DOI 10.5012/bkcs.2011.32.2.620

Dynamic Research of a Potential Carrier for Hydrophobic Compound Model Pyrene Using Amphiphilic Peptide EYK

Liang Wang and Xiao-jun Zhao[†,*]

West China Hospital Nanomedicine Laboratory, Center for Regenerative Medicine and Institute for Nanobiomedical Technology and Membrane Biology, West China Hospital, Sichuan University, Chengdu 610065, Sichuan, P. R. China
[†]*Center for Biomedical Engineering, NE47-379, Massachusetts Institute of Technology, Cambridge, MA 02139-4307, USA*
[*]*E-mail: xiaojunzhao08@gmail.com*
Received November 23, 2010, Accepted December 14, 2010

In recent years, the study of self-assembly peptide used in drug delivery system has been attracted great interest from scientists. In the category are self-assembly peptides in the structure either with one hydrophobic surface and another hydrophilic or a hydrophobic head and a hydrophilic tail. Here, we focus on a novel designed peptide EYK with double amphiphilic surfaces, investigating on the capability of peptide as a carrier for hydrophobic compound model pyrene. The fluorescence data presented the dynamic process of the transfer, showing that the pyrene was in the crystalline form in peptide solution, and molecularly migrated from its peptide encapsulations into the membrane bilayers when the peptide-pyrene suspension was mixed with liposome vesicles. The results indicated that the peptide EYK could stabilize hydrophobic pyrene in aqueous solution and delivered it into EPC liposome as a potential carrier.

Key Words: Amphiphilic peptide EYK, Pyrene, Hydrophobic compound carrier, Fluorescence spectroscopy, Dynamic process

Introduction

It is crucial in pharmaceutical industry for hydrophobic compounds to fix out the problem of lower solubility in aqueous solution and exploit an appropriate vehicle to deliver them to the target cell or tissue. The discovery of the self-assembly peptide, emerged as a promising nanomaterials, has chart a new direction for us. This category nanomaterial has been widely used in the area of nanoscience and biomedical engineering such as cell and tissue cultures,[1-4] biological surface engineering,[5-6] membrane protein stabilizations.[7] In recent years, these peptides have also been attracted great interest from scientists used for drug delivery system.[8-10]

As we know, the self-assembly peptides lead to a unique amphiphilic structure because of numbers of hydrophobic and hydrophilic residues in the amino acid sequence. The property made them wide useful for combination with drug molecules. In order to investigate the drug delivery system of peptide and to improve the compatibility between peptides and hydrophobic compound, a novel type of peptide need to be designed. In this research, we employed a newly designed lego-type peptide EYK with double amphiphilic surfaces, different from the classic ones with one hydrophobic surface and another hydrophilic, for the delivery system.[11] It is expected that the peptide EYK could stabilized the hydrophobic drug model pyrene and transfer the pyrene to EPC vesicle. The steady-state fluorescence spectroscopy could exhibit the dynamic process of transfer.

Experimental Part

Chemicals. The original peptide (EYK short for sequence: AEAEYAKAK; theoretical mass 980.02) used in our study was commercially synthesized and purified (> 95%) by Shanghai Bootech Bioscience & Technology Co. Ltd. Egg Phosphatidylcholine (EPC) was purchased from Sinopharm Chemical Reagents Co., Ltd., Shanghai, China. Pyrene (99%) was purchased from Sigma Aldrich and was recrystallized three times before use. The rest chemicals used in the experiment were acquired from Chengdu Kelong Chemical Reagents Co., Chengdu, China. EPC liposome was prepared by the film-ultrasonic method.[12] The size of liposome was monitored by using a Malvern Zetasizer Nano ZS analyzer and the lipid concentration was determined. All aqueous solutions were prepared using deionized water (Elix Water Purification System, Millipore, MA, U.S.A.).

Preparation of Colloidal Suspensions of Pyrene Crystals. Appropriate pyrene was added into freshly prepared peptide EYK solutions in a 10 mL vial. The mixed peptide-pyrene solutions were prepared to obtain the concentrations of 0.5 mg/mL for pyrene and peptide. The sample was kept on magnetic stirrers until equilibrium was reached in about 72 h. The solution was deemed at equilibrium when their fluorescence spectrums did not change in 24 hours.

