of bFGF *in vivo* is also limited on account of its short lifetime and susceptibility to enzymatic degradation.[14,15]

Various approaches have been proposed for the stablisation of bFGF. These include the chemical modification techniques, encapsulation in gels and powder formulation.[16–19] Vesicles are highly attractive carriers for proteins due to their hydrophilic interior. In this work, the protein was dissolved in the ternary complex solution before raising the temperature to 37 °C for the encapsulation of the proteins within the internal hydrophilic reservoir. The protein-loaded vesicles were formed without the use of excipients such as heparin, incubated at 37 °C and the bioactivity of the protein was monitored over time. This method minimises the risk of protein denaturation as it does not expose the protein to extremes in temperatures or organic solvents during the formulation process. In Figure 4(a), the effect of vesicle encapsulation on

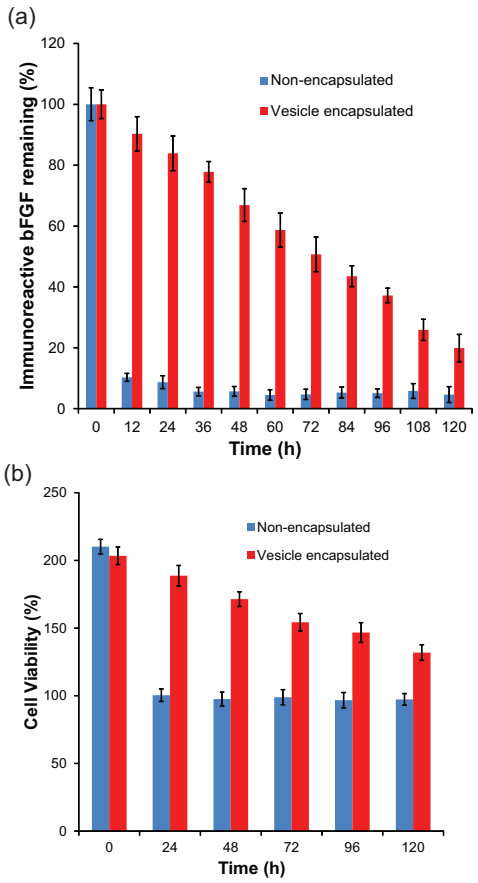

Fig. 4 (a) Assessment of immunoreactive bFGF in vesicles after encapsulation at various times. (b) Bioactivity of bFGF following storage in vesicles after encapsulation at various times.

the immunoreactivity of bFGF incubated at 37 °C are shown. Without any vesicle, the immunoreactivity of bFGF drops to below 10% of the original loaded amount within 1h. When encapsulated in the vesicles, the percentage of bFGF retaining immunoreactivity increases significantly. The remaining immunoreactivity of bFGF remains higher than 60% even after 2 days. Even up to 5 days, the remaining immunoreactivity of the bFGF was three times higher than the unencapsulated counterpart.

The bioactivity of bFGF after 96 h of incubation in the vesicles was quantified by a 3T3 cell proliferation assay Figure 4 (b). The general trend shows that the cell growth is enhanced by up to 200% when bFGF was added without any loss of bioactivity. When bFGF was dissolved in buffer for 1h without the addition of heparin, the bioactivity of bFGF decreases to about zero. There was no appreciable difference in the rates of cell growth with or without the addition of bFGF. The bFGF encapsulated in the vesicles stimulated a significant enhancement in the growth of the fibroblast cells. Even after 5 days, the cell growth was enhanced by over 30% as compared to the controls. Based on both the ELISA and the cell proliferation assay, it can be concluded that the encapsulation of bFGF in the vesicles effectively protects the protein against denaturation without the need for stabilising agents. Even under denaturing conditions such as freeze-thaw cycles, the activity of bFGF is retained, with the cell viability reaching levels of about 200% (Supporting Information Figure S3). When the vesicle-bFGF formulation was subjected to 3 freeze-thaw cycles, there was a minimal reduction of the bioactivity of the bFGF. This was compared with the effect of the concentration of heparin. When heparin was added at a concentration of 10:1 with respect to bFGF, the activity of bFGF was retained at about 200%. When the heparin to bFGF ratio was decreased to 1:10000, the activity of bFGF was decreased to about 120%. The administration of bFGF using these vesicles is potentially useful for patients who do not have high tolerance to anti-coagulants such as heparin. In conclusion, the protein-friendly nature of the vesicles was demonstrated by encapsulating bioactive bFGF into the supramolecular nanocarriers without the use of stabilising agents. This finding suggests that the supramolecular vesicles could potentially be used as an injectable carrier for the release of bioactive cytokines for tissue repair and other related applications.

