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Confocal Laser Scanning Microscope: A Very Useful Tool in Multilayer Capsules

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1. Introduction

Liposomes are widely used as drug carriers to deliver functional compounds into the membrane. [1-3] However, the liposomes from phospholipids are not stable even in the living system. Much attention has been drawn to the mixed polyelectrolyte/lipid system and all of these methods have been commonly recognized to stabilize the mixed film and assigned lipids some new features. [4-6] In order to prepare stabilized vesicles with a defined size, an alternative strategy has been developed by employing layer-by-layer technique. PE multilayers were assembled on the colloidal particles and the colloidal particles were selectivley sacrificed by dissolution to obtain hollow multilayered PE capsules. Phospholipids are adsorbed on these capsules. By this way, giant liposomes can be obtained with a defined size, shape and such giant liposomes are very stable from months to years. [7-19]

Based on our past work on the successful fabricating giant liposomes, a deeper and systematic understanding of the multilayer versatility regarding their functions and properties is clearly necessary. It was possible to change the permeability by pH, salt concentration and temperature for a limited number of systems. [8-10] In this paper, multilayered PE capsules fabricated with Layer-By-Layer (LBL) method were employed as supports and then the lipid bilayers were assembled on them, thus giant liposomes were obtained and their sizes were determined by the supports' size. Their permeabilities will be tuned by the lipid coating and the surface enzyme catalysis reaction. The small fluorescence molecule 6-CF diffusion behavior across the capsule's wall was studied by Confocal Laser Scanning Microscopy (CLSM), which may give information on the permeability control of "artificial cell". All the experimental results were got at room temperature.

2. Materials and methods

2.1 Materials

The sources of chemicals were as follows: poly(styrenesulfonate, sodium salt) (PSS, Mw 70,000) and poly(allylamine hydrochloride) (PAH, Mw 70,000) were from Aldrich. L-a-Dimyristoylphosphatidic acid (DMPA), 6-carboxyfluorescein (6-CF) were purchased from Sigma. All these materials were used as received.

The water used in all experiments was prepared in a three-stage Millipore Milli-Q Plus 185 purification system and its resistivity is higher than 18.2 MΩ cm^{-1}.
Positively charged melamine formaldehyde particles (MF particles) with a diameter of around 2.85 ± 0.09 μm were obtained from Microparticles GmbH, Berlin.

2.2 Polyelectrolyte shells prepared through layer-by-layer adsorption

Multilayer assembly was accomplished by the adsorption of polyelectrolyte at a concentration of 1 mg/ml, 0.5 M NaCl aqueous solutions. Oppositely charged polyelectrolyte species were subsequently added to the suspension followed by repeated centrifugation cycles. After the expected number of layers was adsorbed, 0.1 M HCl was used to remove the core (MF particles) and the obtained hollow polyelectrolyte shells were obtained. [14] PSS is used to form the first layer and the outermost layer is PAH, which is positively charged in order to bind the negatively charged phospholipids in the next step.

2.3 Preparation of phospholipid solution

The phospholipid DMPA was dissolved in a mixed solvent of chloroform and methanol (V_{CHCl3}:V_{MeOH} =1:1) with a concentration of 0.5 mg/ml. After the solvent was evaporated in a rotavap at 30 °C, water was added to a final lipid concentration (C_{DMPA} =0.025 mg/ml), and sonicated for 5 min. Lipid solution was added into polyelectrolyte shells and allowed 5 min for adsorption. The mixed solution was washed for three times with water in order to remove the non-adsorbed materials by centrifugation.

2.4 Measurements of confocal laser scanning microscopy (CLSM)

The images of capsules were obtained by a Leica confocal scanning system. A 100* oil immersion objective with a numerical aperture of 1.4 was used. In the experiment, 6-Carboxyfluorescein (6-CF) was selected as hydrophilic fluorescence dyes. The Raman spectra before and after reaction were also measured with CLSM.

2.5 Measurements of fluorescence recovery after photobleaching (FRAP)

In order to study the penetration of the dyes across the wall, the dyes inside the capsules were photo-chemically bleached. To do this, an argon ion laser from the CLSM emitting at a wavelength of λ= 488 nm was used. The laser beam was focused onto the selected area inside the capsule. The time for bleaching was long enough to ensure that almost all dye molecules in the selected area were bleached. Imaging was typically performed at rather low laser intensity. The interval between each image scan varied with the duration of the recovery established at an initial pilot experiment. Recovery was considered completely when the intensity of the photo-bleached region was stable and the curve was flat. For quantitative analysis, the fluorescence intensity was integrated by tracing a fixed area in the interior (ROI analysis system provided by the CLSM software), giving an intensity value for each time point.[20]
It should be pointed out that there is always a small amount of fluorescence dyes lost during repeatedly scanning and recovering. However, this will not influence the experimental results.