Steady-state Fluorescence Measurements. Fluorescence spectra measurements were performed on a Hitachi F-7000 spectrofluorophotometer with a stir accessory at room temperature. The solutions were operated in a quartz fluorescence cuvette of 1 cm^2 cross-section. The following parameters were used in experiments except special indication. Excitation and emission slits were both set to 2.5 nm. The excitation wavelength was set to 336 nm, and the emission fluorescence spectra were detected from 350 to 600 nm, with scan speed of 240 nm/min; response time, 0.1 s; PMT Voltage = 400 V Excitation spectra were recorded at selected emission wavelength (373 or 470 nm), with scan speed of 240 nm/min.

Pyrene Transfer Experiments. For the experiment of the migration of pyrene from the EYK-pyrene solution into the EPC

liposome, appropriate volume solution of the EYK-pyrene was added to liposome vesicles in quartz fluorescence cuvette (referred to as EPC-EYK-pyrene). All samples should be prepared in less than 20 s before fluorescence measurement initiated. To get the kinetic data for pyrene transfer, a time-dependent fluorescence measurement was required by recording I_m (fluorescence intensity of the pyrene monomer) at 373 nm for 4 hours intervals. In order to avoid the influence of xenon lamp fluctuations, a degassed and sealed solution of pyrene (4×10^{-5} M) dissolved in ethanol was used a standard pyrene solution. Each I_m- versus-time curve was corrected by dividing I_m by I_s.

Atomic Force Microscopy (AFM) Observation. The AFM was employed to detect nanostructures of pyrene crystals and EYK-pyrene complexes. A droplet (about 5 uL) of sample was deposited on a clean mica surface. Each aliquot was left on the mica for 1 min, then washed with deionized water, and dried in air for about 20 min. The samples were covered with Petri dishes to avoid contamination and air-dried for AFM observation. AFM was performed at room temperature using the tapping mode on a SPI4000 Probe Station and SPA-400 SPM Unit (Seiko Instruments Inc., Chiba, Japan). All the height images were recorded with 512 × 512-pixels resolution.

Scanning Electronic Microscopy (SEM) Observation. SEM (JSM-5900LV, JEOL Ltd., Tokyo, Japan) was used in the study of the microstructure and dimension of the peptide-pyrene complexes. An aliquot of 20 uL EYK-pyrene solution was placed on a freshly cleaved mica surface. After 10 minutes, the mica surface was rinsed twice with pure water (each time 200 μL) and air-dried overnight. After gilded, the complexes were imaged using the secondary electron (SE2) mode at 20 kV.

Fluorescence Microscopy. The sample was carried out with the same batch of EYK-pyrene solution used in the AFM and SEM experiments.

Results and Discussion

Interaction between the Peptide and a Model Hydrophobic Compound Pyrene. Pyrene was chosen as the hydrophobic drug model in the research because of its low solubility in water (about $4 \sim 7.0 \times 10^{-7}$ M in the saturated aqueous solution[13-14]) and wide application as hydrophobic fluorescence molecular probe.[15] In order to detect the encapsulation capability of the peptide, pyrene crystals were added into the aqueous solution in presence of the peptide EYK. It was expected to interact with hydrophobic cargo pyrene and stabilize it in aqueous solution because of the amphiphilic property. After a 3-hour-period magnetic stirring, the EYK-pyrene mixture exhibited turbid and milky-white in color while pyrene crystals in pure water either floated on liquid surface or sank to the bottom (Figure 1). The phenomena indicated that the pyrene crystals could be stabilized in the peptide solution *via* absorbing the peptide around the surface of pyrene microcrystals. That might be taken as the initial proof of pyrene encapsulation. Figure 2 revealed that the pyrene crystals in water aggregated together in size of thousands nanometers, while the particles of EYK and pyrene complex with uniform size much smaller those in water. It was further determined that the amphiphilic peptide had the capability of encapsulating pyrene in aqueous solution with small particles.

