Biomimetic Synthesis

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Adenosine Triphosphate Biosynthesis Catalyzed by F_oF_1 ATP Synthase Assembled in Polymer Microcapsules**

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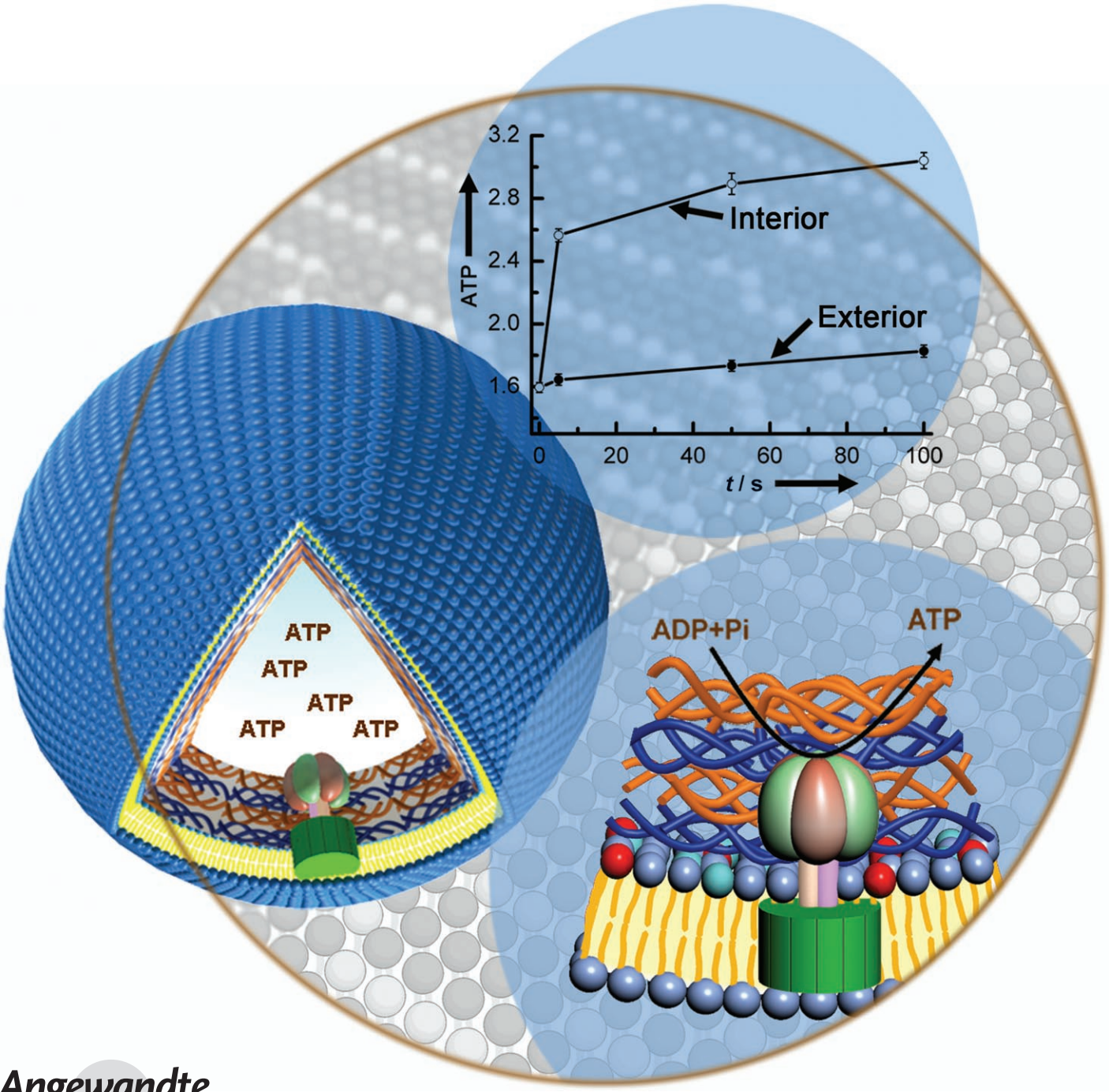

ATP synthase (ATPase), the smallest molecular motor in nature, is composed of two linked multisubunit complexes, a membrane-embedded F_o part and a hydrophilic F_1 part, and can drive the production of adenosine triphosphate (ATP) by utilizing proton gradients. The functionality of ATPase has attracted great interest in the last decade. Numerous potential applications, from the generation of bioenergy to the fabrication of nanodevices, have been suggested.[1–3] As a membrane-bound protein, ATPase has been successfully reconstituted in liposomes acting as biomimetic membranes.[4–8] However, the limitations of the size and stability of the assembled liposome complexes result in difficulty in understanding and analyzing the system. Herein, we report the reconstitution of ATPase in assembled lipid-coated polymer microcapsules to imitate the system in living cells governed by molecular motors.

