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Cyclic Peptide Facial Amphiphile Preprogrammed to Self-Assemble into Bioactive Peptide Capsules

Eun Kyung Chung,[a] Eunji Lee,[a] Yong-beom Lim,*[b] and Myongsoo Lee*[a]

Self-assembled peptide nanostructures have shown great potential as promising biomaterials.[1–8] Peptides have the advantage of being intrinsically biocompatible materials. Peptides, as self-assembling building blocks, have mostly been designed to have head/tail molecular configuration. Since there is a great deal of interest in the precise control of a self-assembly process, novel building blocks significantly different from those with conventional head/tail configuration might provide ample opportunities for constructing elaborate, versatile, and smart nanostructures.

Cyclic peptides, due to their constrained nature, are one of those self-assembly building blocks that have unique topological features compared with conventional linear (head/tail) peptides.[9–11] Another important self-assembling building block that has a special architecture is the facial amphiphiles.[12–15] In facial amphiphiles, the hydrophilic and -phobic groups are located on two opposite faces, rather than at two ends as in head/tail amphiphiles. Herein, we present an approach to construct a novel type of self-assembling building blocks in which the characteristics of cyclic peptides and facial amphiphiles are combined. The self-assembly behavior of this novel cyclic peptide facial amphiphile (CPFA) could be regulated through the judicious design of molecular structure, showing the power of this rational approach for precise nanostructural control.

Capsules or vesicles are one of the most important nanostructural morphologies that can be utilized in many types of bioapplications, including drug, gene, and protein delivery.[16–21] Capsules have hollow interior within which cargo molecules can be entrapped. Upon suitable functionalization of outer shell, capsules can be made to bind or enter the cells. Traditionally, self-assembled capsules have been fabricated by using amphiphilic molecules of head/tail configuration, such as amphiphilic lipids and block copolymers. The supramolecular morphology of the head/tail amphiphiles depends highly on the relative volume fraction between hydrophilic and -phobic blocks.[19,22–24] Therefore, the capsule formation, in most cases, can be possible only by adjusting the volume fraction by trial and error.

To devise a building block preprogrammed to have a predictable self-assembly property for capsule formation, a symmetrical CPFA with a C_3-symmetric triskelion shape was designed to allow the positioning of building blocks in a precise geometry for closed-capsule structure formation. CPFA consists of even-numbered L-amino acids with alternating hydrophilic and -phobic side chains (Figure 1).

In this type of a molecular arrangement, the side chains (R groups at the α-carbon atoms) adopt alternating axial and equatorial positions as a low-energy conformation. In contrast, cyclic peptide structures made up of alternating L- and D-amino acids, as shown in several synthetic cyclic peptides,[11,25] can adopt a flat-ring structure as a low-energy conformation in which all of the side chains lie horizontal to the plane of the ring (equatorial). The six-residue CPFA consists of three arginines and three hydrophobic amino acids as hydrophilic and -phobic residues, respectively. The hydrophilic and -phobic residues are placed at alternating positions in order for the adjacent side chains to be located at different faces (axial or equatorial).

The guanidinium group in arginine has been found to be a crucial residue for cell-surface binding and penetration of cell-penetrating peptides.[18,26,27] The hydrophobic amino acids are tyrosine derivatives modified with long alkyl chains. In addition to CPFA with the perhydrogenated alkyl chain (hCPFA), CPFA with the perfluorinated alkyl chain (fCPFA) was synthesized to take advantage of the "fluorophobic effect". The fluorophobic effect refers to the super-

[a] E. K. Chung, Dr. E. Lee, Prof. Dr. M. Lee
Center for Supramolecular Nano-Assembly and
Department of Chemistry, Seoul National University
Seoul 151-747 (Korea)
Fax: (+82) 2-393-6096
E-mail: myongslee@snu.ac.kr

[b] Prof. Dr. Y.-b. Lim
Department of Materials Science and Engineering
Yonsei University, Seoul 120-749 (Korea)
Fax: (+82) 2-365-5882
E-mail: yblim@yonsei.ac.kr