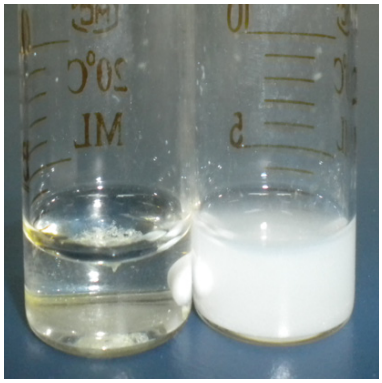

Figure 1. Pyrene crystals ([pyrene] = 2.5×10^{-3} M) in water (left) and with peptide EYK ([pyrene] = 2.5×10^{-3} M, [EYK] = 5.1×10^{-4} M = 0.5 mg/mL) in aqueous solution (right) after stirring both solutions for 3 h.

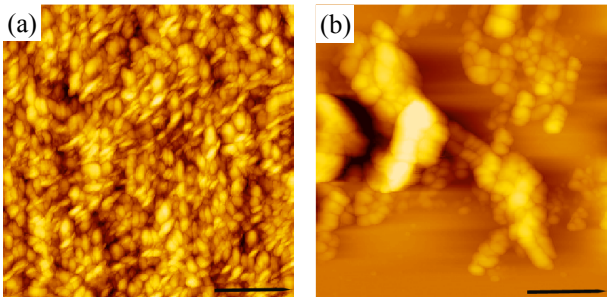

Figure 2. AFM images of pyrene crystals in different aqueous solutions after stirring for 72 h. (a) with peptide EYK in aqueous solution ([pyrene] = 2.5×10^{-3} M, [EYK] = 5.1×10^{-4} M); (b) In water ([pyrene] = 2.5×10^{-3} M); Scale of the black bar is 500 nm.

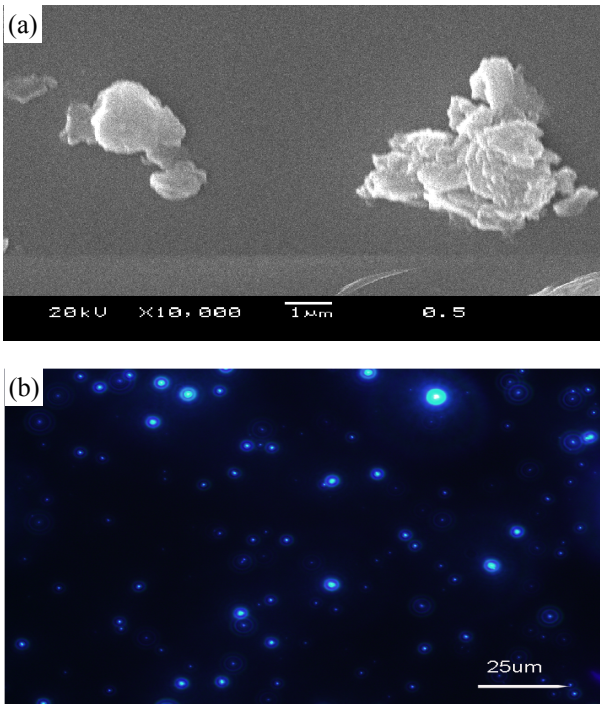

Figure 3. (a) Scanning electron micrographs of the pyrene crystals in peptide solution. (b) Fluorescence microscopy of the EYK-pyrene suspension. The image was observed and captured under excitation of a mercury lamp.

