Journal of Fiber Bioengineering and Informatics 6:1 (2013) 95–102
doi:10.3993/jfbi03201309

Preparation and Characterization of DHAD/HRP Co-loaded Multivesicular Liposomes

Yuangang Liu [a,b,*], Qiyao Zheng [a], Shibin Wang [a,b,*]
Kejing Wu [a], Aizheng Chen [a,b]

[a] *College of Chemical Engineering, Huaqiao University, Xiamen 361021, China*

[b] *Institute of Pharmaceutical Engineering, Institute of Biomaterials and Tissue Engineering Huaqiao University, Xiamen 361021, China*

Abstract

The structure of carriers play an important role in the space distribution of different drugs, therefore this paper studies multivesicular liposomes (MVLs), which have a unique nano-in-micro structure as possible carriers in combination therapy. Horseradish peroxidase (HRP) and mitoxantrone hydrochloride (DHAD) were selected as model drugs for protein and small molecule drug respectively. The results show that the MVLs are spherical and that the internal space is divided up into numerous compartments. The particle size ranged from 15 to 20 μm and the system was stable according to the zeta potentials. In vitro release studies display that the DHAD/HRP co-loaded MVLs has better sustained release profiles than one drug alone, and the MVLs exhibited an orderly release behavior which suggested that MVLs might be used as drug carriers in combination therapy applications.

Keywords: Multivesicular Liposomes (MVLs); Combination Therapy; Protein; Small Molecule Drug

1 Introduction

At present, drug therapy is still one of the most important therapeutic regimens in human health and cure of diseases [1-3]. While in most cases, especially in cancer therapy, single-drug therapy is generally ineffective to completely treat the diseases. The one-dimensional action mechanism often activates and strengthens the alternative pathways, prompting the emergence of chemoresistance mutations and tumor relapse [4, 5]. In order to increase treatment efficacy, drug combination therapy provides an alternative strategy by taking synergetic actions against some diseases. This combination strategy now has been widely studied in many fields such as tissue engineering [6], hypertension [7], diabetes [8], urinary tract symptoms [9], papulosis [10], hepatitis [11] and cancers [12-14]. Woodcock et al. [15] also highlighted the novel combination therapies in a perspective form. Furthermore, some researchers studied both combination therapy and drug delivery systems (DDS) to coordinate the release behavior and extend the release period of different drugs

*Corresponding author.
Email addresses: ygliu@hqu.edu.cn (Yuangang Liu), sbwang@hqu.edu.cn (Shibin Wang).

for better cure efficacy. For example, Jäger et al. [16] prepared core-shell polymeric nanoparticles simultaneously loaded with docetaxel and doxorubicin. The results indicate that the combination provides a more efficient suppression of tumor-cell growth in mice bearing EL-4 T cell lymphoma when compared to the effect of nanoparticles loaded with either docetaxel or doxorubicin separately. Greco et al. [17] also highlighted that combination therapy supplies opportunities and challenges for polymer-drug conjugates such as nanomedicines.

In order to improve the sustained-release properties and control the burst-release effects of carriers, some researchers show interests in designing carriers with a special structure that often plays an important role in the space distribution of different drugs. In our previous work, we prepared nanoparticles embedded microcapsules (NEMs) that had a unique structure for drug allocation in different space, thus led to excellent performances such as sequential release and longer sustained release period without initial burst release effect [18-20]. Therefore, we have particular interests in finding DDS with unique structure. Multivesicular liposomes (MVLs) are unique lipid-based systems containing numerous discontinuous internal aqueous chambers bounded by continuous, non-concentric network [21, 22]. Having considered the structure similarity of NEMs and MVLs, we are curious to whether MVLs can be used in combination therapy. In this paper, MVLs were prepared and characterized using horseradish peroxidase (HRP) and mitoxantrone hydrochloride (DHAD) as model drugs for protein and small molecule drug respectively. We anticipated that the MVLs would show promising use in the future drug combination therapy.