Layer-by-layer assembly of oppositely charged macromolecules onto removable colloidal particles has been utilized to construct ultrathin hollow shells from nano- to microsize.[9–14] The assembled capsules have well-controlled size, shape, and wall thickness. The wall composition can be readily changed to adjust their physicochemical properties and permeability. These hollow capsules are often considered to have potential applications in the delivery and release of drugs, catalysis, biomedicine, and biomaterials. In our previous study,[13] we reported that lipid-coated microcapsules could be constructed through the conversion of liposomes into lipid bilayers to cover the capsule surface, in analogy to the cell membrane.

In the present work, we reconstitute chloroplastic F_oF_1 (CF_oF_1) ATPase in the outer shell of assembled polymer microcapsules containing a lipid bilayer. The concept of a lipid-modified capsule with the incorporation of the CF_oF_1 complex is shown schematically in Figure 1. CF_oF_1 ATPase was first reconstituted into liposomes based on the previously reported method.[4] Briefly, the liposomes were added into a Triton X-100-solubilized CF_oF_1-ATPase buffer solution, and then the Triton X-100 was slowly removed with SM-2 Biobeads. CF_oF_1 proteoliposomes were obtained. The microcapsules were assembled by the alternating adsorption of

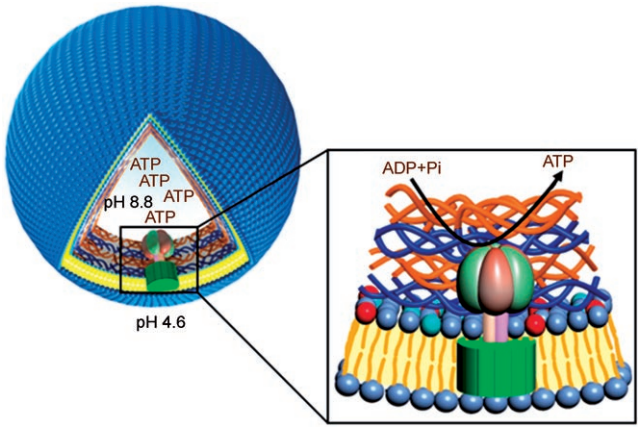

Figure 1. Schematic representation of the arrangement of CF_oF_1 ATPase in lipid-coated microcapsules. ADP: adenosine diphosphate; Pi: inorganic phosphate.

negatively charged poly(acrylic acid) (sodium salt) (PAA) and positively charged poly(allylamine hydrochloride) (PAH) onto 3.93-μm positively charged melamine formaldehyde (MF) particles as templates, followed by removal of the MF templates by using hydrogen chloride (HCl). Combination of the CF_oF_1 proteoliposomes and the microcapsule solution allows the proteolipid to adsorb favorably on the outer shell of the microcapsules through the electrostatic interaction of phosphatidic acid with PAH.[13] Lipid-modified microcapsules with incorporated CF_oF_1 ATPase were thus obtained. It should be noted that only in the case of F_1 subunits of the CF_oF_1 complex extending toward the interior of the assembled capsules ATP synthesis inside the capsules could become possible.

H^+-transporting F_oF_1 ATPase is known to be located in the cytoplasmic membrane of bacteria, the thylakoid membrane of chloroplasts, or the inner membrane of mitochondria. In our experiment, the F_oF_1 ATPase was isolated and purified from spinach chloroplasts. The results from sodium dodecylsulfate (SDS) PAGE analysis (Figure 2a) show the existence of individual units of the CF_oF_1 complex (Figure 2a, lanes 1 and 2) relative to standard protein markers (Figure 2a, lane 3). The surface morphology changes of the polyelectrolyte capsules with and without lipid and with lipid–CF_oF_1 coverage were observed by atomic force microscopy in a dried state, as shown in Figure 2b. From the height profiles, it is estimated that the wall thickness of the hollow capsules without lipid–CF_oF_1 coverage is about 10 nm (left-hand image of Figure 2b), while for both lipid-modified and proteolipid-modified capsules, the wall thicknesses are around 15 nm (middle and right-hand images of Figure 2b).