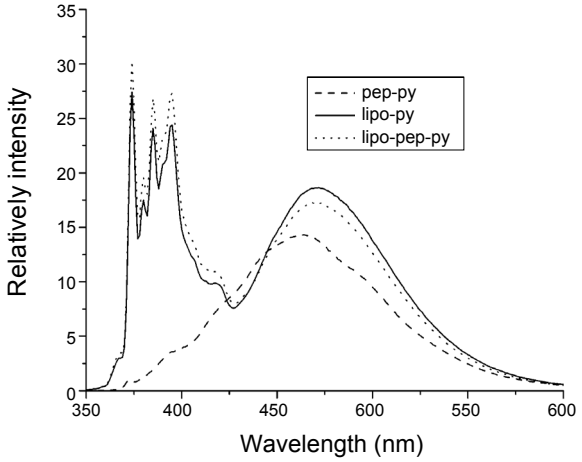

Figure 4. Fluorescence emission spectra of EPC-pyrene solution ([EPC] = 7.4×10^{-4} M, [pyrene] = 10×10^{-5} M), EYK-pyrene solution ([EYK] = 5.1×10^{-4} M, [pyrene] = 2.5×10^{-3} M), and EYK-pyrene solution mixed with EPC liposome ([pyrene] = 9.3×10^{-5} M, [EYK] = 1.92×10^{-5} M, [EPC] = 7.4×10^{-4} M).

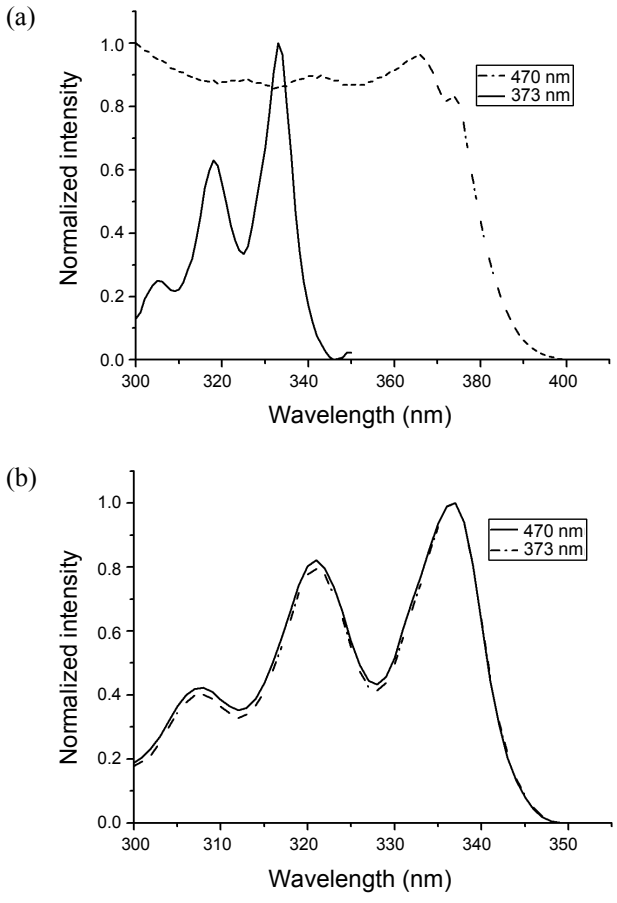

Figure 5. Normalized fluorescence excitation spectra of (a) EYK-pyrene solution ([EYK] = 5.1×10^{-4} M, [pyrene] = 2.5×10^{-3} M), (b) EYK-pyrne solution mixed with EPC vesicles ([pyrene] = 9.3×10^{-5} M, [EYK] = 1.92×10^{-5} M, [EPC] = 7.4×10^{-4} M).

Figure 3 showed us a clear view of pyrene crystals suspended in the peptide solution.

