Final Draft
of the original manuscript:

Li, X.; Haramus, V.M.; Li, N.; Zhe, Z.; Willumeit-Roemer, R.; Zou, A.:
Loading Psoralen into liposomes to enhance its stimulatory effect on the proliferation and differentiation of mouse calvarias osteoblasts.
In: Journal of Dispersion Science and Technology. Vol. 40 (2019) 11, 1531 - 1538.
First published online by Taylor & Francis: 11.09.2019

DOI: /10.1080/01932691.2018.1462196
https://dx.doi.org/10.1080/01932691.2018.1462196

Loading Psoralen into liposomes to enhance its stimulatory effect on the proliferation and differentiation of mouse calvarias osteoblasts

Xiaoran Li[a], Vasil M. Garamus[b], Na Li[c], Zhe Zhe[d], Regine Willumeit-Römer[b], Aihua Zou[a, *]

[a]Shanghai Key Laboratory of Functional Materials Chemistry, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, P.R. China

[b]Helmholtz-Zentrum Geesthacht: Centre for Materials and Coast Research, Institute of Materials Research, Max-Planck-Str. 1, D-21502 Geesthacht, Germany

[c]National Center for Protein Science Shanghai and Shanghai Institute of Biochemistry and Cell Biology, Shanghai 200237, P. R. China

[d]Shanghai municipal Hospital of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, 200237, P.R. China

∗ Corresponding author: Tel.: +86-21-64252231; Fax: +86 -21 -64252231.

E-mail address: aihuazou@ecust.edu.cn

Postal address: East China University of Science and Technology, Shanghai 200237, P.R. China

ABSTRACT

Psoralen (PSR), a well-known traditional Chinese medicine has been claimed for the treatment of osteoporosis. However, its hydrophobicity and the first-pass metabolism confined the potential application of PSR. Thus, the development of PSR-loaded liposome was done to improve the solubility and bioavailability of PSR. The obtained PSR/liposome exhibited a particle size of approximately 110 nm and was quite stable. The entrapment efficiency (EE), drug loading (DL) and zeta potentials of PSR/liposome were 85.0 ± 1.6%, 5.0 ± 1.6 % and -36 mV, respectively. Small angle X-ray scattering (SAXS) and transmission electron microscopy (TEM) measurements suggested that the morphology of obtained PSR/liposome was unilamellar and bilamellar vesicle. The *in vitro* release profile of PSR/liposome exhibited a gradual drug release. Both pure PSR and PSR/liposome promoted osteoblast proliferation in a dose-dependent manner. The proliferation effect was firstly enhanced with drug concentration increased, and then decreased when the concentration was higher than 20 μM. But the PSR/liposome could induce osteoblast proliferation in more gentle way through the sustained release of PSR. Furthermore, for the level of ALP activity, PSR/liposome was 1.2 times higher than pure PSR. Above all, it is expected that PSR/liposome could be used in osteoporosis treatment in the near future.

Keywords: Psoralen, liposome, osteoporosis, proliferation, differentiation.

INTRODUCTION

Osteoporosis is one of the most common human skeletal diseases, which could increase bone fragility and susceptibility to fracture since low bone-mass density and microarchitectural deterioration of bone tissue (Kim, et al., 2011 and Sinningen, et al., 2015). The National Osteoporosis Foundation (NOF) has been reported that approximately 10 million US adults aged 50 years and older had osteoporosis and an additional 33 million had low bone mass (Wright, et al., 2014 and Wright, et al., 2017). In China, the prevalence of osteoporosis has increased from 14.94% before 2008 to 27.96% during the period spanning 2012-2015, which affecting more than one-third of people aged 50 years and older (Chen, et al., 2016). Most osteoporosis is caused by increased bone resorption, many patients with osteoporosis have been treated with anti-resorptive drugs (estrogens, bisphosphonates, calcitonin), which could maintain bone mass by inhibiting osteoclast resorption (Lane, et al., 2003). However, the effect of these drugs on osteoblast formation and function is minor, no more than 2% per year for bone mass increase (Rodan, et al., 2000). In addition, the potential complications also limited their usage for osteoporosis treatment (Ducy, et al., 2000). Traditional Chinese herbal medicine have been widely used to treat osteoporosis for thousands of years, including Herba Epimedii (Yu, et al., 1999 and Meng, et al., 2005), Fructus Cnidii (Zhang, et al., 2010), Tanshinone (Cui, et al., 2004), et al.