2.6 Measurements of single particle light scattering (SPLS)

Measurements were conducted on a home-made photometer equipped with an argon laser, Innova 305 from Coherent, with power track. The equipment has been described earlier. [11]

The dispersion is pressed through a thin capillary with an orifice of 0.1 mm at the end. Hydrodynamic focusing is applied. Particle concentration is adjusted to minimize coincidences. The scattering volume is 1.7×10^{-9} cm^{-3}. Forward scattered light pulses recorded from particles flowing through the scattered volume are detected between 5° and 10°. Intensity distributions are obtained with a resolution of about 0.5%.
The Rayleigh-Debye-Grans theory was used for evaluating the scattering data. The refractive index of the melamine core was assumed as 1.53. This value was obtained by extrapolating concentration dependent measurements of the refractive index of melamine formaldehyde solutions by means of an Abe refractometer. The refractive index of the polyelectrolyte multilayer was taken as 1.47. [22] The scattering intensity distribution of the core particles $\varphi(l)$ is converted into a distribution of the particle radius $\varphi(r)$. To this aim the recorded intensity is calculated as the superposition from the scattered light from the interior of the shell wall.

3. Results and discussions

3.1 Diffusion study of multilayered PE capsules

Fig.1a shows CLSM images of the obtained multilayered $(PAH/PSS)_5$ capsules, the intact capsules are the closed cycles (white arrow). The small fluorescence molecule 6-CF solution was mixed with the capsules' solution in order to study the capsule's permeability. Fig. 1b gives out a further magnification CLSM image. From the picture, one can see that both the inside and the outside of the capsules are bright, i.e., filled with fluorescence molecules, which present that the capsules are permeable to small molecules 6-CF. There are unclosed cycles (black arrow) which present the capsules are imperfect. AFM measurements will give more details about intact and imperfect capsules, shown in Fig.2. The brightest part is because of the water evaporation which results in the wall collapse of the capsules. Comparing Fig.2a with 2b, one can find that the intact capsule is a closed ball while the imperfect capsule is not a closed ball. Since the permeability of intact capsules can only be controlled by environment, we will study them in our research work.

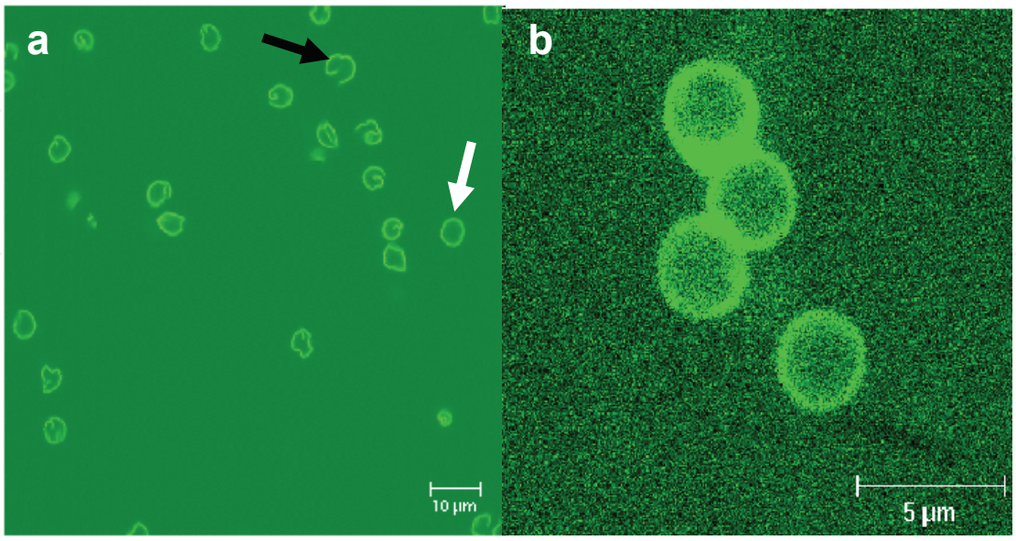


Fig. 1. CLSM images of multilayered PE capsules (a) many imperfact capsules (b) intact capsules