2 Experimental Section

2.1 Materials

Glycerol trioleate (CP), cholesterol (Chol) and oleic acid were all purchased from China National Pharmaceutical Group Chemical Reagent Co., Ltd (China). Soybean lecithin (≥96%) was supplied by Lipoid GmbH (Germany). L-lysine, glucose, dichloromethane and Triton X-100 (CP) were purchased from National Pharmaceutical Group Chemical Reagent Co., Ltd (China). Sucrose was obtained by Shantou Dahao Fine Chemicals Co. (Shantou, China). Mitoxantrone hydrochloride (DHAD) (≥98%) was purchased from Dalian Meilun Biotechnology Co., Ltd (Dalian, China). Horseradish peroxidase (HRP, Rz≥3, 250 U/mg) was purchased from Beijing Biodee Biotechnology Co., Ltd (Beijing, China). Millipore filters (0.22 μm filters, Mixed Cellulose Ester membranes) were purchased from Shanghai Xinya Purification Device Factory (Shanghai, China). All other reagents and chemicals were of analytical grade.

2.2 Preparation of blank or DHAD/HRP co-loaded MVLs

The blank or DHAD/HRP co-loaded MVLs were prepared by the conventional water-in-oil-in-water (w/o/w) double emulsification process [23, 24] with a little modification. The optimized preparation condition in this research are as follows: a lipid combination of 21.25 mM soybean lecithin, 31.04 mM cholesterol, 13.55 mM glycerol trioleate and 3.54 mM oleic acid were dissolved in dichloromethane to form the oil phase, which was emulsified with an equal volume of aqueous solution (the internal aqueous solution) blank or containing drugs (0.5 mg/ml DHAD, 0.5 mg/ml HRP, the DHAD:HRP ratios were varied as follows: 1:1, 1:3 and 3:1 (w/w)) in 5% sucrose, using a high-speed homogenizer (T25, IKA, Germany) at 20 000×g for 10 min, to produce a

water-in-oil (w/o) emulsion. A subsequent emulsification with 7.5% glucose containing 40 mM lysine (the external aqueous solution, 3-fold the volume of the internal aqueous solution), using a vortex mixer (QL-901, Kylin-Bell Lab Instruments Co., Ltd., Jiangsu, China) for 10 s, resulted in a w/o/w double emulsion. The resulting MVLs suspension were obtained by removing the dichloromethane using a rotary evaporator (EYELA NE-1001, EYELA, Japan) at 10×g and at 35°C.

2.3 Analysis of DHAD/HRP in MVLs

A certain concentration of DHAD (HRP) Triton X-100 solution was scanned with UV-vis spectrophotometer (UV-1600PC, Shanghai Meipuda instrument Co., Ltd., China) over the range of 200-800 nm to determine its maximum absorption wavelength (663 nm and 403 nm for DHAD and HRP respectively). Then, according to the Lambert-Beer law A=-lgT=εbc, where A is the absorbency, T is transmittance, ε is the molar absorptivity, b is the sample pathlength in cm, c is the sample concentration in grams per liter, a series of standard solutions with different concentration gradients were prepared and measured at the maximum absorption wavelength (403 nm and 663 nm) to calculate the average value of $\varepsilon_{403}^{\mathrm{DHAD}}$, $\varepsilon_{663}^{\mathrm{DHAD}}$, $\varepsilon_{403}^{\mathrm{HRP}}$ and $\varepsilon_{663}^{\mathrm{HRP}}$.

The DHAD/HRP co-loaded MVLs obtained in section 2.2 were washed three times with saline solution and harvested by centrifugation at 2 500×g for 10 min at 4° to remove the unencapsulated drugs. Then, the supernatant was discarded and 5% (w/v) Triton X-100 was added to the sediments to break the MVLs. After the content was fully reacted, the solution was filtrated through a 0.22 μm millipore filter twice to extrude the residues. The absorbance at 663 nm and 403 nm of an aliquot of the filtrate above were measured using a UV–vis spectrophotometry.

With the determined absorbance and molar absorptivity above, the amount of two drugs can be calculated according to the Lambert-Beer law and the absorbance additivity theory by using the dual wavelength spectrophotometry method [25].