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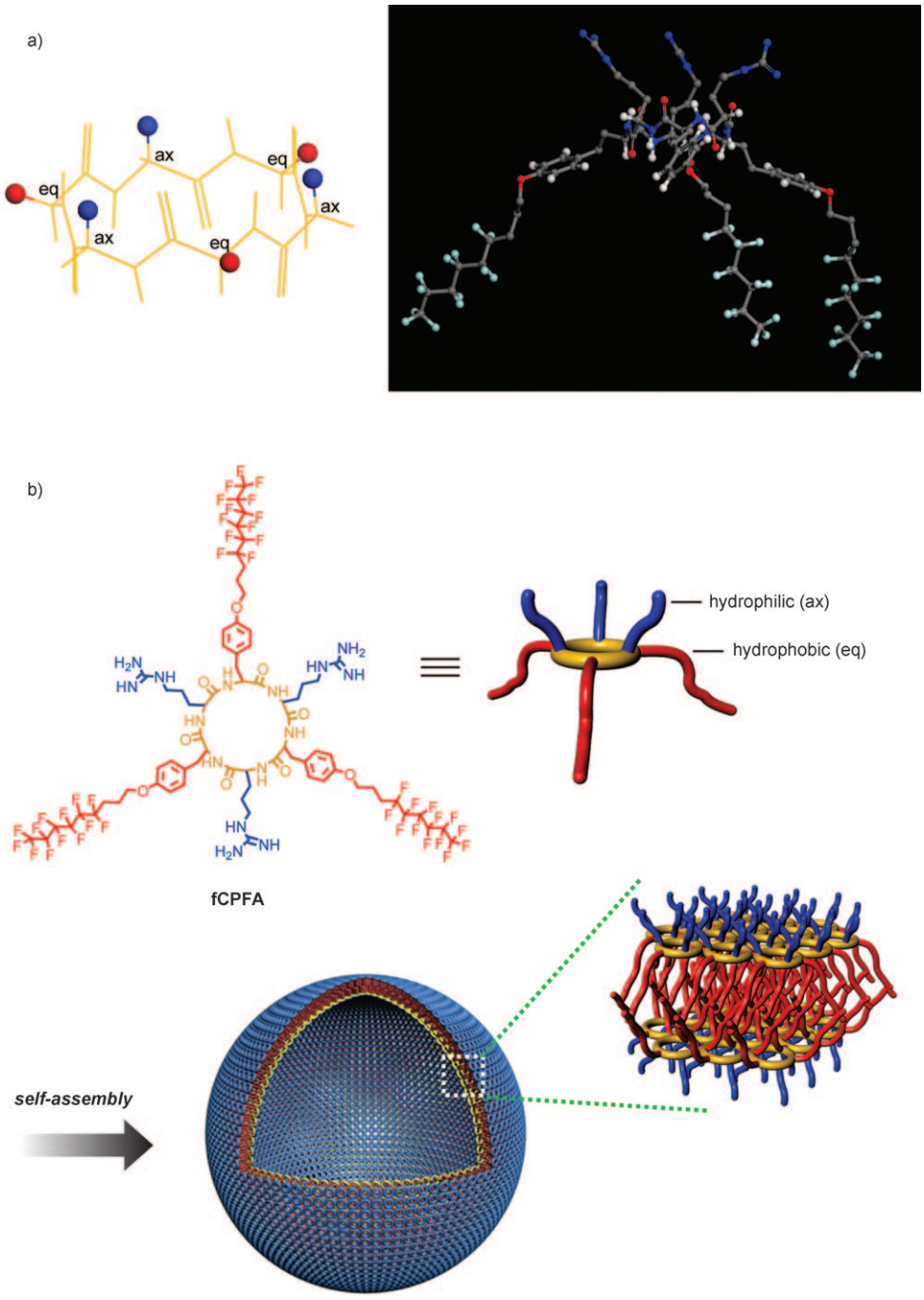

Figure 1. Structure and self-assembly of CPFA. a) Low-energy conformations of 6-residue cyclic peptides. Left: a cyclic peptide consists of only L-amino acids. Right: Molecular modeling of six-residue CPFAs (fCPFAs) by Forcite geometry optimization. The Forcite calculation was performed by using the Accelrys Materials Studio program. ax: axial, eq: equatorial. b) Self-assembly of fCPFAs into a capsule nanostructure.