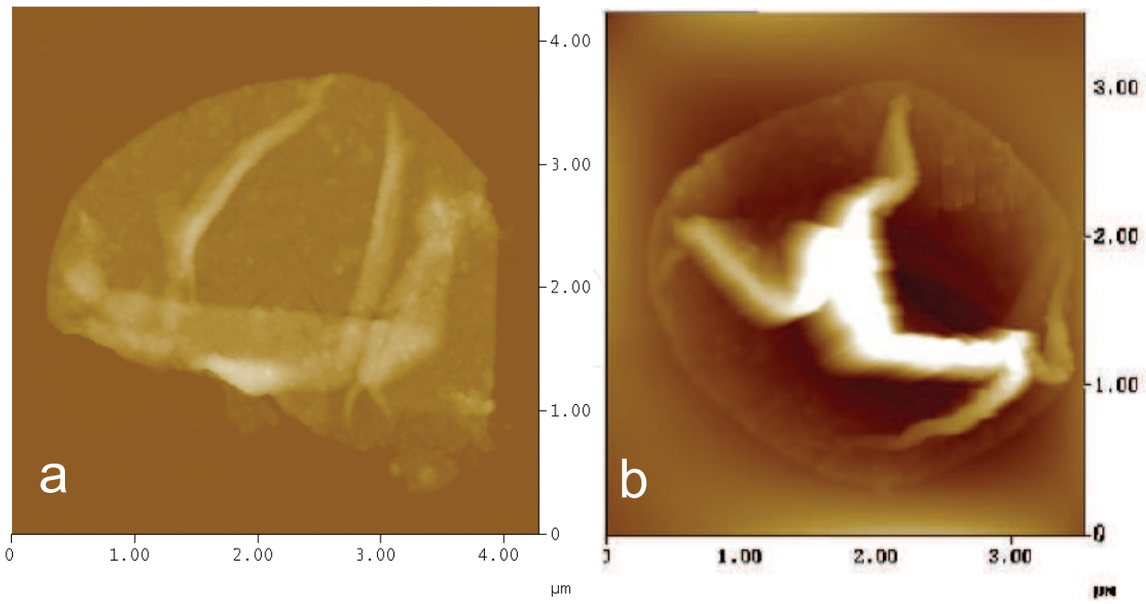


Fig. 2. AFM images of (a) un-intact capsules (b) intact capsules

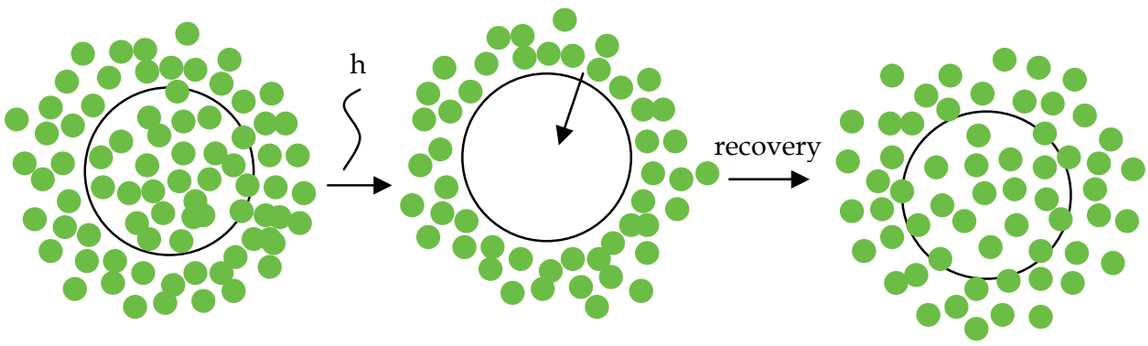


Fig. 3. Scheme for FRAP measurement

To quantify the penetration of the dye molecules, the dyes inside the capsule were bleached photochemically and the fluorescence intensity was recorded due to the penetration of the dye molecules from outside into the capsules with the time. [20] Fig. 3 gives the scheme of the FRAP (fluorescence recovery after photobleaching) experiment. Only permeable capsules can be carried such experiment. Firstly, a permeable capsule was grasped; secondly, the laser was focused onto the spot inside of the capsule, with a maximum bleaching power, most of the dye molecules lost their fluorescence. A dark center inside the capsule would be observed. The third step was to stop bleach the inside of the capsules; and the fourth step is that the outside fluorescence molecules will diffuse through the capsule's wall to reach the inside of the capsules; finally, after a certain time, the fluorescence molecules will arrive an equilibration state between inside and out side of the capsules. The recovery of the fluorescence intensity inside the capsules as a function with time will be recorded automatically by CLSM. Selected CLSM images were shown in Fig.4 during FRAP process and every image was correspond to one step, respectively.