2.4 MVLs Morphology, Size Distribution and Zeta Potential

The morphological examination of the blank or DHAD/HRP (1:1, 1:3 and 3:1(w/w)) co-loaded MVLs was directly performed by biological light microscope (BH-2, Olympus, Japan) using the freshly prepared MVLs suspensions or by transmission electron microscope (TEM, H7650, Hitachi, Japan) by placing samples onto the specific copper grids, negative staining with uranyl acetate and naturally dried for 1-2 days at room temperature before being viewed.

The particle size distribution and zeta potential of the blank or DHAD/HRP (1:1, 1:3 and 3:1(w/w)) co-loaded MVLs were determined by a Mastersizer2000 laser particle analyzer (Malvern Instruments Ltd., Worcestershire, UK) and a ZetaPALS zeta potential meter (Malvern Instruments Ltd., Worcestershire, UK).

2.5 Differential Scanning Calorimetry

The phase transition temperature measurement of the blank or DHAD/HRP co-loaded MVLs was performed by a DSC2910 differential scanning calorimetry (DSC, TA Insruments Ltd., American). The blank or DHAD/HRP co-loaded MVLs was prepared according to the optimized preparation

condition separately, then precooled at −20°C before being lyophilized by a FDU-2100 freeze-dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Some lyophilized MVLs powders were taken to the sample cell. Then the powder was calcined at temperatures between −20°C and 70°C with a heating rate of 5°/min.

3 Results and Discussion

3.1 MVLs Morphology, Size Distribution and Zeta Potential

The DHAD/HRP co-loaded MVLs with different drug ratios were obtained by double emulsification process. The characterization of the MVLs formulation was evaluated by biology light microscope, which could be seen in Fig. 1 (a-c), the MVLs were spherical, having multiple irregular granular internal structures. TEM gave more details of the membrane structure which could be seen in Fig. 1 (d). It shows the internal space that was divided up into numerous compartments by bilayer septums.

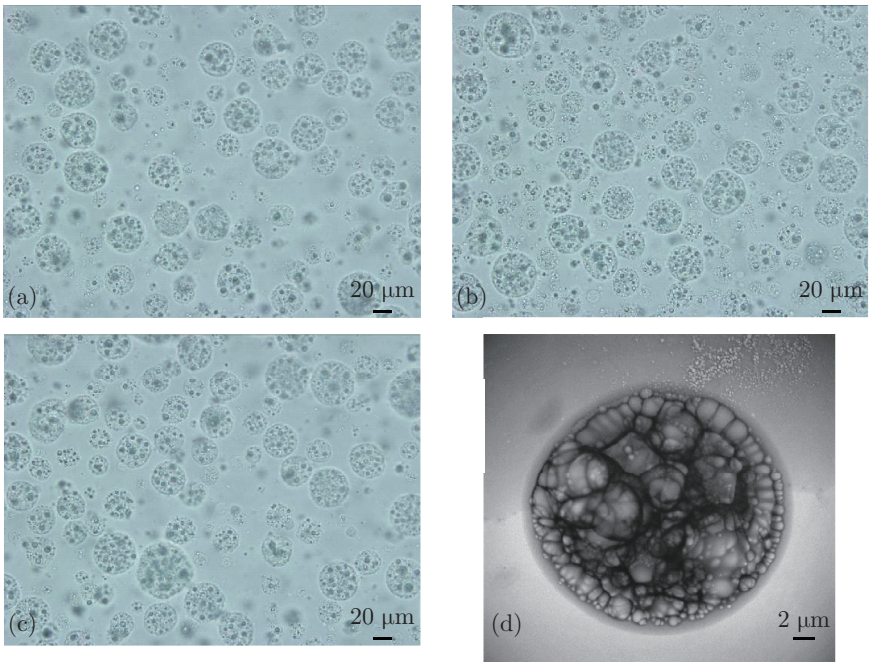

Fig. 1: Different light microscopy photographs of DHAD/HRP co-loaded MVLs with different mass ratios (a, DHAD/HRP 3:1; b, DHAD/HRP 1:1; c, DHAD/HRP 1:3) and the TEM photograph of blank MVLs (d)

