J Mater Sci: Mater Med (2010) 21:551–556
DOI 10.1007/s10856-009-3925-8

Preparation of EGFR monoclonal antibody conjugated nanoparticles and targeting to hepatocellular carcinoma

Peifeng Liu · Zonghai Li · Mingjie Zhu · Ying Sun · Yaogang Li · Hongzhi Wang · Yourong Duan

Received: 1 March 2009 / Accepted: 26 October 2009 / Published online: 17 November 2009

Abstract This study aims to determine the sensitivity, specificity and accuracy of epidermal growth factor receptor monoclonal antibody (EGFRmAb) modified poly(lactic acid-*co*-L-lysine) nanoparticles (PLA-PLL-EGFRmAb) NPs delivery system to EGFR positive cancer cells. In the study, a new PLA-PLL-EGFRmAb NPs was prepared. The cellular cytotoxicity, cellular uptake, and the targeted effect for hepatocellular carcinoma of PLA-PLL-EGFRmAb NPs were investigated. In vitro, the findings of Flow cytometry and Confocal Laser scanning Biological Microscopy showed that PLA-PLL-EGFRmAb NPs can bind to hepatocellular carcinoma cells and were uptaken effectively. In vivo in the SMMC-7721 xenograft mouse model, PLA-PLL-EGFRmAb NPs could target to the tumor effectively, which demonstrated a better targeting. These results showed that the PLA-PLL-EGFRmAb NPs have the potential to be used as a target delivery carrier for tumor therapies.

P. Liu · Y. Li · H. Wang (✉)
State Key Lab for Modification of Chemical Fibers and Polymer Materials, College of Materials Science and Engineering, Donghua University, Shanghai 200051, People's Republic of China
e-mail: wanghz@dhu.edu.cn

Z. Li
National Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai Jiao Tong University, Shanghai, People's Republic of China

P. Liu · M. Zhu · Y. Sun · Y. Duan (✉)
Cancer Institute of Shanghai Jiao Tong University, Shanghai 200032, People's Republic of China
e-mail: yrduan@sci.shmu.edu.cn

1 Introduction

Various nanoparicle systems are currently being exploded for cancer therapeutics such as dendrimers [1], liposomes [2], micelles [3], etc., owing to the improvement of the therapeutic efficacy of anticancer drugs and reduce drug's systemic toxicity [4].

Nanoparticles (NPs) can be delivered to tumor tissues by the enhanced permeability and retention (EPR) effect [5], which is known as a passive targeting. Moreover, their body biodistribution and permeability in tissues can be controlled by size and surface properties [6]. To further obtain higher selectivity and to enhance the uptake of tumor cells for NPs, NPs are often functionalized with ligands such as antibody fragments or peptide. By combining the tumor targeting properties of ligands with NPs, NPs offer the promise of specific and efficient target delivery to tumor cells [7–10].

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase overexpressed in a number of solid tumors [11–14]. The upregulation of EGFR has been found to be strongly correlated with many tumor progressions [15–17], which provides a basis for designing the receptor-targeted approaches for tumor. The difference in the level of EGFR expression between normal cells and tumor cells, suggested that EGFR can be used as a mediator for the targeted delivery [18, 19].

In this study, we prepared PLA-PLL-EGFRmAb NPs, and examined the target efficency of NPs for SMMC-7721 hepatocellular carcinoma cells and tumor bearing mice. The main purpose is to evaluate the targeted efficency of PLA-PLL-EGFRmAb NPs and its potential applications as a target delivery carrier.

2 Experimental

2.1 Materials

N^{ε}-(Carbonylbenzoxy)-L-lysine and D-alanine were purchased from GL biochem (Shanghai, China), L,L-lactide was purchased from GLACO (Jinan, China). *N,N*-Diisopropylethylamine was purchased from J & K chemical. EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and *N*-hydroxylsuccinimide (NHS) were purchase from Acros (New Jersey, USA). Rhodamine B (RB) and the other reagents (analytical grade) were purchased from Sigma (St. Louis, MO, USA). EGFR monoclonal antibody (EGFRmAb) was a gift from Dr. Li Z.H. (National Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai Jiaotong University). The EGFRmAb was prepared according to the paper [20].