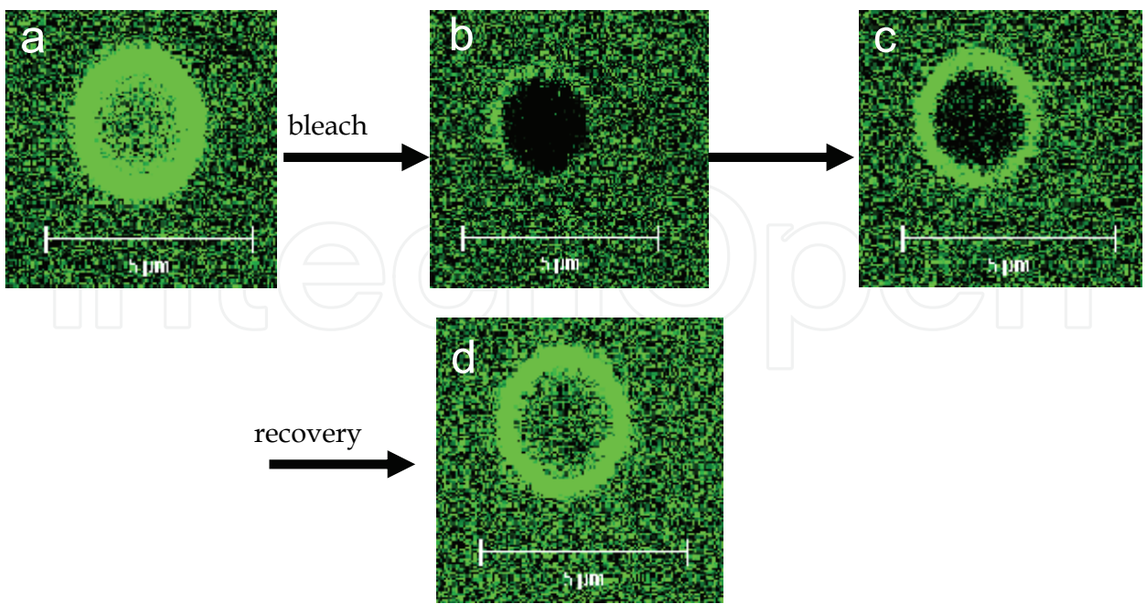


Fig. 4. CLSM images selected from FRAP measurements (a) original permeable capsule (b) bleached capsule (c) recovering capsule (d) recovered capsule

In order to quantity the diffusion coefficient for the small dye 6-CF passing through the multilayered wall, the obtained function between fluorescence intensity to the recovery time will be fitted as shown in Fig.5 (the fitted curve is marked as red line) and they can be described by Equation (1) [20-22]

$$I = I_0(1 - \exp(-t / T_D) \tag{1}$$

Where I_0, I presents the original fluorescence and final equilibrium fluorescence intensity, respectively. T_D is the curve's slope.
In solution, it will obey Fick law, and it can be written as the following Equation (2):

$$\frac{dc}{dt} = -A(C_0 - C) \tag{2}$$

Where C_0, C presents the dye concentration outside the capsules and inside the capsules, respectively. In solution, when small dye molecules pass through a wall with a certain thickness and diameter, A is equal to $1/T_D$. So, for the capsule's diffusion coefficient can be written as the Equation (3):

$$A = 3D / rh \tag{3}$$

Where r is the capsule's radius, h is the capsule's thickness, and D is the diffusion coefficient. Among them, the value for A can be obtained in the fitted function. r can be obtained from the template, h can be obtained by single particle laser scattering (SPLS) or roughly from Atomic Force Microscopy(AFM). For Fig.5, the A=2.05, r is about 2.85 ± 0.09μm, h is about 20 ± 5nm which is measured by SPLS shown in Fig.6 in this study. The diffusion coefficient can be obtained:it is 0.59×10^{-12} cm^2/S, which is higher than the one on

the flat surface but is consistent with the reference. [13] The possible reason behind is that the structure of the capsule's wall (multilayered PE films) was damaged during the removal the core.