Transfer from Pyrene-peptide to EPC Vesicles. In the delivery system, a hydrophobic cargo must into a plasma membrane of the targeted living cell. As lipids are the essential components of cell membranes, we used EPC liposome as the cell mimic. The lipid concentration of the EPC vesicles used in this work was 7.4×10^{-4} M. Figure 4 displayed the fluorescence spectra of pyrene characteristic in different solvent. The pyrene spectroscopy in peptide solution was similar to the reported one in crystalline formation with a maximum intensity at 470 nm wavelength.[10,12,15] The emission spectrum of pyrene and the EYK-pyrene mixture added into EPC liposome were similar, both exhibiting a large amount of pyrene monomer peak at 373 nm and excimer peak at 470 nm. Furthermore, the pyrene has five different peaks between 373 and 470 nm in variation solvent polarity. And the defined emission intensity of I_1/I_3 (the emission intensity at 374 nm and 385 nm) was used as a scale for solvent polarity. This ratio equals 1.96 in polar solvents such as water and decreases with increasing solvent apolarity.[15-17] The ratios of I_1/I_3 for pyrene in liposome and the EYK-pyrene solution mixed with the liposome were 1.03 and 1.05, respectively. It indicated that the pyrene has transferred from the peptide encapsulation into the hydrophobic lipid bilayer. It has been reported that the pyrene excimer could be formed either *via* diffusional encounters between pyrene molecules (dynamic excimer) or by the direct excitation of pyrene crystals (static excimer).[12] The static excimer has a red-shift excimer spectrum while dynamic excimer doesn't.[18] As shown in Figure 5(a), the excitation spectrum at excimer emission had a red-shift about 40 nm compared with that at monomer emission. The result demonstrated that pyrene in the EYK-pyrene solution was in the crystalline form. On the contrary, as shown in Figure 5(b), the excimer spectrums totally inosculated at excimer and monomer emission without red-shit. It indicated that the pyrene excimer of the EYK-pyrene mixed with EPC liposome was formed *via* diffusional encounters of pyrene molecules. The results could further prove that the pyrene had dispersed and transferred from peptide encapsulation to the EPC vesicles.

Dynamics of the Transfer Rprocess. In order to investigate the dynamics of the pyrene transfer process, steady-state fluorescence spectra were employed after adding appropriate amount EYK-pyrene mixture into the EPC liposome. The monomer intensity was recorded at 373nm with four hour interval (Figure 6). Different amounts of EYK-pyrene solution were added to the liposome. The curve a with the final pyrene concentration of 6.2×10^{-5} M, obtained with continuous increase of Im during the first hour and then gradually reached a plateau after about 3-hour. At the lower pyrene concentration, the I_m increased with the pyrene disolved to the liposome. When all the pyrene transfered to vesicles the fluorescence intensity reached a plateau. Curve b of higher pyrene concentration exhibited a rapid increase during the first 10 minutes, and then decrease to a plateau in about 3 hours. When excessive pyrene-EYK solution were added to the EPC liposome, the I_m intensity increased drastically at the beginning. With the increase of pyrene molecules disolved into the liposome, the inner filter effect took place to decrease the I_m till the plateau reached.[19] Additionally, there were multiple scattering and potential quenching of the pyrene monomer disolved inside the EPC vesicles by the pyrene microcrystals. That might be the reason of why

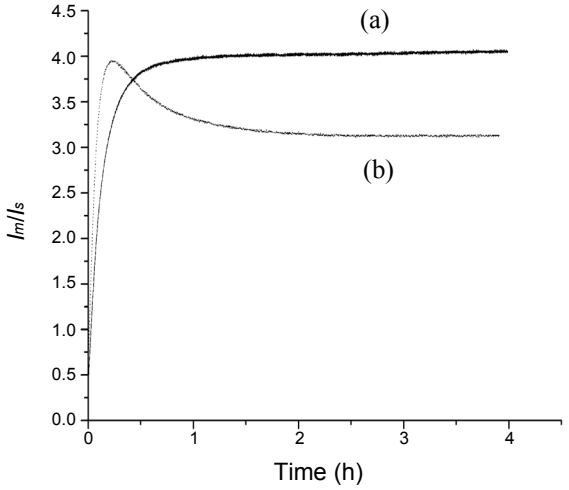

Figure 6. Fluorescence emission of the pyrene monomer at emission wavelength 373 nm when different amounts of EYK-pyrene solution are added to EPC vesicle solutions. The fluorescence intensity (I_m) is divided by that of the standard (I_s). Curve (a), data acquisition over a 4 h time span at the 1 s interval ([pyrene] = 6.2×10^{-5} M, [EYK] = 1.28×10^{-5} M, [EPC] = 7.4×10^{-4} M). Curve (b), data acquisition over a 4 h time span at the 1 s interval ([pyrene] = 9.3×10^{-5} M, [EYK] = 1.92×10^{-5} M, [EPC] = 7.4×10^{-4} M).

the fluorescence intensity was lower in the end of the experiment at higher pyrene concentration than that at lower pyrene concentration.[16,20] It was considered that the quantity of individual molecules dissolved inside the vesicles membrane was the main factor to affect the intensity of fluorescence under the same experiment condition.