The size distribution and zeta potential are shown in Table 1, from which we can see the average particle size ranging from 15 to 20 μm. D_{10}, D_{50}, D_{90}, and SPAN were calculated to characterize the particle size distribution of the MVLs [26]. The MVLs particle size increased slightly with increasing amounts of HRP, while at the same time, the particle size distribution became narrower. We can also see that the average particle size and the particle size distribution of the blank are both the lowest. The reason could be that the composition of the primary emulsion (w/o) plays an important role in the final structure of the product. When drugs were added in, there existed more cores to form multivesicular structure during the process of dichloromethane evaporation,

thus the average particle size increased compared to the blank one. HRP is a macromolecular drug, the coiled molecules are easier to occupy the space of the compartments in MVLs and thus making the compartment bigger in size. The zeta potentials of the blank and drug-loading MVLs were all around the value of −48 mv, which meant that the co-loading system was stable and it might been used in the future drug combination system.

Table 1: Size distribution and zeta potential of DHAD/HRP-MVLs

Sample	Zeta potential (mV)	Average particle size (μm)	D_{10}(μm)	D_{50} (μm)	D_{90} (μm)	SPAN
Blank	−48.1	14.95	3.89	14.14	26.36	1.59
DHAD:HRP 3:1	−49.6	17.17	2.64	11.32	39.70	3.27
DHAD:HRP 1:1	−47.7	18.42	3.46	15.60	36.89	2.14
DHAD:HRP 1:3	−47.3	20.60	6.24	17.43	38.67	1.86

3.2 The Differential Thermal Analysis of DHAD/HRP Co-loaded MVLs

Fig. 2 shows the thermograms of the blank MVLs and the co-loaded DHAD/HRP MVLs with different drug ratios. The blank MVLs has an endothermic peak at 38.06°C and the drug loaded MVLs has an endothermic peak at 38.51°C (DHAD:HRP 3:1), 38.43°C (DHAD:HRP 1:1) and 38.37°C (DHAD:HRP 1:3) respectively, which shows there are no significant changes in the transition temperature between the blank MVLs and the drug loaded MVLs. The results indicate that this system might be safe if used on humans (37°C) as it could avoid the drug leakage from the MVLs.