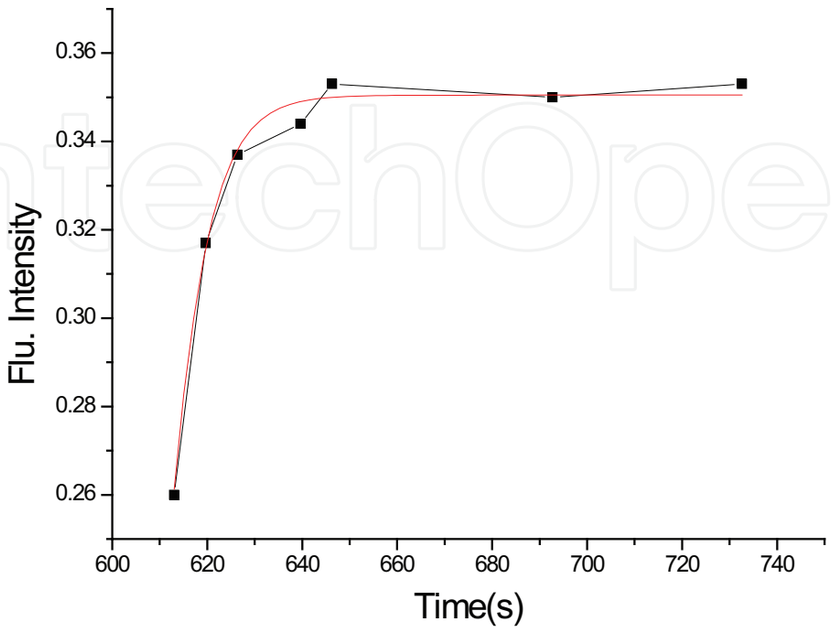


Fig. 5. Recovery profile for pure PE capsule measured by CLSM (black dot is recorded by CLSM, red line is the fitted curve)

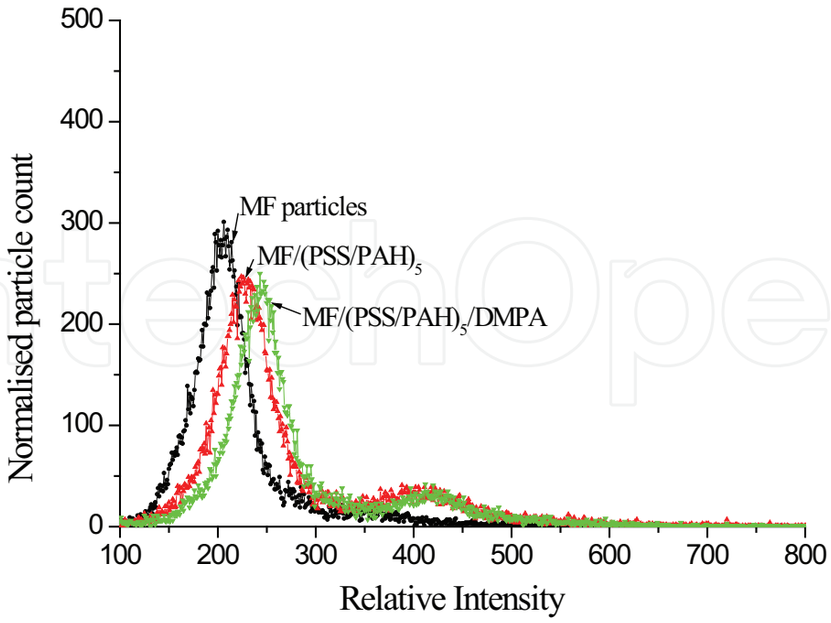


Fig. 6. Normalized light scattering intensity distributions of uncoated MF particles, MF coated with $(PSS/PAH)_5$ and MF/$(PSS/PAH)_5$/DMPA, respectively. The diameter of MF particles used in experiment is 1.08 ± 0.10 μm.

3.2 Assembly of phospholipid vesicles onto polyelectrolyte capsules

According to our previous research, [11-14] one can control the capsules' permeability to small molecules 6-CF by partly coated by phospholipids, which is still permeable to 6-CF but the capsules' surface will become more smooth than the pure multilayered PE capsules, i.e., the mean roughness of the pure multilayered capsules decreases from about 10 nm to 5-6 nm after the phospholipid partly coating on the capsules (the scheme shown in Fig. 7). [12] In this paper, we will study the diffusion behavior for small fluorescence molecules passing through the phospholipid partly coated capsules' wall by CLSM. The relationships between the recovery fluorescence intensity and the recovery time (black line) and the fitted function (red line) are shown in Fig.8. According to the Equation (3), one can obtained the diffusion coefficient is 0.49×10^{-13} cm^2/S. This value is much smaller than that of the pure PE capsules, therefore, the phopholipids on the surface prevent the dye passing through the wall quickly.