hydrophobicity of fluorocarbon atoms and the selective self-association between fluorinated moieties, which have been demonstrated to be effective in stabilizing self-assembled nanostructures and protein structures.[28–31] The head-to-tail cyclization of the corresponding protected linear peptide is conducted under pseudo-high-dilution conditions (for details, see the Supporting Information.)[32]

The self-assembly behavior of CPFA in aqueous solution was investigated with dynamic light scattering (DLS), circular dichroism (CD) spectroscopy, zeta-potential measurements, and transmission electron microscopy (TEM). DLS studies revealed that hCPFA and fCPFA self-assembled into nanoaggregates with an average hydrodynamic radii (R_H) of 420 and 60 nm, respectively (Figure 2aand Figure S1a in the Supporting Information). CD and FTIR results show that both CPFAs are mostly in a random-coil conformation, suggesting that hydrogen-bonding interactions responsible for peptide secondary-structure formation (e.g., α helix and β sheet) are not involved in the self-assembly process of CPFAs (Figures S1b and S2 in the Supporting Information). These results suggest that the driving force for the self-assembly of CPFAs is pure hydrophobic interactions between the perhydrogenated or perfluorinated alkyl chains rather than β-sheet interactions, as found in nanotube-forming cyclic peptides with alternating L- and D-amino acids.[11,25] Analysis of nanostructural morphology with TEM revealed that hCPFA forms overall irregular aggregates (Figure 2c), suggesting that the aggregation strength of perhydrogenated alkyl chains is rather weak. In contrast, fCPFA self-assembled into discrete nanocapsules, as shown in Figure 2d. The concaveness observed in the negatively stained TEM image indicates the shape of shrunken capsules formed during the drying process, providing the evidence of the hollow nature of the vesicular structures. Moreover, the cryo-TEM image clearly shows the formation of hollow nanocapsules with high contrast between the wall and the hollow interior. Therefore, the strengthened hydrophobic interactions by the fluorophobic effect are important for stabilizing the capsule nanostructures, which further support the notion that CPFAs self-assemble through purely hy-