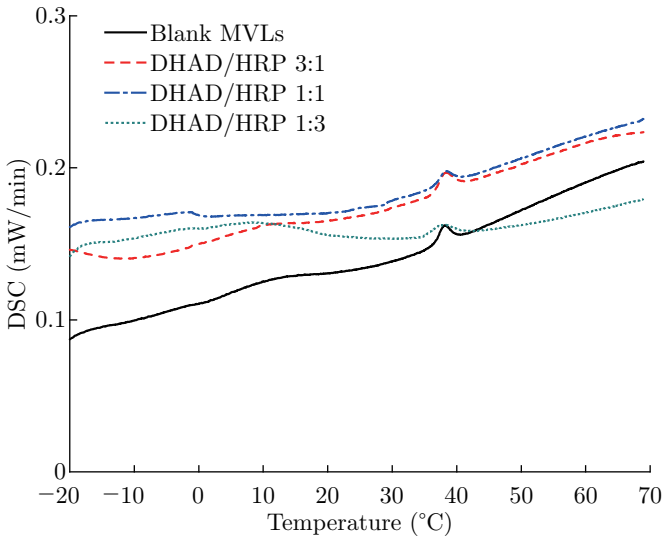

Fig. 2: The thermograms of DHAD/HRP-MVLs with different drug ratios

3.3 The in Vitro Release of DHAD/HRP from MVLs

The encapsulation efficiency of HRP and DHAD varied from 67.5% to 77% and from 64% to 70% respectively when we changed DHAD/HRP ratios. These data indicate that the MVLs could have relatively high encapsulation efficiency in co-loading drugs. The in vitro release of drugs from MVLs with different drug ratios was studied in normal saline. Fig. 3 (a) shows that when the drug ratio is 3:1, the cumulative release rate of DHAD and HRP from MVLs are 14.92% and 13.39% in 0.5 h, and 80.92% and 75.80% in 168 h. The drug co-loading MVLs have better sustained release performance than the single loading MVLs (for DHAD and HRP, the cumulative release rate is 80.68% and 65% respectively in 120 h). The reason could be due to the electrostatic interaction between DHAD and HRP, which could avoid the drug outflow. It is similar to the result of co-delivery of an antisense oligonucleotide and 5-fluorouracil reported by Hussain et al [27]. Fig. 3 (b) shows that when the drug ratio is 1:1, the cumulative release rate of DHAD and HRP from MVLs are 11.92% and 9.54% in 0.5 h, and 88.24% and 84.47% in 168 h. When the release time passed 24 hrs, the release rate difference between two drugs increased; after 96 hrs the release rate of HRP became faster, while the release rate difference between two drugs decreased. Fig. 3 (c) shows that when the drug ratio is 1:3, the cumulative release rate of DHAD and HRP from MVLs are 9.86% and 5.37% in 0.5 h, and 94.34% and 83.17% in 168 h. During the whole release period, there was no obvious burst release of both DHAD and HRP with different drug ratios according to the China Pharmacopoeia 2010. We can also see from Fig. 3 that the release rate of small molecule drug DHAD was always faster than that of protein drug HRP, suggesting an orderly release of DHAD and HRP from MVLs, which might be used as a drug delivery vehicle for carrying two different drugs in the future applications.

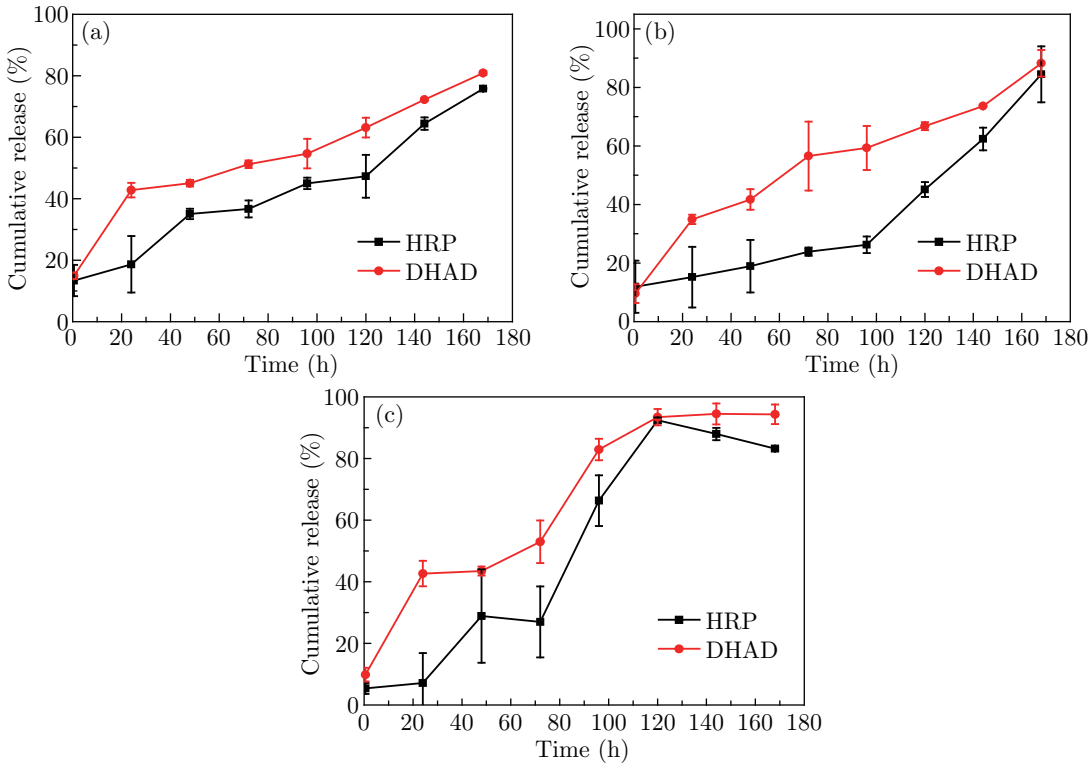

Fig. 3: In vitro release of DHAD/HRP co-loaded MVLs with different drug ratios (a) DHAD/HRP 3:1; (b) DHAD/HRP 1:1; (c) DHAD/HRP 1:3) in normal saline

