ELSEVIER

International Journal of Pharmaceutics 184 (1999) 45–51

international journal of pharmaceutics

A genetically modified recombinant tumor necrosis factor-α conjugated to the distal terminals of liposomal surface grafted polyethyleneglycol chains

Michalakis Savva [a,*], Ernò Duda [b], Leaf Huang [a]

[a] *Departments of Pharmaceutical Sciences and Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[b] *Institute of Biochemistry, Biological research Center, Hungarian Academy of Sciences, temesváry krt, Hungary*

Received 2 December 1998; received in revised form 2 March 1999; accepted 2 March 1999

Abstract

A genetically modified recombinant tumor necrosis factor (TNF)-α (rKRKTNF) was conjugated to the terminal carboxyl groups of liposome grafted polyethyleneglycol (PEG) chains. The long-circulating liposomes were composed of egg phosphatidylcholine, cholesterol (chol) and 7% carboxyl PEG-phosphatidylethanolamine. The conjugation efficiency of the genetically modified rKRKTNF under the conditions described in the text was approximately 55%. The biological activity of liposomal rKRKTNF, as tested with an in vitro cytotoxicity assay was reduced compared to the free, unconjugated rKRKTNF. In vivo biodistribution studies showed that conjugation of as little as 0.13% of the grafted PEG chains resulted in a rapid elimination of the formulation from the blood stream. It is speculated that both non-selective conjugate chemistry and inherent recognition of the TNF by the components of the reticuloendothelial system (RES) are responsible for the short blood half life of the rKRKTNF-PEG-liposomes. The result suggest that conjugating a rapidly clearing recombinant cytokine to long-circulating liposomes provides little advantage in modifying the pharmacokinetic parameters of the cytokine.

Keywords: Liposome; TNF-α; Polyethyleneglycol; Cytotoxicity

1. Introduction

Tumor necrosis factor-α (TNF) is a cytokine which can elicit a wide range of cellular responses depending on the cell type (Beutler, 1992). Structurally, it is composed of three subunits hydrophobically joined to form a trimeric cone. The overall shape of a single 157-amino-acid subunit (17 kDa) is wedge-like. The N-terminus is highly flexible and does not seem to play a major role in its biological activity (Goh and Porter, 1991). On the contrary, alteration of the C-terminal amino acid sequences leads to a marked loss of cytotoxic activity (Gase et al., 1990). The tumoricidal activity exerted by TNF was the reason why many

* Corresponding author. Current address: Schering-Plough Research Institute, K-11-2-J4, Kenilworth, NJ 07033, USA. Tel.: +1-908-740-5231; fax: +1-908-740-2802.
E-mail address: michalakis.savva@spcorp.com (M. Savva)

0378-5173/99/$ - see front matter
PII: S0378-5173(99)00092-7

investigators used it as an experimental antitumor drug. However, initial clinical trials have shown that TNF does not appear to be promising for the treatment of human cancer. This is due to its wide range systemic toxicity (fever, anorexia, hypotension, renal and hepatic toxicity) which precludes the administration of sufficient doses required to obtain blood levels high enough to cause cancer cell death.

During the last several decades, liposomes have found a great many applications in the field of therapeutics. Most liposome-targeted drugs have had reasonable success as pharmacological agents in vitro. In vivo, however, their success was limited due to rapid uptake by the reticuloendothelial system (RES) which prevented them from reaching other target tissues. The advent of long-circulating liposomes prepared by incorporation of a small percentage of phospholipid modified polyethyleneglycol (PEG) derivatives (2–5 kDa) in the phospholipid bilayer allowed them to overcome the above limitation of conventional liposomes.

A variety of biologically active proteins (Blume et al., 1993; Mori et al., 1996; Bally et al., 1997) including whole antibodies (Allen et al., 1994; Maruyama et al., 1995; Goren et al., 1996), $F(ab)_2$, Fab′ (Kirpotin et al., 1997; Maruyama, 1997) and smaller molecules such as scFv fragment, oligopeptides and folic acid have been used as targeting ligands (Zalipsky et al., 1995). The half life in the blood of these PEG-grafted liposomes bearing targeting ligands on the PEG terminals generally depends on: (1) the size of the liposomes; (2) the type of the conjugated ligand; and (3) the liposomal surface density of the conjugated ligand (Allen et al., 1994; Maruyama et al., 1995; Goren et al., 1996; Bally et al., 1997).

In the present study, rKRKTNF has been conjugated, a genetically modified TNF, on the distal terminals of PEG previously anchored on liposomes. This was an attempt to develop a delivery system of prolonged blood circulation time which would ideally be accompanied by substantial increase in passive and active accumulation of TNF in the tumor.