The copolymers PLA-PLL (poly(lactic acid-*co*-L-lysine)) were synthesized in three steps according to our previous literature [21]. Briefly, I to prepare the monomer of 3-(N^{ε}-benzoxycarbonyl-L-lysine)-6-L-methyl-2,5-morpholinedione; II to prepare diblock copolymer poly(lactic acid-*co*-(Z)-L-lysine) (PLA-PLL(Z)) by ring-opening polymerization of monomer and L,L-lactide with stannous octoate as initiator; III to prepare diblock copolymer PLA-PLL by deprotected the copolymer PLA-PLL(Z) in HBr/HoAc solution.

2.2 Preparation of antibody conjugated NPs

2.2.1 Preparation of NPs

Rhodamine B loaded PLA-PLL NPs (RB-PLA-PLL) were prepared as described previously [22]. Briefly, 20 μl of a 10 mg/ml RB solution were emulsified with 200 μl mixture of methylene dichloride and acetone (3:2, v/v) containing 5 mg of polymers (PLA-PLL) with an ultrasonic processor (400 W, 10×2 s) (JY92-II ultrasonic processor, Ningbo Scientz Biotechnology Co., Ltd., China). Then, the emulsion was poured into 2 ml aqueous solution of Pluronic F-68 (1%, w/v). And the mixture was sonicated for 1 min (400 W, 10×2 s), subsequently stirred at room temperature for 4 h to evaporate the organic phase. The resultant NPs were purified and obtained by centrifugation at 14,000 rpm for 30 min. The PLA-PLL NPs were prepared according to the above mentioned method except omitting the RB.

Size and z potential of NPs were measured by laser light scattering using a Nicomp 380/ZLS zeta potential analyzer (Particle Sizing System, USA).

2.2.2 Conjugation of EGFRmAb to NPs

EGFRmAb conjugation were developed using the coupling strategy between EGFRmAb and RB-PLA-PLL or PLA-PLL NPs with EDC and NHS as the coupling agent. Briefly, 1 ml of RB-PLA-PLL or PLA-PLL NPs suspension (5 mg/ml, Ph 7.1) was incubated with 50 μl of 50 mg/ml EDC and 50 μl of 50 mg/ml NHS for 4 h at room temperature with gentle stirring. After that, the EGFRmAb was added into the NPs suspension and incubated for 4 h. After the conjugation reaction, the EGFRmAb modified RB-PLA-PLL (RB-PLA-PLL-EGFRmAb) or PLA-PLL NPs (PLA-PLL-EGFRmAb) were purified by centrifugation (L-100XP, Beckman, USA) at 14,000 rpm × 30 min twice and redispersed in phosphate buffer solution (PBS). Finally, RB-PLA-PLL-EGFRmAb or PLA-PLL-EGFRmAb NPs were washed with PBS several times and kept at 4°C in PBS.

2.2.3 Cell culture

SMMC-7721 hepatocellular carcinoma cells were obtained as a kind gift from Dr. Li Z.H. (National Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai Jiaotong University) and grown in RPMI1640 medium (Gibco BRL, Paisley, UK) containing 10% fetal bovine serum (FBS) at 37°C in humidified environment of 5% CO_2.

2.2.4 In vitro cellular cytotoxicity assays

The cytotoxicity of NPs was evaluated by MTT assay on SMMC-7721 cells. Briefly, SMMC-7721 cells were seeded in 96 well plates at a density of 2×10^4/well and incubated (37°C, 5% CO_2) for 24 h in RPMI1640. The cells were incubated for 4 h with different concentrations of PLA-PLL or PLA-PLL-EGFRmAb NPs (5–40 μg/ml), then washed 3 times with PBS and further cultured in medium for 24 h. Subsequently, 100 μl MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide, 5 mg/ml) was added to the culture medium, and cells were incubated for 4 h. After that, 150 μl of DMSO was added to dissolve the crystal. Absorbance was measured at 570 nm using a microplate reader.

2.2.5 Cellular uptake of NPs

Rhodamine B (RB) was encapsulated in NPs as a probe for the uptake study. SMMC-7721 cells were seeded in 6-well plates and incubated for 24 h. After the cell culture reached about 80% confluence, RB-PLA-PLL and RB-PLA-PLL-EGFRmAb NPs were added at equivalent RB per well and incubated with cells for 2 h at 37°C, and then washed three times with PBS, followed by fixation with 4% *p*-formaldehyde (PFA) for 20 min.