4 Conclusion

In order to improve the treatment efficacy of combination therapy, MVLs which has a unique structure containing numerous discontinuous internal aqueous chambers bounded by continuous, non-concentric networks were prepared. The special nano-in-micro structure endowed the DHAD/HRP co-loaded MVLs better sustained release profiles than one drug alone. The results also indicate that the co-loading drugs exhibit an orderly release property. Therefore, the MVLs may have potential applications in the future drug combination therapies.

Acknowledgments

Financial supports from NSFC (31000441 and 31170939), Natural Science Foundation of Fujian Province (2011J01223), the Scientific Research Foundation for the Returned Overseas Chinese Scholars (State Education Ministry), the Open Research Fund of State Key Laboratory of Bioelectronics (Southeast University) and the Fundamental Research Funds for the Central Universities (JB-JC1009, JB-SJ1009) are gratefully acknowledged.

References

[1] Li JS, Li Y, Zhao Z, Li G, Zhang J. Review on drug delivery of 5-fluorouracil for anti-cancer tissue engineering. Textile Bioengineering and Informatics Symposium Proceedings. The 5th International Symposium of Textile Bioengineering and Informatics, Shinshu University, Ueda, Japan, edited by Li Y, Takatera M, Kajiwara K, Li JS, 2012; p. 234-252.

[2] Xie RJ, Wu HY, Xu JM, Deng QM. The preparation of silk fibroin drug-loading microspheres. J Fiber Bioengineering Informatics 2008; 1: 73-80.

[3] Chen AZ, Wang GY, Wang SB, Feng JG, Liu YG, Zheng W. Preparation of ibuprofen-loaded poly-(methyl vinyl ether-co-maleic anhydride) nanoparticles by solution-enhanced dispersion by supercritical CO_2. J Fiber Bioengineering Informatics 2012; 5: 309-320.

[4] Lin CI, Whang EE, Moalem J, Ruan DT. Strategic combination therapy overcomes tyrosine kinase coactivation in adrenocortical carcinoma. Surgery 2012; 152: 1045-1050.

[5] Hu CMJ, Zhang L. Nanoparticle-based combination therapy toward overcoming drug resistance in cancer. Biochem Pharmacol 2012; 83: 1104-1111.

[6] Ma CYJ, Panoskaltsis N, Kumar R, Xu XY, Mantalaris A. Simulation of ex vivo bone marrow culture: Application to chronic myeloid leukaemia growth model. Biochem Eng J 2012; 61: 66-77.

[7] Byrd JB, Zeng C, Tavel HM, Magid DJ, O'Connor PJ, Margolis KL, Selby JV, Ho PM. Combination therapy as initial treatment for newly diagnosed hypertension. Am Heart J 2011; 162: 340-346.

[8] Vora J, Bain SC, Damci T, Dzida G, Hollander P, Meneghini LF, Ross SA. Incretin-based therapy in combination with basal insulin: A promising tactic for the treatment of type 2 diabetes. Diabetes Metab 2012; DOI: 10.1016/j.diabet.2012.08.002

[9] Kaplan SA, He W, Koltun WD, Cummings J, Schneider T, Fakhoury A. Solifenacin plus tamsulosin combination treatment in men with lower urinary tract symptoms and bladder outlet obstruction: a randomized controlled trial. Eur Urol 2012; DOI: 10.1016/j.eururo.2012.07.003

[10] Wang XL, Wang HW, Guo MX, Huang Z. Combination of immunotherapy and photodynamic therapy in the treatment of Bowenoid papulosis. Photodiagn Photodyn 2007; 4: 88-93.