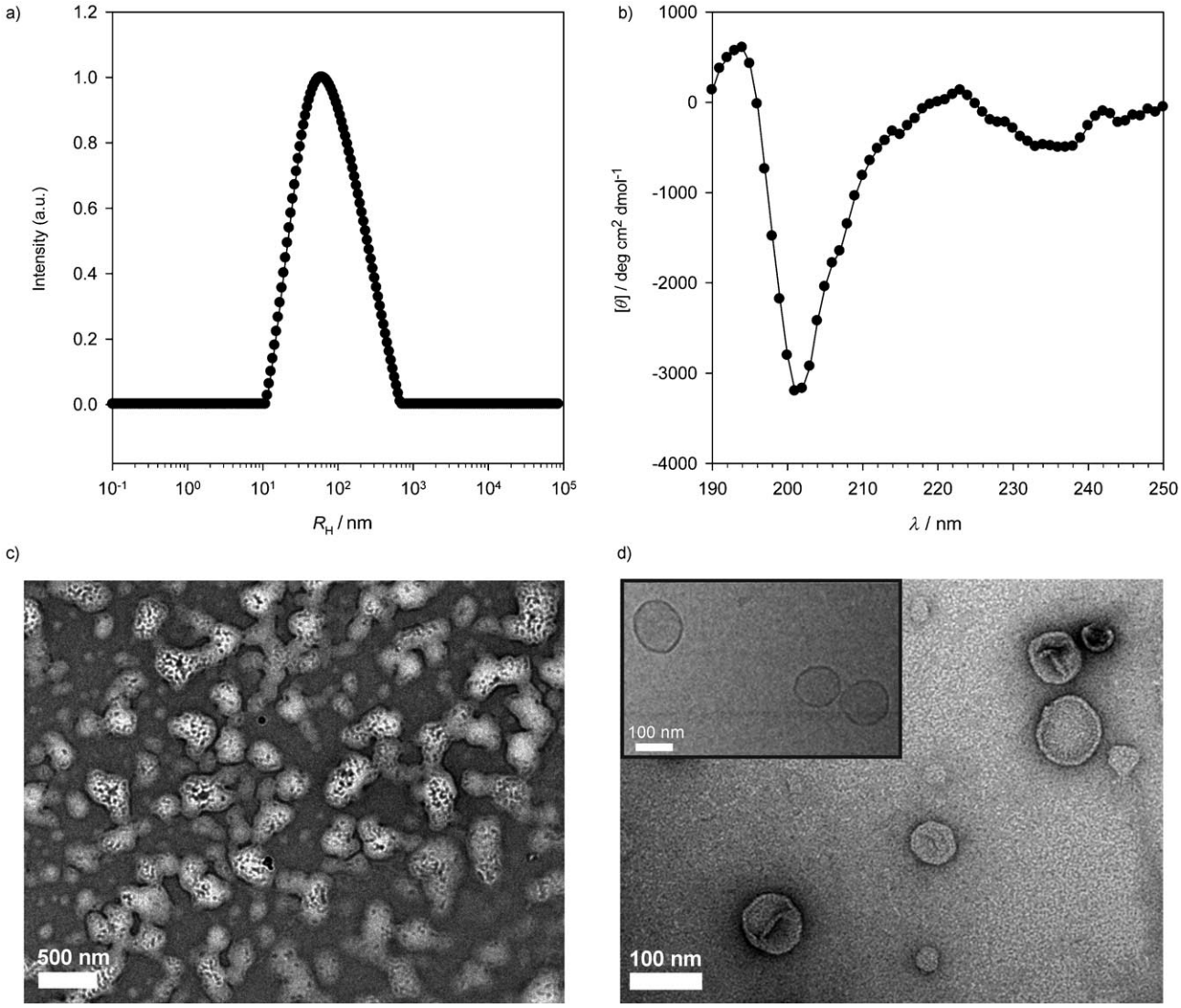

Figure 2. Self-assembly of CPFAs. a) DLS and b) CD analyses of fCPFA. Negatively stained TEM images of c) hCPFA and d) fCPFA. Inset in d): cryo-TEM image. Samples (30 μM) were prepared in a 75 mM aqueous solution of KF.

drophobic interactions. The wall thickness (ca. 5.9 nm) corresponds well to the calculated thickness of the fCPFA bilayer (5.5 nm), indicating that the wall consists of the hydrophobic interior of alkyl chains and the hydrophilic exterior of guanidinium groups (see Figure 1b). Zeta-potential (ζ) measurement showed that fCPFA capsules had positive ζ potential values ($(+54\pm4)$ mV), which further demonstrates the formation of the positively charged surface by the guanidinium group coating. All these data clearly show that C_3-symmetric and facially amphiphilic CPFAs predestined for capsule formation do form the anticipated nanostructures.

To further demonstrate the importance of C_3-symmetric and cyclic properties of supramolecular building blocks for round and closed-capsule nanostructure formation, analogous molecules were designed and synthesized. The first analogue (fLPA) is a linear amphiphilic peptide, which is a precursor of fCPFA. As shown in Figure 3a, TEM analysis shows the formation of nanosheets from fLPA in aqueous solution. CD and FTIR experiments reveal the presence of strong β-sheet hydrogen bonds in fLPA aggregates, indicating that the fLPA self-assembly process is not solely driven by hydrophobic interactions (Figures S2 and S3 in the Supporting Information). The result clearly illustrates that the C_3-symmetric and cyclic nature of the supramolecular building block is a necessary condition for capsule formation. To further address the importance of a C_3-symmetric structure for capsule formation, fCPA was synthesized and its self-assembly behavior was investigated (Figure 3b). fCPA has a cyclic structure like that in fCPFA; however, fCPA has a C_2-symmetric structure, since the molecule has four arginines and two amino acids with perfluorinated alkyl chains. Investigation of the nanostructural morphology with TEM reveals that fCPA self-assembles into a bundle of nanofibers with the diameter of the individual nanofiber being approximate-