As a well-known traditional Chinese medicine, natural coumarin compound Psoralen (PSR) is isolated from the dried fruit of *Psoralea corylifolia L*. PSR has many pharmacological effects, such as anti-bacteria effect, antitumor effect and broadening coronary artery effect (Ji, et al., 1999). In addition, it also could be used to treat vitiligo and

psoriasis. PSR ultraviolet A (PUVA) therapy has been approved for clinical use by US Food and Drug Administration (FDA) (Robert, et al., 2007). Recently, there were some research worker reported that PSR had the effect of stimulating new bone formation (Tang, et al., 2011; Li, et al., 2011; Xu, et al., 2011 and Wong, et al., 2011). Tang et al. found that PSR could promote osteoblast differentiation by up-regulating the expressions of genes and increasing alkaline phosphatase activity (Tang, et al., 2011). Li et al. reported that PSR was essential for osteoblast proliferation and differentiation (Li, et al., 2014). Xu et al. showed that PSR could activate chondrocytes from articular cartilage of the rat knee (Xu, et al., 2015). Wong et al. demonstrated that PSR mixed with collagen matrix had the effect of increasing new bone-forming locally (Wong, et al., 2011). However, the low solubility of PSR in biological fluids and extensive first pass metabolism had largely limited its potential application. Zhang et al. prepared PSR-loaded liposome with the method of ethanol injection to treat psoriasis, and it showed greater drug delivery and skin deposition than PSR tincture (Zhang, et al., 2014). Fang et al. developed PSR-loaded solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) to treat psoriasis, which increasing the skin permeability and the release controllability of PSR (Fang, et al., 2008). However, to the best of our knowledge, no report has been published about PSR/liposome on the proliferation and differentiation of osteoblast.

The aim of this study was to optimize the formulation of PSR-loaded liposome (PSR/liposome) with the thin film hydration followed by high-pressure homogenization method. In order to select the optimal formula, the L9 (3^3) orthogonal experiments were designed. The morphology of obtained optimal PSR/liposome was determined by

transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS). The release property of PSR from the liposome was studied *in vitro*. In addition, the regulatory effects of PSR/liposome on the proliferation and differentiation of mouse osteoplastic cells were examined.

MATERIALS AND METHODS

Materials

Psoralen (PSR, >98%, Chengdu Herbpurify Co., Ltd, China); soybean phosphatidylcholine (SPC, 95%, Shanghai Taiwei Pharmaceutical Co., Ltd., China); cholesterol (95%, J&K Scientific Co., Ltd., China); chloroform (Aladdin Chemical Reagent Co., Ltd., China); cell counting kit(CCK-8, Dojindo Laboratories, Japan); an alkaline phosphatase (ALP) activity kit (Nanjing Jiancheng Biological Engineering Institute, China); BCA Protein Assay Kit (Shanghai Beyotime Biotechnology, China).

Preparetion of liposome

Liposome was prepared by thin film hydration followed by high-pressure homogenization method (Mourtas, et al., 2011). In detail, a certain amount of SPC, cholesterol and drugs were placed into a flask and dissolved in the 30 mL chloroform. The flask was placed in rotary evaporator and dried for 30 min to form a thin film layer. Residual chloroform was removed under vacuum for 2 h. The lipid film was hydrated with PBS buffer (pH 7.4) and followed by shaken mechanically for 1 hour at 40 °C. This dispersion was homogenized through a high-pressured homogenizer (HPH ATS Engineering, Canada) with 5 cycles at 600 bars. In the end, liposome dispersion was placed at temperature above the transition temperature of lipid for 1 h to avoid stiff gel formation and all the liposome

suspension was kept at 4 °C until further testing (Kastellorizios, et al., 2012).

In order to optimize the formula of PSR/liposome, the drug entrapment efficiency (EE) was taken as an important index to evaluate the formula. Three factors and three levels were developed to study the optimum levels of these variables. Three factors included the phospholipid concentration ($mg \cdot mL^{-1}$, A), the weight ratio of phospholipid to cholesterol (w/w, B), and the weight ratio of phospholipid to drug (w/w, C). Those factors have been proved to be the most influential factors by single factor experiments. The factors and levels of orthogonal design were shown in Tables S1 and Tables S2. All tests were performed in triplicate.

Characterization of liposome

The stability of optimal PSR/liposome dispersion was characterized by size distribution, PDI and zeta potential using dynamic light scattering (DLS) with a DelsaTM Nano C Particle Analyzer (Beckman Coulter, USA) at proper time intervals (after 1, 7, 15 and 30 day storage). Each test was performed three times. The morphology of liposome was examined on a JEM-2100 transmission electron microscope (TEM, JEOL, Japan). Placed sample on carbon film copper grid and negative staining with 1.0% phosphotungstic acid. Small angle X-ray scattering (SAXS) experiments were performed to obtain the mean or global structure features of the liposome, using synchrotron light on the BL19U2 beamline of the National Center for Protein Science Shanghai at Shanghai Synchrotron Radiation Facility (Lv, et al., 2016).