Quantitative Release Curves of the Transfer Process. The quantity of pyrene molecules dissolved inside the EPC liposome membrane could be determined by the method described in ref. [1]. The calibration curve of I_m-*versus*-concentration of pyrene in EPC was shown in Figure 7(a). I_m was divided by I_s to avoid xenon lamp fluctuations.

The calibration curves also revealed the inner filter effect as I_m/I_s passes through a maximum, with little changes for pyrene concentrations between 2.5×10^{-5} and 3.5×10^{-5} M. Therefore, a gap was observed in the time-dependent concentration profile for pyrene located inside the vesicles, where no data points were reported for pyrene concentrations in this range. The concentrations of released pyrene molecules from different EYK-pyrene complexes into lipid membranes are shown in Figure 7(b). As time went on, more pyrene molecules were transferred from the EYK-pyrene complex into the lipid membranes. The final pyrene concentrations of profiles 1 and 2 were about 1.86×10^{-5} M and 9.3×10^{-5} M, respectively. Although it is difficult to establish a model describing the uniqueness of the pyrene transfer, the profiles shown in Figure 7(b) could be fit with Eq. (1).

$$[Py]_{(t)} = [Py]_{eq} - ([Py]_{eq} - [Py]_0)\exp(-k_{trans} \times t) \quad (1)$$

In Eq. (1), $[Py]_{(t)}$, $[Py]_{eq}$, $[Py]_0$, and k_{trans}, represented the pyrene concentration inside the EPC vesicles at time t, at equilibrium (infinite time), at time t = 0 s, and transfer rate constant, respectively. The release rate constants k of profile 1 and profile 2 were 1.93 ± 0.002 h^{-1} and 2.83 ± 0.04 h^{-1}, respectively.

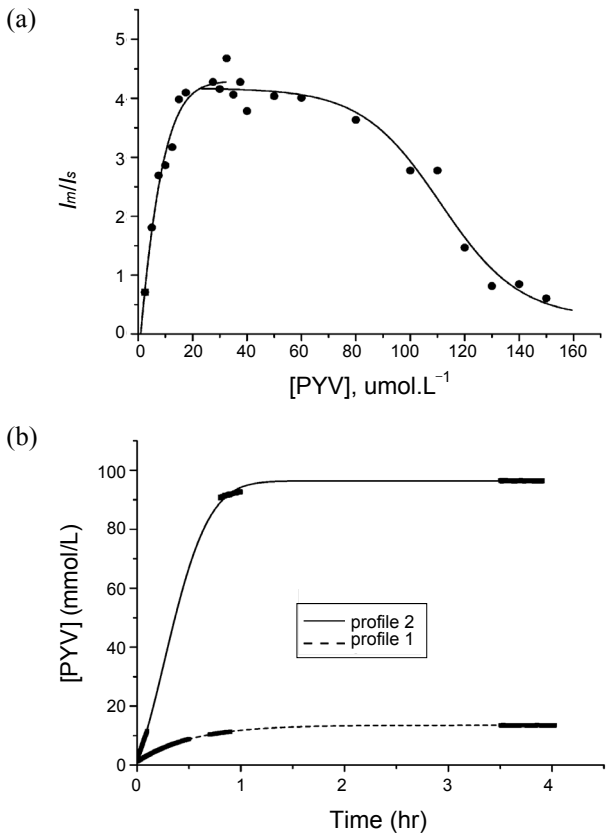

Figure 7. (a) Calibration curve for different pyrene concentrations in EPC vesicles ([EPC] = 7.4×10^{-4} M). The concentration of pyrene ranged from 2 μM to 160 μM. The fluorescence intensity (I_m) is divided by that of the standard (I_s). (b) Profiles for the release of molecular pyrene from peptide coated pyrene microcrystals into a solution of EPC vesicles according to the fluorescence results. The final pyrene concentrations are as follows: profile 1 ([pyrene] = 1.86×10^{-5} M); profile 2 ([pyrene] = 9.3×10^{-5} M). All release experiments reported in Figure 6 were carried out with the same batch of Peptide and the same stock solution of lipid used in the calibration curve.