In the competitive binding experiments, 50-fold moles excess of free EGFRmAb were added into the medium 2 h before the addition of targeted NPs. After 2 h incubation at 37°C, the cells were washed three times with PBS to remove

unbound NPs. The bound fluorescent NPs were visualized by Confocal Laser Scanning Biological Microscope (FV1000, Olympus, Japan).

2.2.6 Cellular binding of NPs

To investigate the binding ability of NPs, 1×10^6 SMMC-7721 cells were cultured in 6 well plates the day before the experiments, Subsequently, the cells were treated with RB-PLA-PLL, RB-PLA-PLL-EGFRmAb and 50-fold moles of free EGFRmAb + RB-PLA-PLL-EGFRmAb NPs for 2 h at 37°C, and washed twice with PBS, then trypsinized and harvested, washed and resuspended in PBS. The fluorescence was measured by FAC Scan flow cytometry (Becton-Dickinson, USA).

2.2.7 The establishment of tumor model

Subcutaneous tumors were establishment in 4- to 6-week-old BALB/c female athymic nude mice that were purchased from Cancer Institute of Shanghai Jiaotong university (Shanghai, China) by injecting 5×10^6 SMMC-7721 cells into their dorsal subcutaneous space. With the establishment of tumor model, the tumor bearing mice were divided into three groups, (1) RB, (2) RB-PLA-PLL, (3) RB-PLA-PLL-EGFRmAb. The animal study protocols were approved by the Animal Study Committee of Cancer Institute of Shanghai Jiao Tong University.

2.2.8 Targeting study

To verify that the PLA-PLL-EGFRmAb NPs could specifically target to human 7721 xenografts tumor in vivo, Rhodamine B (RB) was encapsulated in NPs as a probe for the study of target delivery. RB, RB-PLA-PLL, RB-PLA-PLL-EGFRmAb NPs were injected into tumor bearing mice through the tail vein in SMMC-7721 xenograft mice, respectively. Fluorescence images of tumor bearing mice were captured at various time points after injection using Small Animal In vivo Fluorescence Imaging System (LB 983, Berthold Technologies Gmbh & Co KG, Germany).

3 Results and discussion

3.1 Preparation and physicochemical characterization of NPs

In the present study, RB-PLA-PLL NPs were prepared by using emulsion-solvent evaporation method. Orthogonal design was applied to optimize the preparation technology on the basis of the single factor evaluation. The optimal conditions for preparation of NPs were as follows: 25 mg/ml and 10 mg/ml were the concentration of PLA-PLL and RB, respectively. The methylene dichloride/acetone ratio was 3:2 (v/v), the concentration of Pluronic F-68 was 1% and the volume ratio of O/W was 1/10 (v/v).

Table 1 The particle size and zeta potential of NPs

	RB-PLA-PLL NPs	RB-PLA-PLL-EGFRmAb Nps
Particle size (nm)	126 ± 20.4	147 ± 26.2
Zeta potential (mV)	11.2 ± 4.1	14.0 ± 7.2

Data are expressed as the mean ± SD ($n = 6$)

The RB-PLA-PLL, RB-PLA-PLL-EGFRmAb NPs size distribution and zeta potential were shown in Table 1. The size of RB-PLA-PLL-EGFRmAb NPs increase 21 nm as compared with RB-PLA-PLL NPs, presumably owing to the presence of antibody on the NPs surface. The mean zeta potential of RB-PLA-PLL NPs was 11.2 ± 4.1 mV, whereas the zeta potential of RB-PLA-PLL-EGFRmAb NPs is 14.0 ± 7.2 mV. It further demonstrated that the increase in zeta potential may be attributed to the presence of antibody on the NPs surface.

3.2 In vitro cytotoxicity

In vitro cytotoxicity of NPs was evaluated using an MTT assay in SMMC-7721 cells treated with increasing doses of PLA-PLL and PLA-PLL-EGFRmAb NPs. The NPs were found to be non-toxic at each of the tested concentrations. We did not observe a significant difference in the toxicity of PLA-PLL and PLA-PLL-EGFRmAb NPs, although there was a slight reduction in cell viability at higher concentrations. Average cell viability ranged from 80% to 98% of control viability at the concentrations studied, suggesting