In addition, the roughness of the selected area is about 1 nm for the pure hollow capsules, 2 nm for the lipid-modified capsules, and 2.5 nm for the proteolipid-modified capsules. The analysis of surface roughness indicates that the capsule surfaces are getting rougher as the lipid and lipid–CF_oF_1 are added. We can also deduce that the lipid layer coated on the capsule surface is about 5 nm, which corresponds to the thickness of a lipid bilayer. The results are similar to those

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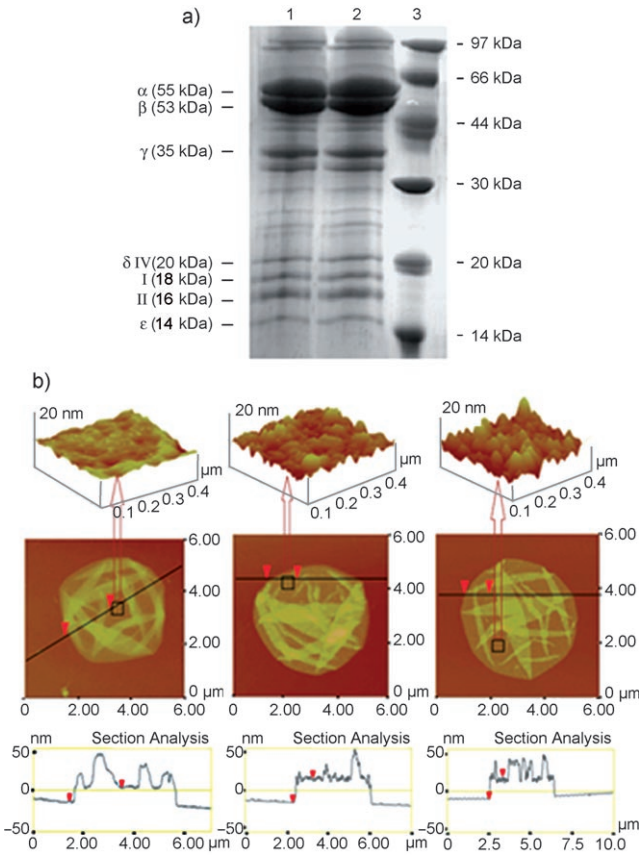

Figure 2. a) SDS polyacrylamide gel electrophoresis of the purified CF_oF_1 complex (lanes 1 and 2). shows the existence of individual units from CF_oF_1; the expected molecular weights (from comparison with the standard protein molecular-weight markers (lane 3)) are indicated on the left. b) Atomic force microscopy images (from left to right): $(PAA/PAH)_3$ microcapsules, microcapsules coated with lipids, and microcapsules coated with lipid–CF_oF_1, respectively. The corresponding surfaces for an indicated area are given at the bottom with a size of $0.5 \times 0.5\ \mu m^2$.

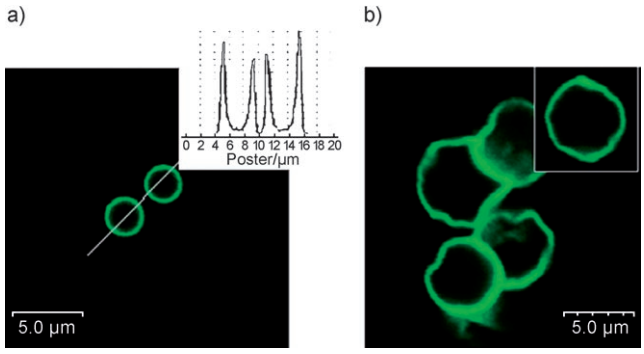

Figure 3. CLSM images of assembled $(PAA/PAH)_3$ microcapsules covered by a) lipids (inset image: fluorescence profile along the line indicated in the confocal image) and b) lipid–CF_oF_1. The walls of the microcapsules with the incorporated CF_oF_1 complex exhibit some distortion.

obtained by single-particle light-scattering measurements in our previously published work[12] and by freeze–fracture electron microscopy measurements.[15]