Conclusion

This study has shown that the peptide EYK could stabilize the hydrophobic drug model pyrene in aqueous solution. The pyrene was in crystalline form in the colloidal suspension after being stirred with the peptide. However, pyrene molecularly migrated from its peptide encapsulations into the membrane bilayers when the peptide-pyrene suspension was mixed with EPC liposome vesicles. The dynamic process was investigated by the steady-state fluorescence spectra and the concentration of pyrene transferred into EPC liposome was determined as a time-dependant function. The transfer rate was employed to determine to quantify of the dynamic transfer behavior of pyrene. In summary, the peptide could be used as a potential carrier for the hydrophobic drugs and broaden the field of drug delivery system.

Acknowledgments. This work was financially supported by the National "985 Project" of the Ministry of Education of China to Sichuan University.

References

1. Zhang, S.; Gelain, F.; Zhao, X. *Semin. Cancer Biol*. **2005**, *15*, 5.
2. Mi, K.; Wang, G.; Liu, Z.; Feng, Z.; Huang, B.; Zhao, X. *Macromol. Biosci*. **2009**, *9*, 5.
3. Song, H.; Zhang, L.; Zhao, X. *Macromol. Biosci*. **2009**, *10*, 1.
4. Gelain, F.; Bottai, D.; Vescovi, A.; Zhang, S. *PLoS ONE*. **2006**, *1*, 1.
5. Qiu, F.; Chen, Y.; Tang, C.; Zhou, Q.; Wang, C.; Shi, Y. K.; Zhao, X. *Macromol. Biosci*. **2008**, *8*, 11.
6. Vauthey, S.; Santoso, S.; Gong, H.; Watson, N.; Zhang, S. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 8.
7. Zhao, X.; Nagai, Y.; Reeves, P. J.; Kiley, P.; Khorana, H. G.; Zhang, S. *Proc. Natl. Acad. Sci.* **2006**, *103*, 47.
8. Fung, S. Y.; Yang, H.; Chen, P. *Colloids and Surfaces B: Biointerfaces* **2007**, *55*, 2.
9. Segers, V. F. M.; Lee, R. T. *Drug Discovery Today* **2007**, *12*, 13-14.
10. Wang, J.; Tang, F.; Li, F.; Lin, J.; Zhang, Y.; Du, L.; Zhao, X. *Journal of Nanomaterials* **2008**, *2008*, 2008.
11. Wang, L.; Zhao, X. *Bull Korean Chem. Soc*. in press,
12. Keyes-Baig, C.; Duhamel, J.; Fung, S.-Y.; Bezaire, J.; Chen, P. *J. Am. Chem. Soc*. **2004**, *126*, 24.
13. Yekta, A.; Xu, B.; Duhamel, J.; Adiwidjaja, H.; Winnik, M. A. *Macromolecules* **1995**, *28*, 4.
14. Štěpánek, M.; Krijtová, K.; Procházka, K.; Teng, Y.; Webber, S. E.; Munk, P. *Acta Polymerica* **1998**, *49*, 2-3.
15. Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc*. **1977**, *99*, 7.
16. Pandeya, S.; Reddena, R. A.; Hendricksa, A. E.; Fletchera, K. A.; Palmer, C. P. *Journal of Colloid and Interface Science* **2003**, *262*, 2.
17. L'Heureux, G. P.; Fragata, M. *Biophysical Chemistry* **1988**, *30*, 3.
18. Gao, C.; Qian, H.; Wang, S.; Yan, D.; Chen, W.; Yu, G. *Polymer* **2003**, *44*, 5.
19. French, S. A.; Territo, P. R.; Balaban, R. S. *Am. J. Physiol. Cell Physiol.* **1998**, *275*, 3.
20. Daems, D.; Zegel, M.; Boens, N.; Schryver, F. C. *Eur. Biophys. J*. **1985**, *12*, 2.