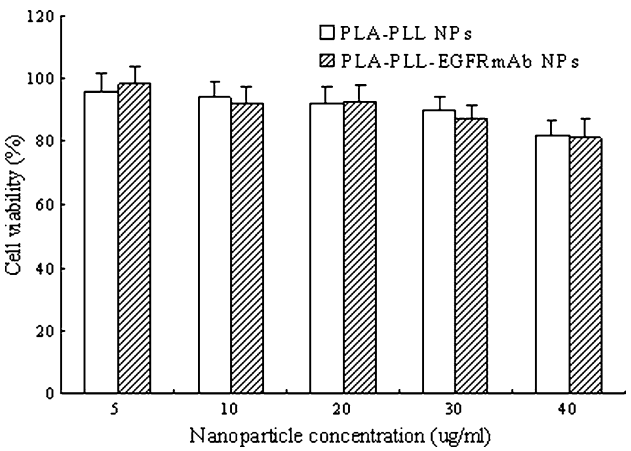

Fig. 1 Cytotoxicity of PLA-PLL (*white bars*) and PLA-PLL-EGFRmAb NPs (*hatched bars*) in SMMC-7721 cells. The cells were incubated using different concentrations NPs in the range from 5 to 40 μg/ml for a time period of 24 h (mean ± SD; $n = 6$)

that PLA-PLL-EGFRmAb NPs may be used as a target delivery carrier (Fig. 1).

3.3 Binding and internalization of antibody modified NPs

Flow cytometry (FACS) was used to observe the binding ability of RB-PLA-PLL and RB-PLA-PLL-EGFRmAb NPs to tumor SMMC-7721. As shown in Fig. 2A, a little fluorescence was detected in cells incubated with RB-PLA-PLL NPs, whereas much higher fluorescence intensity was shown in cells treated with RB-PLA-PLL-EGFRmAb NPs, which could be caused by only a little RB-PLA-PLL NPs that were internalized into the cells by endocytosis or phagocytosis. While in the case of RB-PLA-PLL-EGFRmAb NPs, the ligand-receptor recognition could help to increase the internalization of NPs.

Confocal microscopy was used to observe the intracellular distribution of the uptaken NPs. After incubation with either RB-PLA-PLL or RB-PLA-PLL-EGFRmAb NPs, the fluorescence was mainly localized in cytoplasm (Fig. 2B). Fluorescence intensity of cells treated with RB-PLA-PLL-EGFRmAb NPs was much higher than that of those cells treated with RB-PLA-PLL NPs, which was consistent with above result of cellular binding.

In order to demonstrate the specificity of the interaction of RB-PLA-PLL-EGFRmAb NPs with the cells, a competition assay was performed by using free EGFRmAb. As shown in Fig. 2A and B, the binding of RB-PLA-PLL-EGFRmAb NPs to SMMC-7721 cells was inhibited by EGFRmAb, which demonstrated the RB-PLA-PLL-EGFRmAb NPs was internalized by ligand-mediated approaches.

3.4 In vivo ligand-mediated target delivery

Accurate delivery of antitumor drug-loaded target delivery carriers to tumor is crucial for increasing antitumor efficacy. Therefore, a distinct capacity to target tumor is important in tumor therapeautics. In recent years, target delivery carriers have received considerable attentions, in view of their ability to target tumor. Various targeted delivery carriers that were functionalized with different ligand were explored for further enhance drug or gene delivery. Some target moieties such as folate or RGD peptide have been developed