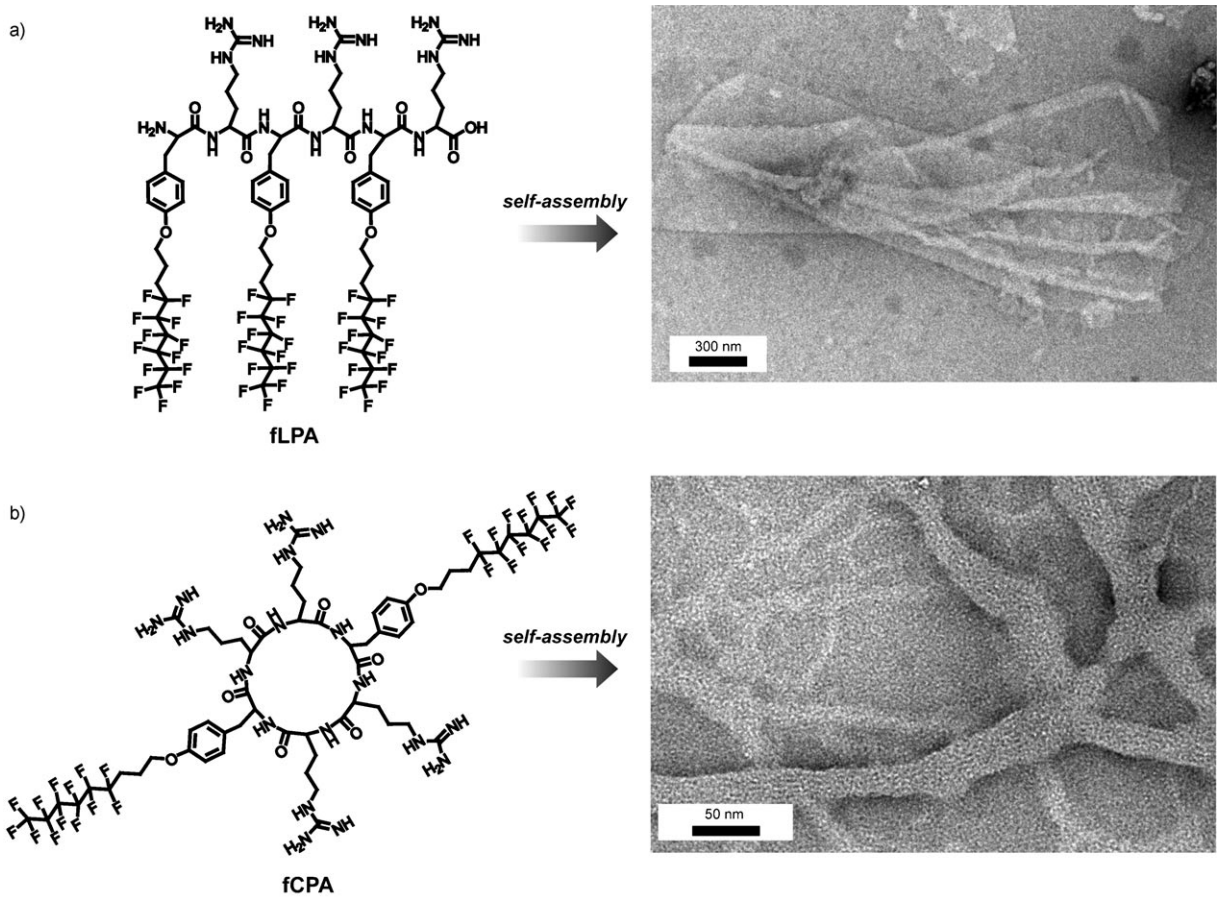

Figure 3. Self-assembly of analogue building blocks. Negatively stained TEM images. Samples (30 μM) were prepared in a 75 mM aqueous solution of KF.

ly 5 nm. Again, this result demonstrates that, as in the case of the clathrin protein and analogous triskelion molecules, the building blocks should have C_3-symmetry in order for them to grow (self-assemble) in every direction.

The primary driving force underlying the formation of a curved capsule structure is believed to be the combination of the strong tendency of the C_3-symmetric facial amphiphile to grow in a two-dimensional way and the lack of directional hydrogen-bonding interactions imposed by the cyclic nature of the amphiphilic molecule. At the initial stage of the self-assembly process, a 2D bilayered patch of a certain size starts to bend to reduce its total energy, thereafter further addition of building blocks generates a closed-capsule structure.[24,33] In that process, the absence of hydrogen-bonding interactions between amide C=O and N–H groups should play an important role, which would otherwise complicate the self-assembly process.