References

1 J. D. Hartgerink, E. Beniash and S. I. Stupp, *Science*, 2001, **294**, 1684–1688.
2 S. E. Paramonov, H. W. Jun and J. D. Hartgerink, *J. Am. Chem. Soc.*, 2006, **128**, 7291–7298.
3 X. J. Loh, Y. L. Wu, W. T. J. Seow, M. N. I. Norimzan, Z. X. Zhang, F. Xu, E. T. Kang, K. G. Neoh and J. Li, *Polymer*, 2008, **49**, 5084–5094.
4 E. A. Appel, X. J. Loh, S. T. Jones, C. A. Dreiss and O. A. Scherman, *Biomaterials*, 2012, **33**, 4646–4652.
5 U. Rauwald, J. del Barrio, X. J. Loh and O. A. Scherman, *Chem. Commun.*, 2012, **47**, 6000–6002.
6 U. Rauwald and O. A. Scherman, *Angew. Chem., Int. Ed.*, 2008, **47**, 3950–3953.
7 X. J. Loh, J. del Barrio, P. P. C. Toh, T. C. Lee, D. Z. Jiao, U. Rauwald, E. A. Appel and O. A. Scherman, *Biomacromolecules*, 2012, **13**, 84–91.
8 Y. J. Jeon, P. K. Bharadwaj, S. W. Choi, J. W. Lee and K. Kim, *Angew. Chem., Int. Ed.*, 2002, **41**, 4474–4476.
9 K. M. Park, D. W. Lee, B. Sarkar, H. Jung, J. Kim, Y. H. Ko, K. E. Lee, H. Jeon and K. Kim, *Small*, 2010, **6**, 1430–1441.
10 Q. B. Zhou, H. Wang, T. Gao, Y. Yu, B. K. Ling, L. Mao, H. B. Zhang, X. G. Meng and X. H. Zhou, *Chem. Commun.*, 2011, **47**, 11315–11317.
11 D. Jiao, J. Geng, X. J. Loh, D. Das, T.-C. Lee and O. A. Scherman, *Angew. Chem., Int. Ed.*, 2012, **51**, 9633–9637.
12 R. Langer and J. P. Vacanti, *Science*, 1993, **260**, 920–926.
13 E. R. Edelman, E. Mathiowitz, R. Langer and M. Klagsbrun, *Biomaterials*, 1991, **12**, 619–626.
14 O. Jeon, S. H. Ryu, J. H. Chung and B. S. Kim, *J. Controlled Release*, 2005, **105**, 249–259.
15 I. Wilcke, J. A. Lohmeyer, S. Liu, A. Condurache, S. Kruger, P. Mailander and H. G. Machens, *Langenbecks Arch. Surg.*, 2007, **392**, 305–314.
16 T. H. Nguyen, S.-H. Kim, C. G. Decker, D. Y. Wong, J. A. Loo and H. D. Maynard, *Nat. Chem.*, 2013, **5**, 221–227.
17 D. Dyondi, T. J. Webster and R. Banerjee, *Int. J. Nanomedicine*, 2013, **8**, 47–59.
18 B. M. Ibrahim, S. W. Jun, M. Y. Lee, S. H. Kang and Y. Yeo, *Int. J. Pharm.*, 2010, **385**, 66–72.
19 O. Ziv-Polat, H. Skaat, A. Shahar and S. Margel, *Int. J. Nanomedicine*, 2012, **7**, 1259–1274.