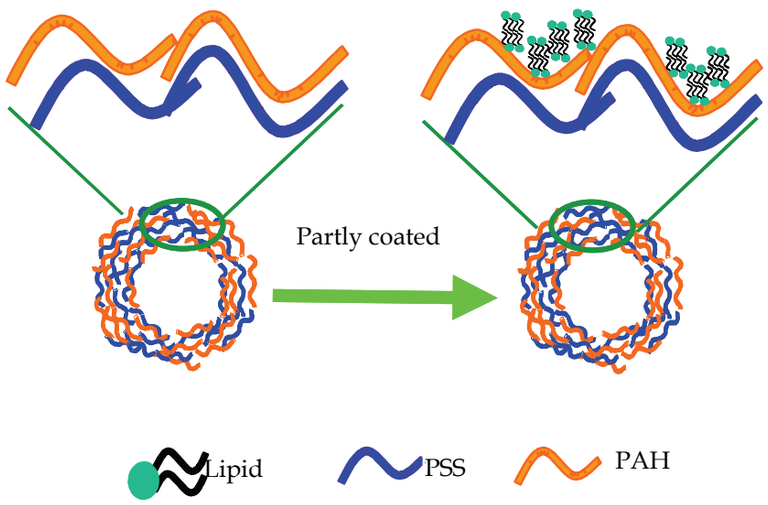


Fig. 7. Scheme for lipid partly coated PE capsules

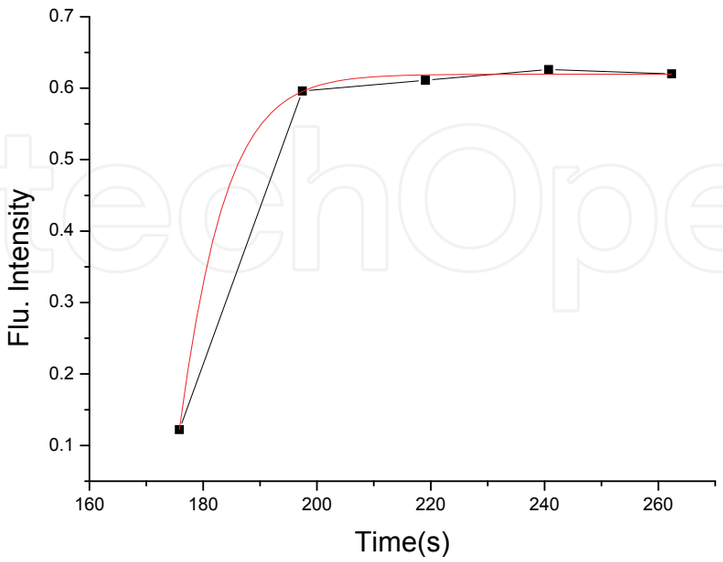


Fig. 8. Recovery profile for lipid partly coated PE capsules measured by CLSM (black dot is recorded by CLSM, red line is the fitted curve)

3.3 Lipid fully coated PE capsules in EtOH solution

According to our previous results [11-14], pure PE capsules are permeable to small molecules 6-CF, the fully coated with phospholipids capsules are impermeable to 6-CF. i.e., one can control the capsule's permeability to 6-CF by fully coated with phospholipids. The phospholipid fully coated capsules' surfaces become much smoother than the pure multilayered PE capsules', [12] the mean roughness decreased from about 10nm for the pure PE capsules to 3-4 nm for the lipid fully coated capsules measured by AFM. However, if such phospholipid fully coating multilayered capsules were treated with EtOH, the intact structure of the phopholipids on the surface will be destroyed and the capsules become permeable again to 6-CF. As a result one can realize the task to tune of the capsule's permeability. Small molecules 6-CF to such capsules' diffusion behavior was studied by CLSM and the recovery fluorescence intensity with a function of time was recorded in fig. 9 as black line and the red line is fitted function. According to Equation (3), the diffusion coefficient is 0.67×10^{-12} cm^2/S, it is similar to that of pure multilayered PE capsules. From this result, one also can see that after treated with EtOH, the intact structure of the lipids was destroyed completely which changes the capsule's permeability.

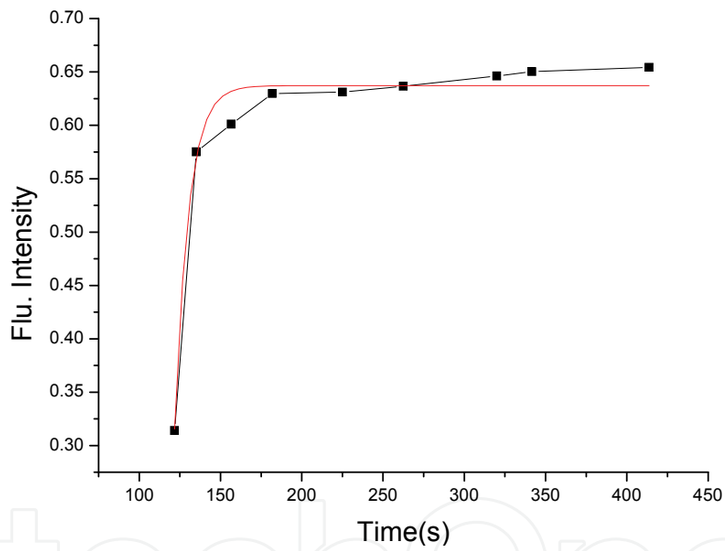


Fig. 9. Recovery profile for the product from EtOH treated lipid completely coated PE capsules (black dot is recorded by CLSM, red line is the fitted curve)