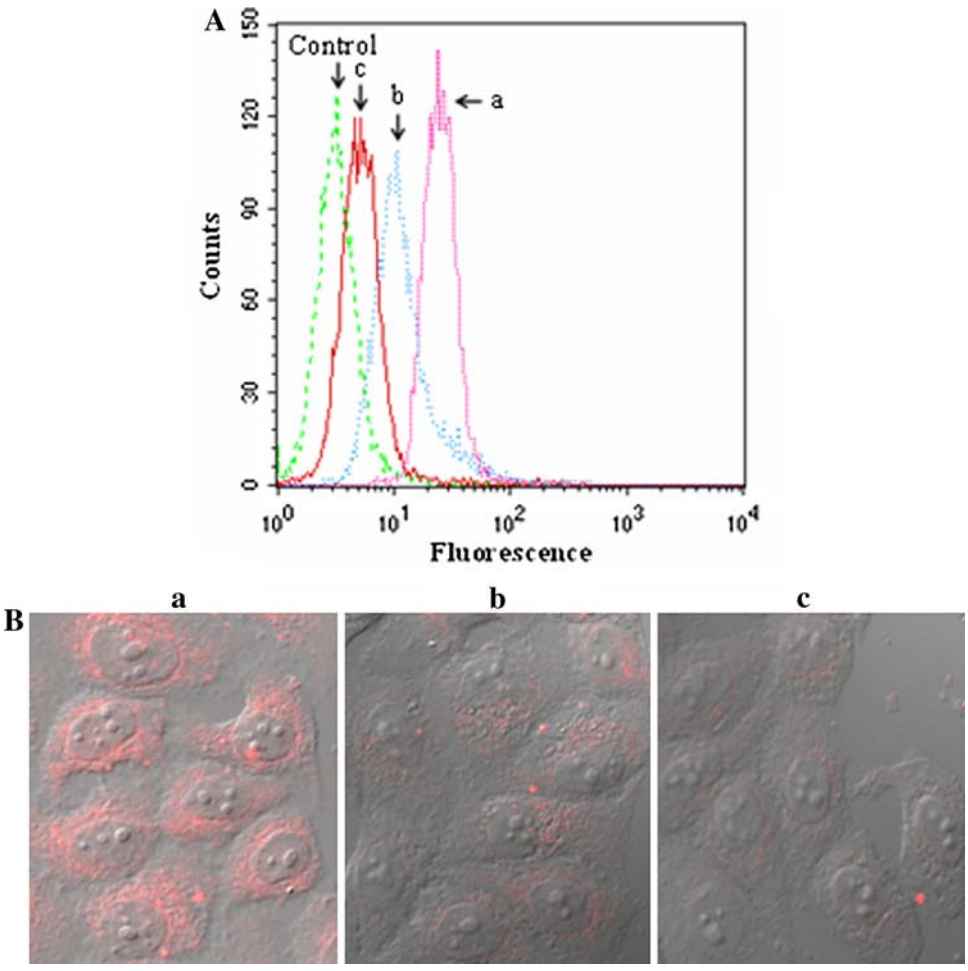

Fig. 2 Flow cytometry images (**A**) and confocal images (**B**) of SMMC-7721 cells after treatment of RB-PLA-PLL-EGFRmAb NPs (*a*), RB-PLA-PLL NPs (*b*), Competition of RB-PLA-PLL-EGFRmAb NPs to SMMC-7721 cells at 37°C in the presence of 50 × moles excess free EGFRmAb (*c*)

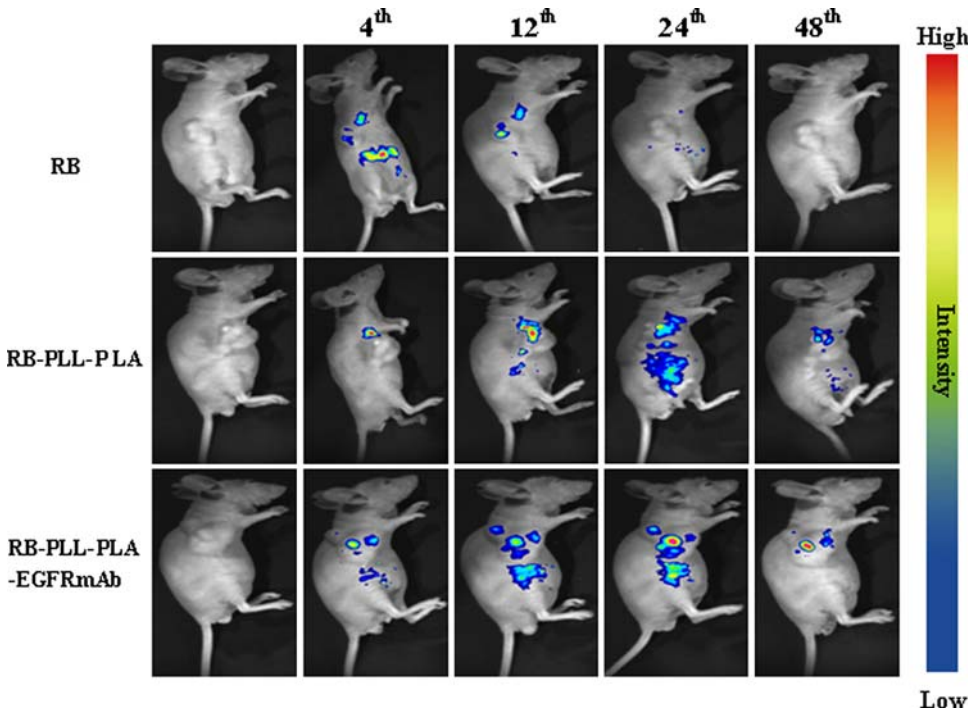

Fig. 3 In vivo fluorescence image of RB, RB-PLA-PLL, RB-PLA-PLL-EGFRmAb NPs in tumor-bearing mice. Images captured were taken at 4, 12, 24 and 48 h after injection

to help achieve specific delivery due to their high binding affinity for the folate receptors or $\alpha v\beta 3$ integrin, respectively [23–26]. For the experiments presented here, EGFRmAb was used to target nanoparticles to tumors.