To further prove the adsorption of lipids onto the capsule shells, we performed experiments by confocal laser scanning microscopy (CLSM) with the addition of 4-nitrobenzo-2-oxa-1,3-diazole-labeled 1,2-dipalmitoylphosphoethanolamine (DPPE) (5%) to the mixture of lipids. Figure 3a shows that continuous fluorescence with green color and an almost constant intensity occurs over the entire surface of the capsules. It further proves that the lipids are adsorbed on the capsule shells. The CLSM image in Figure 3b also shows the bent surface of the assembled microcapsules with incorporated CF_oF_1 complex. It is supposed that such a distortion might be caused by the assembly of CF_oF_1 ATPase on the microcapsules. The transmembrane electrochemical potential difference existing in CF_oF_1-containing microcapsules may lead to the distortion of the capsule surface due to the ionic intensity changes between the interior and exterior buffer solutions. The rotary torque of the γ subunit attached to the fluorescent actin filament can exceed 40 pN nm^{-1}[1] and this may also partially affect the morphology of capsules, a theory that needs to be further investigated.

In the present case, the driving force for ATP synthesis catalyzed by CF_oF_1 ATPase is provided by an acid/base transition between the interior and exterior of the capsules.[4,6] To realize this, we added buffer solutions with pH values of 8.8 and 4.6 successively to create a proton gradient between the inside and outside of the capsules. To detect proton transportation across the membrane through CF_oF_1 ATPase during the synthesis of ATP from ADP and phosphate, a pH-sensitive fluorescent probe, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (pyranine), was introduced in the interior of the capsules to monitor the changes in pH value within them. Generally, the F_1 subunit has a preferred orientation extending into the liposome bulk aqueous phase, as described for the reconstitution procedure by Richard et al.[4] But, for the reconstitution of ATPase into capsules, we consider that the experimental conditions of liposome fusion will ensure that the F_1 subunit partly extends into the inner wall of the capsules during the adsorption of lipids on the outer shells of the capsules. To prove this, we have performed the following experiments.

An aqueous microcapsule solution was firstly mixed with buffer at pH 8.8 containing 100 mM Tricine, 2.5 mM $MgCl_2$, 5 mM NaH_2PO_4, 0.2 mM ADP, and 0.5 mM pyranine. After this mixture had been gently stirred for a few minutes, CF_oF_1 proteoliposomes were added into the capsule solution and allowed to adsorb for 30 min; this was followed by three rounds of centrifugation and washing with buffer solution. As a result, pyranine and buffer solution at pH 8.8 were encapsulated in the cavity of the capsules. Figure 4a shows the CLSM images of microcapsules containing encapsulated pyranine and the corresponding fluorescence intensity profile. It can be seen that the fluorescence intensity of the inner core of the capsules is obviously higher than that of the surrounding solution. The self-deposition of pyranines in the interior of capsules could be derived from the charged species already existing within the interior of the capsules. These charged species are likely to be a complex formed from PAA, the

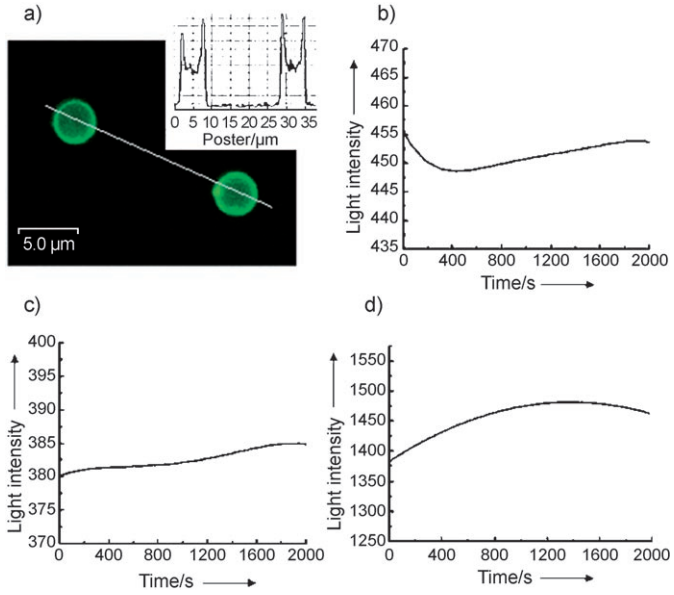

Figure 4. a) CLSM image of proteolipid-coated capsules containing pyranine in a buffer solution at pH 8.8 (inset as in Figure 3). b–d) The change of light intensity as a function of reaction time for b) microcapsules modified by a lipid bilayer with CF_oF_1 ATPase, c) microcapsules modified by a lipid bilayer without CF_oF_1 ATPase in buffer solution at pH 8.8, and d) proteoliposomes with CF_oF_1 ATPase in buffer solution at pH 4.6.