3.4 Lipid fully coated PE capsules catalyzed by PLA_2

PLA_2 is a small, stereo-selective, calcium-dependent enzyme that hydrolyzes the sn-2 ester linkage of phosphatidylcholine.[23-24] In the present work, we chose the mixed-lipid system, l-α-dimyristoylphosphatidic acid (DMPA), which is less hydrolyzed by PLA2 but is better suited for the attraction to the charged polyelectrolyte by electrostatic interaction, and l-α-dipalmitoylphosphatidylcholine (l-DPPC), which is easily cleaved by PLA2 (Fig.10), to cover the capsule's surface and obtained lipid coated multilayered capsules which are impermeable to 6-CF. By making use of the selectivity of the cleavage reaction, there are channels produced on the surface of hollow polyelectrolyte capsules to enable controlled the

capsule's permeability, i.e., the product multilayered capsules are permeable to 6-CF. Confocal laser raman spectra (Fig.11) were employed to confirm that after enzyme catalyst reaction one of the products: fatty acid was removed after washing by centrifugation because no special -COOH stretch peak at 1750 cm^{-1} of fatty acid was observed. To study the 6-CF molecules passing through the product capsule's wall, FRAP measurement by CLSM was carried out and the result was shown in Fig.12 (black line is the recovery fluorescence intensity with a function of time, red line is fitted function for the black one). According to Equation (3), the diffusion coefficient is 0.55 × 10^{-13} cm^2/S. This value is about an order of

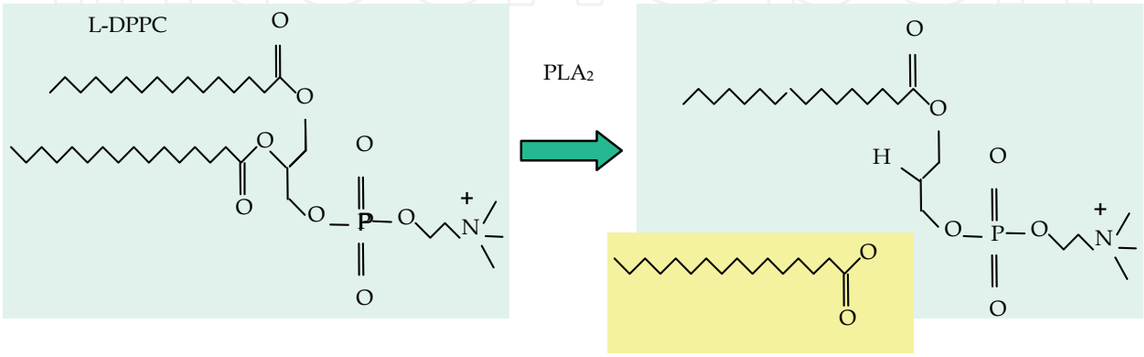


Fig. 10. Scheme for PLA_2 catalyzed l-DPPC reaction

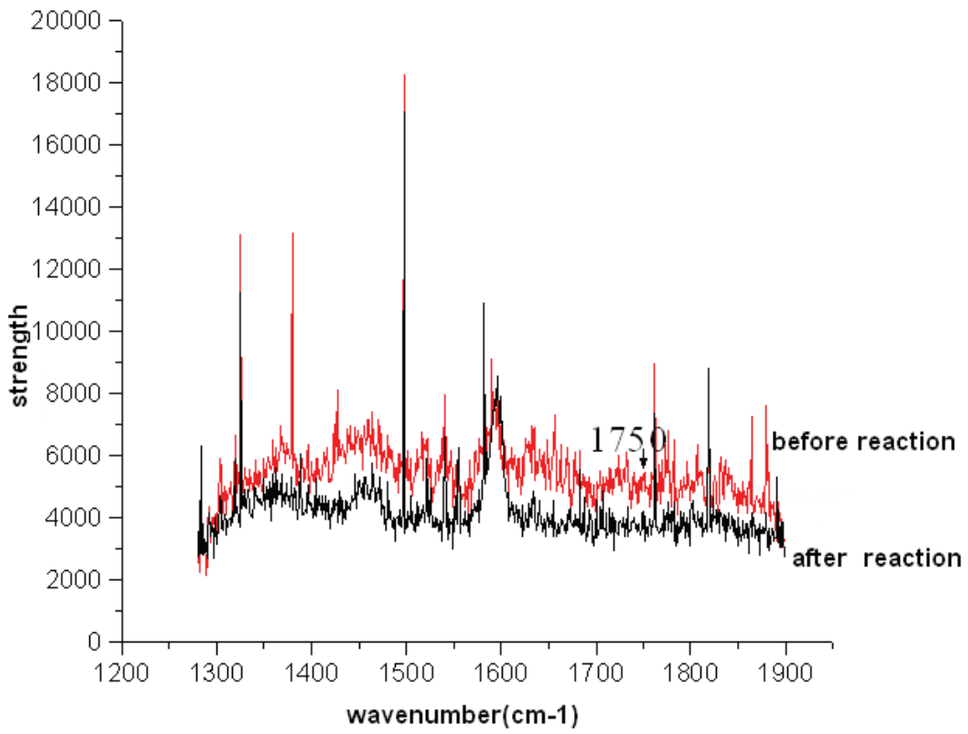


Fig. 11. Confocal laser raman spectra of capsules before and after reaction

magnitude lower than the value measured for the diffusion of dyes across polyelectrolyte multilayer capsules. It indicates that there may be some sealing of holes in the polyelectrolyte by the products of the reaction that have not been removed. [11]