Fluorescence dye RB was encapsulated in NPs so as to investigate the efficacy of target delivery. As shown in Fig. 3, with the passing of time, the tumor fluorescence intensity of mice injected with RB and RB-PLA-PLL NPs were gradually decreased. In sharp contrast, the tumor fluorescence intensity of mice treated with RB-PLA-PLL-EGFRmAb NPs does not decrease significantly with time post-injection. At 24 h of post-injection, the tumor fluorescence intensity of mice treated with RB has decreased significantly as compared with mice treated with RB-PLA-PLL or RB-PLA-PLL-EGFRmAb NPs. At 48 h of post-injection, the differences of tumor fluorescent intensity of mice treated with RB, RB-PLA-PLL and RB-PLA-PLL-EGFRmAb NPs were more significant. It was clear that for RB-PLA-PLL-EGFRmAb NP-treated mice, the fluorescent intensity of tumor was relatively stronger and could lasted longer, when compared with the RB or RB-PLA-PLL NPs-treated mice. The main reason resulting in the difference of fluorescence intensity between RB-PLA-PLL and RB-PLA-PLL-EGFRmAb NPs was the difference in tumor cells uptake to NPs.

RB was quickly showed up in the kidney and bladder region, and then eliminated. Therefore, the accumulation in tumor tissue was lower (Fig. 3). As compared with RB, the RB-PLA-PLL NPs passively target to tumor by enhanced permeability and retention effect (EPR), and showed more accumulation in tumor. This was mainly because that the NPs, generally controlled under 200 nm, can help to leak preferably through out the tumor vasculature [27]. The RB-PLA-PLL-EGFRmAb NPs could actively target to tumor via the ligand-receptor recognition, which helped the further enhance delivery of NPs.

4 Conclusions

The PLA-PLL-EGFRmAb NPs can efficiently target to tumor and were specifically internalized in EGFR over-expressing tumor cells. The PLA-PLL-EGFRmAb NPs also showed better targeting for tumor as compared with the PLA-PLL NPs. The better targeting of PLA-PLL-EGFRmAb NPs was attributed to more cellular uptake by tumor cells, which were mediated by ligand-receptor recognition. These results suggested that the PLA-PLL-EGFRmAb NPs could be used as an efficient target delivery carrier.

Acknowledgments This research was supported by National 973 Basic Research Program of China (No. 20108529902), National 863 Research Program of China (No. 2007AA021203), National Natural Science Foundation of China (No. 50673058), Shanghai Municipal Public Health Bureau (No. 2008Y088) and Nano-Science and Technology from Science and Technology Commission of Shanghai Municipality (No. 0852nm05800).

References

1. Wolinsky JB, Grinstaff MW. Therapeutic and diagnostic applications of dendrimers for cancer treatment. Adv Drug Deliv Rev. 2008;60:1037–55.