Entrapment efficiency (EE)

The entrapment efficiency of the liposome was determined by the

centrifugal-ultrafiltration method using Amicon Ultra-4 centrifugal filter units (MWCO = 10 KD). In detail, 4 mL samples were placed in the upper chamber of centrifuge tube and centrifuged for 30 min at 8000 rpm at 4 °C. The un-encapsulated PSR from the ultra-filtrate was calculated according to the calibration curve by HPLC method. The mobile phase consists of methanol: water = 60:40 at a flow rate of 1 $mL \cdot min^{-1}$. The detection wavelength of UV detector was set to 246 nm (Zhang, et al., 2014).

The entrapment efficiency (EE %) was calculated by the formula given below:

$$EE\ \% = (1-\frac{C_U}{C_T})\times 100$$

Where C_U is the amount of un-entrapped drug, C_T is the total amount of drug used.

Psoralen release kinetics from the liposome

The *in vitro* release profile of PSR from liposome was performed using dialysis bag method. Briefly, PSR/liposome and pure PSR solution were taken into two dialysis bags (MWCO = 8-14kDa), respectively. The dialysis bags were then placed into the PBS release media (pH 7.4) and kept in a thermostatic shaker bath at 37 °C. 1mL of the release medium was sampled at predetermined time points (0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60 h), and replaced with 1mL of the fresh release medium. The amount of released PSR was analyzed by the method of HPLC.

Biological studies

Cell culture

Primary mouse calvarias osteoblasts MC3T3-E1 was purchased from Wuhan Boster Biological Technology co., ltd. Cells were cultured on 25 cm^2 flasks containing Dulbecco's Modified Eagle Medium (DMEM, Wuhan Boster Biological Technology co., ltd, China)

supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin (100 $U \cdot mL^{-1}$) at 37 °C in a humidified 5% CO_2 atmosphere.

Cell proliferation assessment

The effect of pure PSR, PSR/liposome and blank liposome on the proliferation of MC3T3-E1 cells were evaluated by CCK-8 assay. In brief, osteoblasts were seeded into 96-well plates at a density of 8×10^3 cells/well and incubated in 100 μL medium to allow cells attachment. After incubation 24 h, cells were treated with pure PSR, PSR/liposome (the concentrations of PSR was 0, 2.5, 5.0, 10 and 20 μM) and blank liposome (the same volume as that used for PSR/liposome). After cells were cultured for 48 h, 10 μL of CCK-8 was added to each well and plates were incubated for 1 h at 37 ºC. Finally, the absorbance was measured at 450 nm by using microplate reader.

Cell differentiation assay

ALP activity, as a marker of osteoblasts differentiation, was normalized for total protein content of the cell lysates (Wang, et al., 2012 and Zhang, et al., 2015). In detail, cells were cultured in the 6-well microplates at a density of 1×10^5 cells/well and incubated in 2 mL medium for 24 h to allow cells attachment. Cells were treated with pure PSR, PSR/liposome (the concentrations of PSR was 0, 1, 10, 100 μM) and blank liposome (the same volume as that used for PSR/liposome). After 48 h of incubation, cells were washed with PBS for three times, then lysed with lysis buffer consisted by 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, and 1% triton X-100 at -20 °C for 12 h to ensure cell membrane was collapsed completely (Liu, et al., 2015). ALP activity was assessed using the ALP assay kit and the protein concentration was determined using BCA protein assay kit.

Statistical analysis

All the experiments were repeated three times independently and the results were showed as mean ± standard error of mean. Results were analyzed using ANOVA and Student's t-test. When compared with the control group, $P < 0.05$ considered were statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

RESULTS AND DISCUSSION

Liposome preparation and characterization

Liposome was prepared using thin film hydration followed by high-pressure homogenization method (Mourtas, et al., 2011). The L9 (3^3) orthogonal experiments were designed in order to get the optimal formula (Tables S1, Tables S2). As shown in the Tables S2 and Tables S3, the entrapment efficiency was 60% ~ 85% and the drug loading was 3.02% ~ 4.23%, the particle size changed slightly for all orthogonal experiment groups during 30 days storage, so the preparation method was feasible. In order to determine the optimal formula composition of PSR/liposome, the encapsulation efficiency and the drug loading was taken as an important index, range analysis was used as method (Gao, et al., 2012). As shown in table S2, for the factors of the phospholipid concentration ($mg \cdot mL^{-1}$, A) and the weight ratio of phospholipid to cholesterol (w/w, B), the mean value K of 3st level was the highest. For the weight ratio of phospholipid to drug (w/w, C), 1st level was the highest. According to the analytical results, the optimal formula should be A3B3C1, i.e. the concentration of phospholipids was $3mg \cdot mL^{-1}$, the weight ratio of phospholipid to cholesterol was 4:1 and the weight ratio of phospholipid to drug was 50:1, which was selected as the optimal formula composition for further investigation.