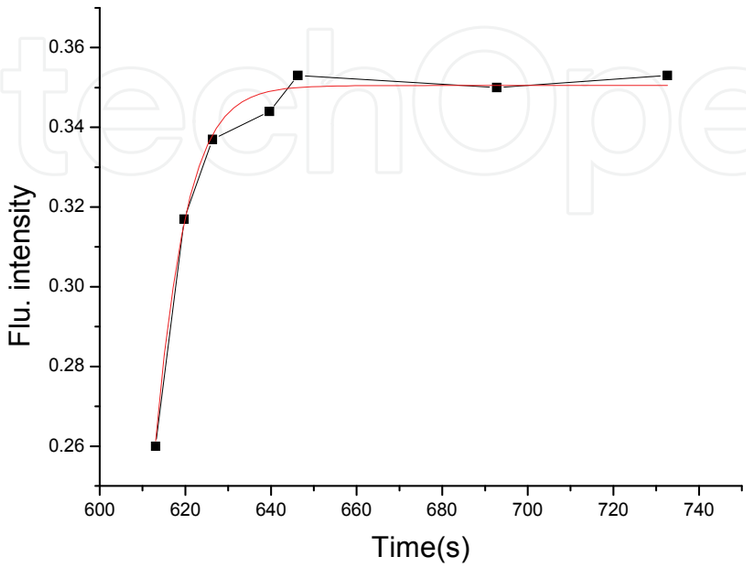


Fig. 12. Recovery profile for the product from PLA_2 catalyzed reaction on PE capsules (black dot is recorded by CLSM, red line is the fitted curve)

4. Conclusions

In this paper, Layer-By-Layer (LBL) self-assembly technique was employed to fabricate multilayered polyelectrolyte capsules, which are permeable for small fluorescence 6-CF molecules. In order to quantify dye's diffusion behavior across the multilayered polyelectrolyte films, FRAP measurement was carried out by CLSM and the diffusion coefficient was obtained as 0.59×10^{-12} cm^2/S, which is much larger than the one on the flat surface but is consistent with the reference. It might due to the damage of capsule wall structure during the removal the core. Lipid coating on the PE capsules makes capsule's permeability to 6-CF changing from permeable to impermeable. There are two ways to tune such lipid coated multilayered PE capsules' permeability, there are two ways: the first one is treated it with EtOH; the other one is to be catalyzed by phospholipids A_2. Both of them can reverse lipid coated multilayered PE capsules' permeability from impermeable to permeable to 6-CF. FRAP measurements were carried out to such two products and diffusion coefficients were obtained, 0.67×10^{-12} cm^2/S, 0.55×10^{-13} cm^2/S, respectively. Comparing it with the one of pure multilayered PE capsules and the one of lipid partly coated multilayered PE capsules, one can find that the diffusion coefficient of the product by EtOH treatment is similar to the one of the pure multilayered PE capsules. Thus, we can conclude that after treated with EtOH, the intact structure of the lipids was destroyed completely and

there are almost no lipids on the surface. While the diffusion coefficient of the product by PLA_2 catalyzed reaction, it is similar to the one of lipid partly coated capsules, this indicates that there might be some sealing of the holes in the polyelectrolyte by the products of the reaction which have not been removed.

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Ever since the invention of laser by Schawlow and Townes in 1958, various innovative ideas of laser-based applications emerge very year. At the same time, scientists and engineers keep on improving laser's power density, size, and cost which patch up the gap between theories and implementations. More importantly, our everyday life is changed and influenced by lasers even though we may not be fully aware of its existence. For example, it is there in cross-continent phone calls, price tag scanning in supermarkets, pointers in the classrooms, printers in the offices, accurate metal cutting in machine shops, etc. In this volume, we focus the recent developments related to laser scanning, a very powerful technique used in features detection and measurement. We invited researchers who do fundamental works in laser scanning theories or apply the principles of laser scanning to tackle problems encountered in medicine, geodesic survey, biology and archaeology. Twenty-eight chapters contributed by authors around the world to constitute this comprehensive book.

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