[11] Petersen J, Ratziu V, Buti M, Janssen HLA, Brown A, Lampertico P, Schollmeyer J, Zoulim F, Wedemeyer H, Sterneck M, Berg T, Sarrazin C, Lutgehetmann M, Buggisch P. Entecavir plus tenofovir combination as rescue therapy in pre-treated chronic hepatitis B patients: An international multicenter cohort study. J Hepatol 2012; 56: 520-526.

[12] Diyabalanage HVK, Granda ML, Hooker JM. Combination therapy: Histone deacetylase inhibitors and platinum-based chemotherapeutics for cancer. Cancer Lett 2012; DOI: 10.1016/j.canlet.2012.09.018

[13] Sosman J, Puzanov I. Combination targeted therapy in advanced renal cell carcinoma. Cancer 2009; 115: 2368-2375.

[14] Pishvaian MJ, Slack R, Koh EY, Beumer JH, Hartley ML, Cotarla I, Deeken J, He AR, Hwang J, Malik S, Firozvi K, Liu M, Elston B, Strychor S, Egorin MJ, Marshall JL. A Phase I clinical trial of the combination of imatinib and paclitaxel in patients with advanced or metastatic solid tumors refractory to standard therapy. Cancer Chemoth Pharm 2012; 70: 843-853.

[15] Woodcock J, Griffin JP, Behrman RE. Development of novel combination therapies. N Engl J Med 2011; 364: 985-987.

[16] Jäger E, Jäger A, Chytil P, Etrych T, ŘihováB, Giacomelli FC, Štěpánek P, Ulbrich K. Combination chemotherapy using core-shell nanoparticles through the self-assembly of HPMA-based copolymers and degradable polyester. J Control Release 2012; DOI: 10.1016/j.jconrel.2012.11.009

[17] Greco F, Vicent MJ. Combination therapy: Opportunities and challenges for polymer–drug conjugates as anticancer nanomedicines. Adv Drug Deliver Rev 2009; 61: 1203-1213.

[18] Liu Y, He P, Wang S, Sun X, Chen A. Preparation of embolic NEMs loading capecitabine. J Mater Sci-Mater M 2013; 24: 155-160.

[19] Liu Y, Sun X, Wang S, Xie M, Chen A, Long R. Preparation of nanoparticles embedded microcapsules (NEMs) and their application in drug release. Mater Lett 2012; 75: 48-50.

[20] Liu YG, Mao HH, Wang SB, Sun XZ. Preparation and characterization of a novel embolic nano-in-micro drug delivery system. Chem J Chin Univ 2011; 32: 2574-2580.

[21] Mantripragada S. A lipid based depot (DepoFoam technology) for sustained release drug delivery. Prog Lipid Res 2002; 41: 392-406.

[22] Langston MV, Ramprasad MP, Kararli TT, Galluppi GR, Katre NV. Modulation of the sustained delivery of myelopoietin (Leridistim) encapsulated in multivesicular liposomes (DepoFoam). J Control Release 2003; 89: 87-99.

[23] Ye Q, Asherman J, Stevenson M, Brownson E, Katre NV. DepoFoam technology: a vehicle for controlled delivery of protein and peptide drugs. J Control Release 2000; 64: 155-166.

[24] Zhong H, Deng Y, Wang X, Yang B. Multivesicular liposome formulation for the sustained delivery of breviscapine. Int J Pharmaceut 2005; 301: 15-24.

[25] Modi M, Shah R, Mashru RC. Development and validation of spectrophotometric methods for simultaneous estimation of metoprolol succinate and telmisartan in combined pharmaceutical formulation. Int J Pharm Sci Res 2012; 3: 1348-1354.

[26] El-Mahdy M, Ibrahim ES, Safwat S, El-Sayed A, Ohshima H, Makino K, Muramatsu N, Kondo T. Effects of preparation conditions on the monodispersity of albumin microspheres. J Microencapsul 1998; 15: 661-673.

[27] Hussain M, Beale G, Hughes M, Akhtar S. Co-delivery of an antisense oligonucleotide and 5-fluorouracil using sustained release poly (lactide-co-glycolide) microsphere formulations for potential combination therapy in cancer. Int J Pharmaceut 2002; 234: 129-138.