The particle size means average hydrodynamic diameter. PDI indicates the width of the particle size distribution and higher value of it indicates samples easily aggregated (Das, et al., 2012). In order to evaluate the stability of PSR/liposome, the variation of particle size, polydispersity index (PDI) and zeta potential with time was measured. As shown in Fig. 1A, the mean particle size of fresh PSR/liposome was 110 ± 0.20 nm and slightly changed to 120.0 ± 0.30 nm after 30 days storage (Fig. 1A). The PDI values were all less than 0.25 during 30 days storage (Fig. 1B), which indicating that samples had relatively narrow size distribution and comparatively stable. Surface charge was also used to assess the physical stability of colloidal nanoparticle system in a medium. As shown in Fig. 1C, the zeta potential value decreased from -36.8 ± 0.21 mV to -32.1 ± 0.16 mV after 30 days of storage, while the absolute zeta potential value was still more than 30 mV. The higher absolute value of zeta potential is, the better dispersion and stability of colloidal system is (Fan, et al., 2013 and Zhang, et al., 2010). According to the above results, it can be concluded that the obtained PSR/liposome was exceptionally stable.

The morphology of blank liposome and PSR/liposome were determined by TEM. It can be seen from Fig. 2, both liposome systems was nearly spherical or oval in the shape. As shown in Fig. 2A, most of the blank liposomes were beautiful onion like multilamellar vesicles. While for PSR/liposome, both multilamellar vesicles and unilamellar vesicles could be found in Fig. 2B. Compared with blank liposome, more unilamellar vesicles formed in the system PSR/liposome. The diameters of both blank liposome and PSR/liposome systems were approximately 100 nm according to TEM measurements, which were slightly smaller than the hydrodynamic diameter determined by DLS (~ 110 nm). The reason can be attribute

to the shrinkage of liposome during sample preparation for TEM measurements (He, et al., 2014;Qi, et al., 2012; Tian, et al., 2014 and Wu, et al., 2014).

In this study, SAXS measurement was used to get a better insight into the lamellarity of vesicles after PSR loading (Pabst, et al., 2010; Dong, et al., 2010 and Schilt, et al., 2016). As shown in Fig. 3A, a small but sharp peak at $q_{max} \sim 0.87$ nm^{-1} was observed for blank liposome, indicating that both the unilamellar and multilamellar liposome was present. The effect of PSR on the structure of blank liposome could be obtained i.e. an absence of sharp peak and a decrease in scattering intensity (Fig. 3B). It was indicated that the fraction of multilamellar vesicles versus unilamellar vesicles in the case of PSR/liposome was less than that in the sample of blank liposome. The low q part of SAXS curves was further analyzed by exploring the slope of log I (q) ~ log (q) plots. As shown in the Fig. 3C and Fig. 3D, the values of slope for blank liposome and PSR/liposome were -2.9 and -2.3, respectively. The slope of both samples demonstrated that samples were composed by polydisperse mixture of unilamellar and mulitilamellar vesicles as well. While the slope change from 2.9 to 2.3 indicated that the fraction of unilamellar vesicles was increased after the loading of PSR to the blank liposome. This result was consistent with the above TEM images. It was reasonable since hydrophobic compounds insert into the lamellae can interact with the lipid bilayer, influencing the structural properties of the liposome (Fadda, 2015). Bouwstra et al. has obtained the SAXS scattering curves of liposome with different DPPC: CHEMS ratio. With the increase of the DPPC: CHEMS ratio, the mean number of phospholipid bilayers also increased. Simultaneously, the peak of the scattering curves became sharper (Bouwstra, et al., 1993) In a word, polydisperse liposomes have been obtained and the effect of PSR on the

structure of liposome were confirmed by SAXS measurement.

***In vitro* release behavior of PSR/liposome**

The *in vitro* release behavior of PSR/liposome was studied by using dialysis method in pH 7.4 PBS buffer at 37 °C. Pure PSR solution was investigated as a control. Fig. 4 showed that the release of pure PSR solution was very fast, where almost 80% PSR was released after 1 h and completely released within 24 h. For PSR/liposome system, there showed a burst release in the initial time and then a cumulative release after 1 h. There was only released 65% within 24 h. When prolonged the release time up to 48 h, approximately released 85%. The burst phase was mostly associated with the release of un-encapsulated PSR, whereas the gradual phase was determined by the release of PSR within the liposomes. In short, the above results indicated that PSR/liposome could prolong the release of PSR more effectively.