2. Vasir JK, Labhasetwar V. Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv Drug Deliv Rev. 2007; 59:718–28.
3. Singh R, Lillard JW Jr. Nanoparticle-based targeted drug delivery. Exp Mol Pathol. 2009;86:215–23.
4. Haley B, Frenkel E. Nanoparticles for drug delivery in cancer treatment. Urol Oncol. 2008;26:57–64.
5. Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. Science. 2004;303:1818–22.
6. Stayton PS, Hoffman AS, Murthy N, Lackey C, Cheung C, Tan P, et al. Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics. J Control Release. 2000;65:203–20.
7. Wartlick H, Michaelis K, Balthasar S, Strebhardt K, Kreuter J, Langer K. Highly specific HER2-mediated cellular uptake of antibody-modified nanoparticles in tumour cells. J Drug Target. 2004;12:461–71.
8. Anhorn MG, Wagner S, Kreuter J, Langer K, von Briesen H. Specific targeting of HER2 overexpressing breast cancer cells with doxorubicin-loaded trastuzumab-modified human serum albumin nanoparticles. Bioconjug Chem. 2008;19:2321–31.
9. Mitra A, Coleman T, Borgman M, Nan A, Ghandehari H, Line BR. Polymeric conjugates of mono- and bi-cyclic alphavbeta3 binding peptides for tumor targeting. J Control Release. 2006;114: 175–83.
10. Xiong XB, Huang Y, Lu WL, Zhang X, Zhang H, Nagai T, et al. Enhanced intracellular delivery and improved antitumor efficacy of doxorubicin by sterically stabilized liposomes modified with a synthetic RGD mimetic. J Control Release. 2005;107:262−75.
11. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129–39.
12. Hirsch FR, Scagliotti GV, Langer CJ, Varella-Garcia M, Franklin WA. Epidermal growth factor family of receptors in preneoplasia and lung cancer. Perspectives for targeted therapies. Lung Cancer. 2003;41(suppl 1):S29–42.
13. Maihle NJ, Baron AT, Barrette BA, Boardman CH, Christensen TA, Cora EW, et al. EGF/ErbB receptor family in ovarian cancer. Cancer Treat Res. 2002;107:247–58.
14. Di-Lorenzo G, Bianco R, Tortora G, Ciardiello F. Involvement of growth factor receptors of the epidermal growth factor receptor family in prostate cancer development and progression to androgen independence. Clin Prostate Cancer. 2003;2:50−7.
15. Meert AP, Verdebout JM, Martin B, Ninane V, Feoli F, Sculier JP. Epidermal growth factor receptor expression in pre-invasive and early invasive bronchial lesions. Eur Respir J. 2003;21: 611–5.
16. Piyathilake CJ, Frost AR, Manne U, Weiss H, Bell WC, Heimburger D, et al. Differential expression of growth factors in squamous cell carcinoma and precancerous lesions of the lung. Clin Cancer Res. 2002;8:734–44.
17. Selvaggi G, Novello S, Torri V, Leonardo E, De Giuli P, Borasio P, et al. Epidermal growth factor receptor overexpression correlates with a poor prognosis in completely resected non-small-cell lung cancer. Ann Oncol. 2004;15:28–32.
18. Sorkin A. Internalization of the epidermal growth factor receptor: role in signalling. Biochem Soc Trans. 2001;29:480–4.
19. Zhou Y, Drummond DC, Zou H, Hayes ME, Adams GP, Kirpotin DB, et al. Impact of singlechain Fv antibody fragment affinity on nanoparticle targeting of epidermal growth factor receptorexpressing tumor cells. J Mol Biol. 2007;371:934–47.
20. Gao HP, Jiang H, Wang HM, Li ZH. Preparation and identification of anti-EGFRvIII/EGFR monoclonal antibody 9B9. Chin J Cancer Biother. 2009;16:391−5.
21. Yu H, Guo XJ, Qi XL, Liu PF, Shen XY, Duan YR. Synthesis and characterization of arginine-glycine-aspartic peptides conjugated poly (lactic acid-co-L-lysine) diblock copolymer. J Mater Sci Mater Med. 2008;19:1275–81.
22. Duan YR, Xu S, Wang Q, Liu J, Zhang ZR. Optimization of preparation of DHAQ-loaded PEG-PLGA-PEG nanoparticles using central composite design. J Mater Sci Mater Med. 2006; 17:559–63.
23. Eliyahu H, Barenholz Y, Domb AJ. Polymers for DNA delivery. Molecules. 2005;10:34–64.
24. Veiseh O, Kievit FM, Gunn JW, Ratner BD, Zhang M. A ligand-mediated nanovector for targeted gene delivery and transfection in cancer cells. Biomaterials. 2009;30:649–57.
25. Yoshizawa T, Hattori Y, Hakoshima M, Koga K, Maitani Y. Folate-linked lipid-based nanoparticles for synthetic siRNA delivery in KB tumor xenografts. Eur J Pharm Biopharm. 2008; 70:718–25.
26. Pan J, Feng SS. Targeting and imaging cancer cells by folate decorated, "quantum dots loaded nanoparticles of biodegradable polymers". Biomaterials. 2009;30:1176–83.
27. Chatterjee DK, Fong LS, Zhang Y. Nanoparticles in photodynamic therapy: an emerging paradigm. Adv Drug Deliv Rev. 2008;60:1627–37.