Since an fCPFA nanocapsule has cell-binding/penetrating guanidinium groups and a hollow interior, it might trap hydrophilic drugs and be developed as an intracellular nanocarrier.[18,34] To explore such a possibility, entrapment experiments were performed with a model hydrophilic compound, calcein. For the entrapment of calcein within the nanocapsule, fCPFA and calcein were mixed in an aqueous solution and sonicated. The calcein-loaded nanocapsules were separated from the free calcein by using gel filtration chromatography with Sephadex G-25. Analyses of the nanocapsules by DLS showed that the sizes of the nanocapsules after calcein entrapment are almost similar to those of the original nanocapsules (Figure 4a). TEM investigations revealed that the spherical shape is preserved even after calcein entrapment (inset in Figure 4a). The intracellular delivery experiment was performed with a mammalian cell line, HeLa. The result shows the successful intracellular delivery of calcein (Figure 4b). The image shows the bright green fluorescence of calcein in every cell, indicating the highly efficient intracellular delivery capability of fCPFA nanocapsules.

In conclusion, novel and unique self-assembling peptide building blocks, CPFAs, were designed, synthesized, and their self-assembly behavior was investigated. The significance of this approach lies in the fact that nanocapsule formation could be predicted and controlled by the precise molecular design, rather than experimental adjustment of the relative volume fraction as in amphiphilic block molecules. The guanidinium-group-coated fCPFA developed in this study should be useful for the intracellular delivery of cargo molecules. Moreover, the results from this study indicate that any type of hydrophilic-ligand-coated nanocapsules

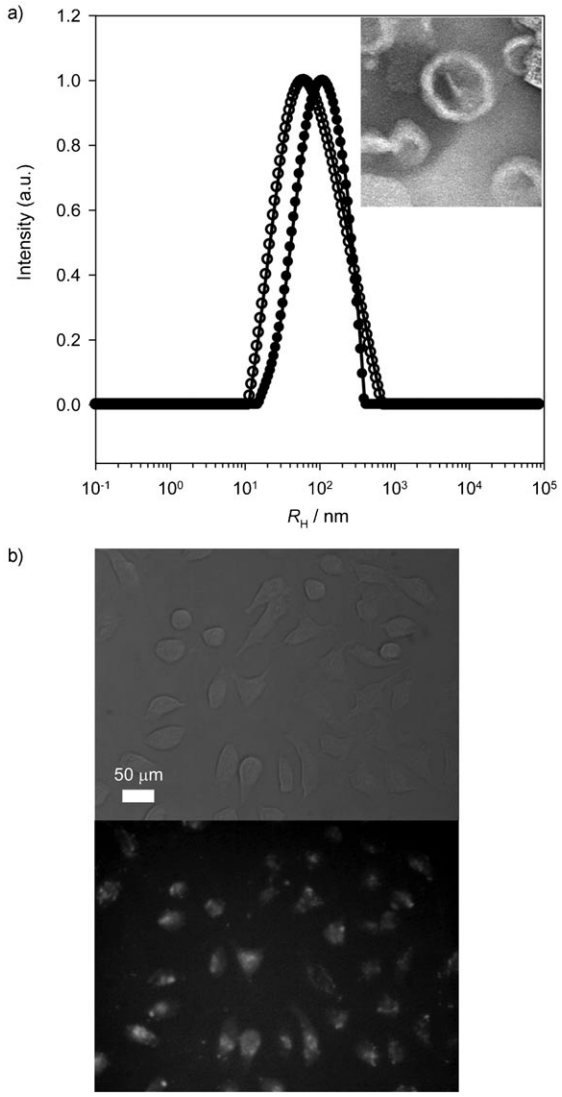

Figure 4. fCPFA nanocapsules as intracellular nanocarriers. a) DLS analyses of hydrodynamic radii of the original (open circles) and the calcein-loaded nanocapsules (closed circles). b) Intracellular delivery of the calcein-loaded nanocapsules in HeLa cells. Cells were treated with the nanocapsules for 3 h.

might be constructed if the molecular configuration of supramolecular building blocks follows that of CPFA.

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