Cell proliferation assessment

The influence of PSR/liposome on the proliferation of MC3T3-E1 was assessed using CCK-8 assays. Firstly, the effect of pure PSR on cell proliferation was measured at different concentrations (0, 2.5, 5, 10, 20 μM). Fig. 5 showed that the blank liposome had no obvious proliferation effect towards MC3T3-E1 cells and would not affect cells viability at all concentrations we used. As shown in Fig. 5, it could be found that pure PSR promoted osteoblast proliferation to MC3T3-E1 cells in a dose-dependent manner. The proliferation effect of pure PSR was firstly enhanced with the increase of drug concentration, and then slightly decreased when PSR concentration was higher than 20 μM. This result was agreed well with the previous work reported by Tang et al. (Tang, et al., 2011). Similar phenomenon of proliferation effect by PSR/liposome concentration was observed. While it was interesting

to find that the proliferation effect of PSR/liposome was higher than pure PSR when the drug concentration up to 20 μM (Fig. 5). This phenomenon suggested that PSR/liposome induced osteoblast proliferation in more gentle way through the sustained release of PSR.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) is an early marker of osteoblast differentiation. To study the effect of PSR/liposome on differentiation of mouse primary calvarias osteoblasts, the activity of ALP was measured and normalized according to the total protein concentration of cell lysate. As shown in Fig. 6, blank liposome showed no effect on cells differentiation. After exposed pure PSR and PSR/liposome to MC3T3-E1 cells for 48 h, it was clear that PSR/liposome displayed much higher level in ALP activity than pure PSR. ALP activity level of PSR/liposome to MC3T3-E1 cells was approximately increased by 2.5-fold ($P < 0.001$), which was 1.2 times higher than that with pure PSR (Fig. 6). In spite of PSR/liposome caused a dose-dependent increase in ALP activity, but the concentration of PSR/liposome should be limited when it was applied to cure osteoporosis. According to the cell proliferation assessment, the proliferation effect of PSR will decrease when its concentration was more that than 20 μM (Tang, et al., 2011), but the PSR/liposome could induce osteoblast proliferation in more gentle way through the sustained release of PSR. Monteiro reported that Dexamethasone-loaded liposome could be coating to the surface of bio-functionalized scaffolds to induce the differentiation of Human mesenchyme stem cells (Monteiro, et al., 2015). Therefore, the strategy of encapsulation PSR into liposome can be applied to tissue-engineering and regenerative approaches to treat osteoporosis.

CONCLUSION

In this study, PSR-encapsulated liposome was prepared by the method of thin film hydration followed by high-pressure homogenization. Optimal formula was obtained using orthogonal design. The optimal PSR/liposome formula exhibited a high colloidal stability during 30 days storage. The release profile of PSR/liposome showed an initial burst release and a sustained release subsequently. Biological experiments illustrated that PSR/liposome could promote the proliferation and differentiation of MC3T3-E1 cells in more gentle way at appropriate concentrations through the sustained release of PSR. These findings suggest that liposome can play a promoting role in PSR as promising drug for osteoporosis treatment and it could be grafted on the surface of scaffolds for the further study of bone regeneration.

ACKNOWLEDGEMENT

We gratefully acknowledge the support of this work by the National Natural Science Foundation of China (No. 31200617), Shanghai Natural Science Foundation (No. 15ZR1409900) and the Knowledge Innovation Program of CAS (No. 2013KIP103). Thanks to the staff of the BL19U2 beamline at the National Center for Protein Science Shanghai and the Shanghai Synchrotron Radiation Facility for assistance during data collection.

Reference

1. Kim, T.H., Jung, J.W., Ha, B.G., Hong, J.M., Park, E.K., Kim, H.J. and Kim, S.Y. (2011) *J. Nutr. Biochem.*, 22: 8-15.
2. Sinningen, K., Albus, E., Thiele, S., Grossklaus, S., Kurth, T., Udey, M.C., Chavakis, T., Hofbauer, L.C. and Rauner, M. (2015) *Bone***,** 76: 107-114.
3. Wright, N.C., Looker, A.C., Saag, K.G., Curtis, J.R., Delzell, E.S., Randall, S. and Dawson-Hughes, B. (2014) *J. Bone. Miner. Res.*, 29: 2520-2526.
4. Wright, N.C., Saag, K.G., Dawson-Hughes, B., Khosla, S. and Siris, E.S. (2017) *Osteoporos Int*., 28: 1225-1232.
5. Chen, P., Li, Z. and Hu, Y. (2016) *BMC Public Health*. 16: 1039.
6. Lane, N.E. and Kelman, A. (2003) *Arthritis Res. Ther*., 5: 214-222.
7. Rodan, G.A. (2000) *Science*, 289: 1508–1514.
8. Song, X.W., Shen, P.Z. and Chen, B.X. (2000) *J. Shanghai.Tiedao. Univ*., 21: 38–40.
9. Wang, Z.L., Sun, J.Y., Wang, D.N., Xie,Y.H., Wang, S.W. and Zhao, W.M. (2006) *Phytomedicine*,13: 718–723.
10. Ji, Y.B. (1999) *Haerbin: Hei Long Jiang Scientific and Technological Press*.,118–121.
11. Robert, S. (2007) *N. Engl. J. Med*., 357: 682-690.
12. Tang, D.Z., Yang, F., Yang, Z., Huang, J., Shi, Q., Chen, D. and Wang, Y.J. (2011) *Biochem. Biophys. Res. Commun*., 405: 256-261.
13. Li, W.D., Wu, Y. (2014) *Phytomedicine*, 21: 400–405.
14. Xu, K., Pan, X., Sun, Y., Xu, W., Njunge, L. and Yang, L. (2015) *Pharm. Biol*., 53: 1010-1015.