negatively charged inner wall material, and the degradation product of MF, the positively charged core template.[14]

ATP synthesis was begun by quick injection of the same volume of buffer solution at pH 4.6, which contained 2.5 mM $MgCl_2$, 5 mM NaH_2PO_4, and 0.2 mM ADP. This was followed by scanning of the fluorescence emission spectrum of pyranine (excitation wavelength 460 nm, emission wavelength 513 nm). It should be noted that the change of fluorescence intensity is caused by the change in pH value. The intensity change directly reflects the change in proton gradient, which corresponds to the activity of ATPase. In the first 300 s, the light intensity decreases with the time elapsed, after which it slowly approaches a constant value, as shown in Figure 4b. For comparison, lipid-modified capsules without CF_oF_1 ATPase were also prepared. The results in Figure 4c show that no intensity decrease occurs.

These results suggest that proton transportation occurs across the capsule walls through CF_oF_1 ATPase from the exterior solution to the interior solution due to the proton gradient. As the proton gradient vanishes inside and outside the capsule solution, the ATP synthesis reaction stops. As a control experiment, the proton gradient in liposomes with reconstituted CF_oF_1 ATPase was measured. We took the above-mentioned proteoliposomes to dilute with pH 4.6 buffer solutions and then quickly injected the same volume of buffer solution at pH 8.8 containing 0.5 mM pyranine. We then recorded the change of florescence intensity of pyranine outside the liposomes to detect the proton gradient. The experimental results in Figure 4d show that the change of fluorescence intensity for proteoliposomes with CF_oF_1 ATPase is about 7.5%, estimated from $(I_{max}-I_{min})/I_{min}$, which is higher than that in ATPase/lipid-coated capsules (about 1.5%). This might be ascribed to the fact that the head group of the ATPase is hindered during ATP synthesis due to steric constraints caused by interactions between the cationic-polymer layer and the enzyme. The slope of Figure 4d is the opposite of that in Figure 4b. This is because the head group of the ATPase reconstituted in liposomes faces toward the exterior.[4–8]

To detect the activity of CF_oF_1 ATPase reconstituted in the capsule's outer shell, we measured ATP amounts by employing the luciferin–luciferase system. We terminated the reaction after 1 min by addition of trichloroacetic acid (final concentration of 0.4%) to the microcapsule suspensions. For ATP measurement, the above solutions (5 μL) were added into a luminometer cuvette and then mixed with luciferin–luciferase reagent (50 μL); this was immediately followed by measurement of the luminescence on a recorder. ATP production was measured at different reaction times. For comparison, we destroyed the lipid bilayer on the outer shell of the capsules by adding Triton X-100 (0.1%) to a capsule suspension. In this case, the permeability of the capsules was enlarged and ATP encapsulated in the interior of the capsules could be released. The changes of ATP production with the reaction time are shown in Figure 5. It can be seen that the ATP concentration continuously increases over the 100-s reaction period and, more importantly, it is obviously higher after addition of Triton X-100, a result indicating that ATP has been synthesized inside the microcapsules.

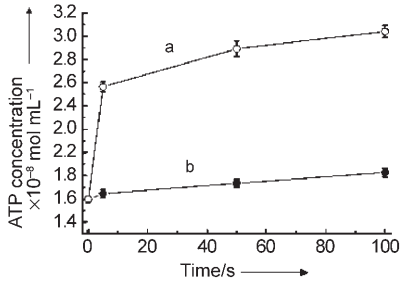

Figure 5. ATP biosynthesis as a function of reaction time in CF_oF_1/lipid-modified polyelectrolyte microcapsules a) in the interior and exterior solutions after addition of 0.1% Triton X-100 and b) in the exterior solution.

As a desirable property, the permeability of the microcapsules can be easily adjusted in various ways, such as pH and temperature change.[16,17] So we can synthesize and encapsulate the ATP that is released under well-defined conditions.

In summary, we describe a novel biomimetic system with the assembly of F_oF_1 ATPase in lipid-modified microcapsules. The well-defined microcapsules may serve as containers for the storage of the synthesized ATP as an energy currency. By using this system, it becomes possible to study the function of ATPase in a biomimetic unit in detail. Furthermore, as vital activities need energy, ATP could be released from the assembled capsules to provide energy on demand. Hence, we

have built a micrometer-sized energy-storage device suitable to power biological activity.

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