15. Wong, R.W. and Rabie, A.B. (2011) *J. Orthop. Res*., 29: 158-164.

16. Wright, N.C., Looker, A.C., Saag, K.G., Curtis, J.R., Delzell, E.S., Randall, S. and Dawson-Hughes, B. (2014) *J. Bone Miner Res*., 29: 2520-2526.

17. Kastellorizios, M., Michanetzis, G.P., Pistillo, B.R., Mourtas, S., Klepetsanis, P., Favia, P., Sardella, E. and Antimisiaris, S.G. (2012) *Int. J. Pharm*., 432: 91-98.

18. Song, X.W., Shen, P.Z. and Chen, B.X. (2000) *J. Shanghai Tiedao. Univ*., 21: 38–40.

19. Zhang, Y.T., Shen, L.N., Wu, Z.H., Zhao, J.H. and Feng, N.P. (2014) *Int. J. Pharm*., 471: 449-452.

20. Fang, J.Y., Fang, C.L., Liu, C.H. and Su, Y.H. (2008) *Eur. J. Pharm. Biopharm*., 70: 633-640.

21. Mourtas, S., Kastellorizios, M., Klepetsanis, P., Farsari, E., Amanatides, E., Mataras, D., Pistillo, BR., Favia, P. and Antimisiaris, S.G. (2011) *Colloids Surf. B Biointerfaces*., 84: 214-220.

22. Kastellorizios, M., Michanetzis, G.P., Pistillo, B.R., Mourtas, S., Klepetsanis, P., Favia, P., Sardella, E. and Antimisiaris, S.G. (2012) *Int. J. Pharm*., 432: 91-98.

23. Lv, W.H., Zhao, S.N., Yu, H.I., Li, N., Garamus, V.M., Chen, Y.Y., Yin, P.H., Zhang, R.G., Gong, Y.B. and Zou, A.H. (2016) *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 504: 312-319.

24. Zhang, Y.T., Feng, N.P., Shen, L.N. and Zhao, J.H. (2014) *Int. J. Nanomedicine*, 9: 669-678.

25. Wang, R., Luo, J.G. and Kong, L.Y. (2012) Chromatographia*,* 75: 607-615.

26. Gao, D., Tang, S. and Tong, Q. (2012) *Int. J. Nanomedicine*, 7: 3517-3526.

27. Das, S., Ng, W.K. and Tan, R.B. (2012) *Eur. J. Pharm. Sci*., 47:139-151.

28. Zhang, X., Liu, J., Qiao, H., Liu, H., Ni, J., Zhang, W. and Shi, Y. (2010) *Powder Technology*, 197: 120-128.

29. Fan, X., Chen, J. and Shen, Q. (2013) *Int. J. Pharm*., 458: 296-304.

30. Tian, H., Guo, Z., Lin, L., Jiao, Z., Chen, J., Gao, S., Zhu, X. and Chen, X. (2014) *J. Control Release*, 174: 117-125.

31. He, Q. and Shi, J. (2014) *Adv. Mater*, 26: 391-411.

32. Qi, R., Liu, S., Chen, J., Xiao, H., Yan, L., Huang, Y. and Jing, X. (2012) *J. Control Release*, 159: 251-260.

33. Wu, W., Zhang, Q., Wang, J., Chen, M., Li, S., Lin, Z. and Li, J. (2014) *Polymer Chemistry*, 5: 5668.

34. Pabst, G., Kucerka, N., Nieh, M.P., Rheinstädter, M.C., Katsaras, J. (2010) *Chem. Phys. Lipids*, 163: 460-479.

35. Dong, Y.D., Boyd, B.J. (2011) *Int. J. Pharm*., *417*: 101-111.

36. Schilt, Y., Berman, T., Wei, X., Barenholz, Y., Raviv, U. (2016) *Biochim Biophys Acta*, 1860: 108-119.

37. Bouwstra, J.A., Gooris, G.S., Bras, W. and Talsma, H. (1993) *Chem. Phys. Lipids*, 64: 83–98.

38. Fadda, A.M. (2015) *Current Drug Targets*, 16: 1438-1447.

39. Monteiro, N., Martins, A., Ribeiro, D., Faria, S., Fonseca, N.A., Moreira, J.N., Reis, R.L. and Neves, N.M. (2015) *J .Tissue Eng. Regen Med*., 9: 1056-1066.

Figure Legends:

Fig.1. The variation of particle size (Fig. 1A), PDI (Fig. 1B) and zeta potential (Fig. 1C) of PSR/liposome were measured with time (1, 7, 15, 30 days). The values were presented as mean ± SD, n = 3.

Fig.2. TEM images of blank liposome (A) and PSR/liposome (B).

Fig.3. Small angle X-ray diffraction profiles of blank liposome and PSR/liposome.

Fig.4. *In vitro* PSR release behavior from PSR/liposome and pure PSR solution at 37 ± 0.5 °C. Values are presented as mean ± SD, n = 3.

Fig.5. The effect of pure PSR, PSR/liposome, blank liposome on cell proliferation of osteoblasts MC3T3-E1 at different concentrations. Cells were seeded into 96-well plates at density of 8×10^3 cells/well and treated for 48 h (**$P < 0.01$, ***$P < 0.001$ when compared with the control group).

Fig.6. The effect of pure PSR, PSR/liposome and blank liposome on alkaline phosphatase activity of primary mouse calvarias osteoblasts MC3T3-E1. Cells were seeded into 6-well plates at density of 1×10^5 cells/well and treated for 48 h (*$P < 0.05$, ***$P < 0.001$ when compared with the control group).

Fig.1

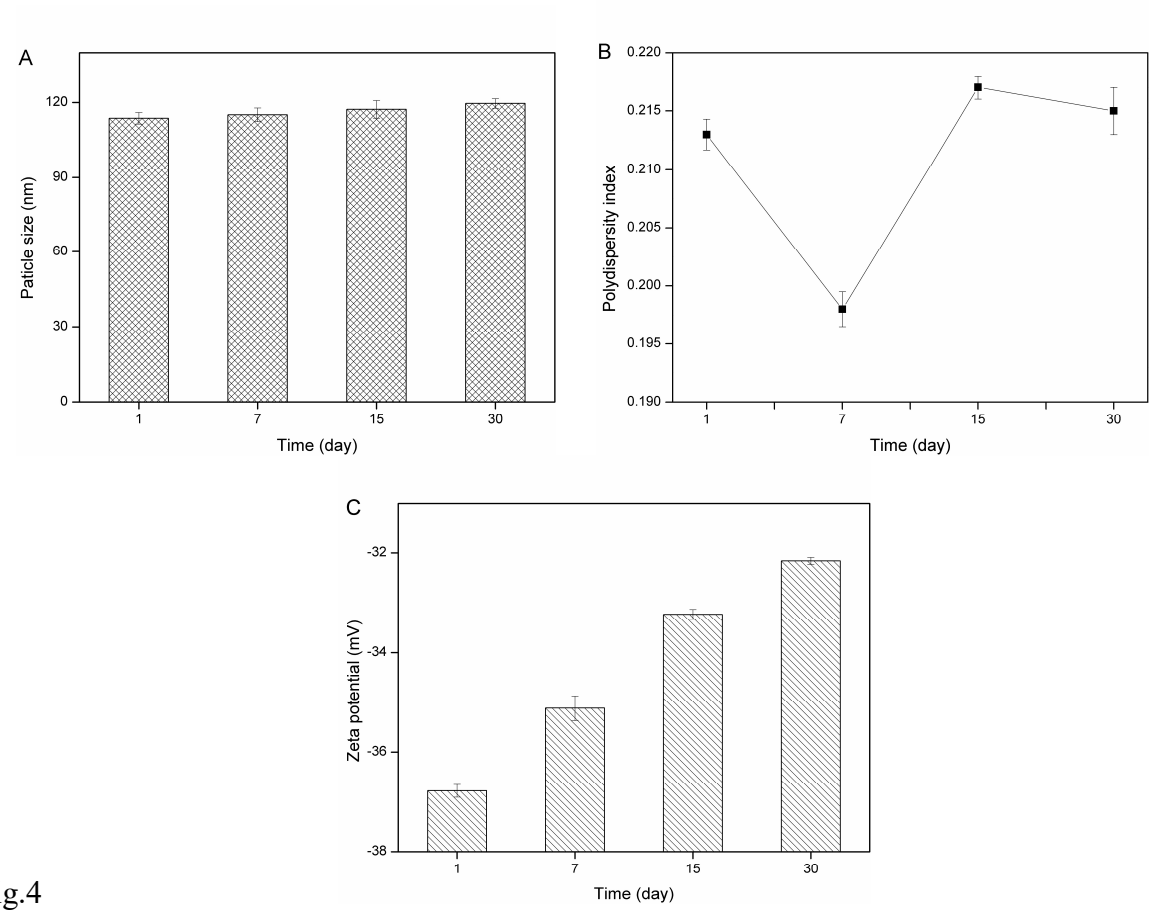

A
Paticle size (nm)
0
30
60
90
120
1
7
15
30
Time (day)
B
Polydispersity index
0.190
0.195
0.200
0.205
0.210
0.215
0.220
1
7
15
30
Time (day)
C
Zeta potential (mV)
-38
-36
-34
-32
1
7
15
30
Time (day)

Fig.4

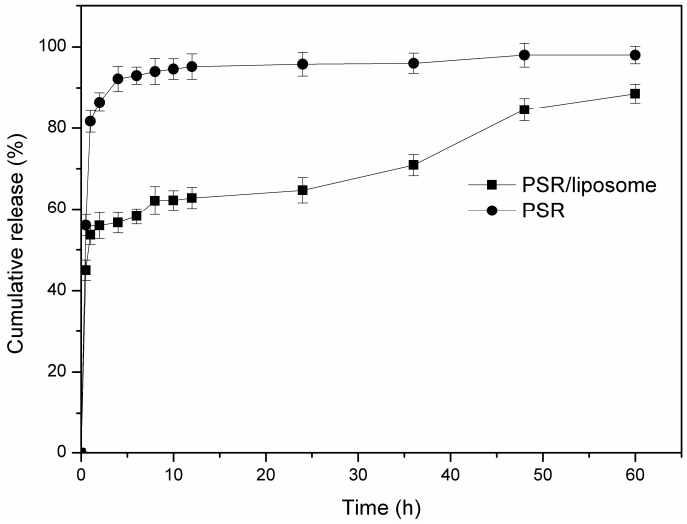

Cumulative release (%)
0
20
40
60
80
100
0
10
20
30
40
50
60
Time (h)
PSR/liposome
PSR

Fig.5

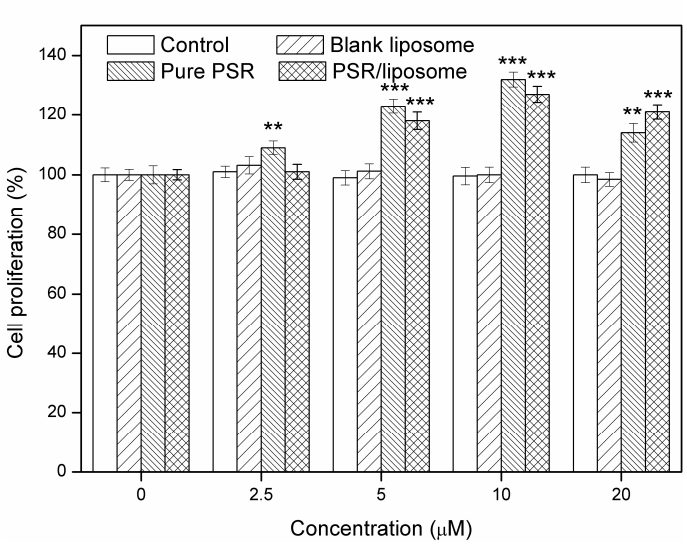
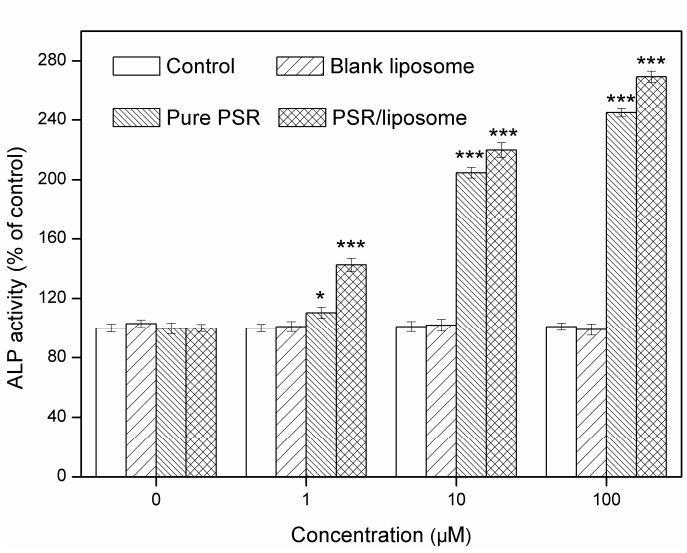

Control
Blank liposome
Pure PSR
PSR/liposome
Cell proliferation (%)
Concentration (μM)
0
2.5
5
10
20
**
***

Fig.6


Control
Blank liposome
Pure PSR
PSR/liposome
ALP activity (% of control)
Concentration (μM)
0
1
10
100
*
***