2. Materials and methods

PEG bis succinic acid ($PEG(COOH)_2$) molecular weight (MW) 3500 was a general gift of Nippon Oil and Fats (Tsukuba, Japan). *N*,*N*′-dicyclohexyl carbodiimide (DCC), 1 ethyl-3(3-dimethylamino propyl) carbodiimide (EDC), *N*-hydroxysuccimide (NHS), stearyl amine (SA), diethylenetriamine pentaacetic dianhydride 98% (DTPA) and cholesterol (chol) were from Aldrich (Milwaukee, WI). *N*-hydroxysulfosuccinimide (S-NHS) and Iodo-Gen were purchased from Pierce (Rockford, IL). Egg phosphatidylcholine 99% (ePC) and dioleoyl phosphatidylethanolamine were from Avanti Polar Lipids (Birmingham, AL). 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Recombinant mouse TNF (rKRKTNF) was prepared by insertion of the three amino acids K-R-K at position 2 of the wild type mouse TNF as shown: M-**K-R-K**-V-S-S-S- and was produced with a specific activity of 20–50 U/ng protein in *Escherichia coli* as previously described (Mori et al., 1996). The modification was done to facilitate and direct chemical coupling to the N-terminus of the cytokine.

rKRKTNF was radiolabeled with ^{125}I using the IodoGen method to a specific activity of 1.07×10^5 cpm/μg protein. Liposomes were labeled with ^{111}In-DTPA-SA synthesized as described elsewhere (Hnatowich et al., 1981).

2.1. Synthesis of HOOC-PEG-DOPE

Synthesis of HOOC-PEG-DOPE was performed by mixing $PEG(COOH)_2$ with DCC and NHS in chloroform at a ratio of $PEG(COOH)_2$:DCC:NHS (1:3:3 mol/mol). After overnight incubation at room temperature, the reaction mixture was cooled to 0°C and then filtered to remove the precipitated urea derivative. The product PEG bis succinimidyl succinate (SuO-PEG-Osu), was mixed with DOPE, followed by addition of triethylamine (TEA) (SuO-PEG-Osu:DOPE:TEA = 3:1:3.5 mol/mol) (Fig. 1A). Purification of HOOC-PEG-PE was performed by

dialysis against water for 1 week at 4°C using a MWCO 300 000 dialysis membrane (Spectrum Medical Industries, CA).

2.2. Preparation of proteoliposomes

ePC/chol/HOOC-PEG-PE (62:31:7 mol/mol) were mixed in chloroform and dried to a thin film first with N_2 gas and then under vacuum for several hours. Hydration was performed with HBS (20 mM HEPES, 2 mM EDTA, 150 mM NaCl) pH 7.4 and the lipid dispersion was extruded several times through two stacked 100 nm nucleopore membranes using liposoFast™ extruders (Avestin, Ottawa, ON, Canada) to generate a monodisperse population of liposomes. Liposome particle size distribution was analyzed by photon correlation spectroscopy using a Coulter model N4plus multiangle submicron particle analyzer (Miami, FL).

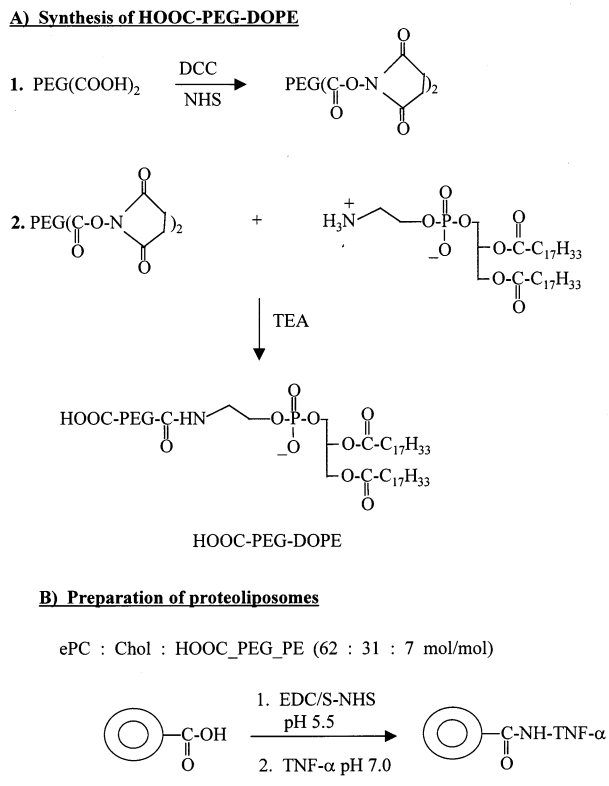

Fig. 1. Synthetic scheme for preparation of tumor necrosis factor (TNF)-polyethyleneglycol (PEG)-liposomes.

Activation of the terminal carboxyl group was performed by sequential addition of EDC and S-NHS into 300 μl liposomes (~ 4.84 μmol total lipid) in MES buffer pH 5.5. After 5–10 min incubation the mixture was neutralized with 1 N NaOH to pH 7.0. Fifty μg of rKRKTNF was then added and the whole mixture was incubated at 4°C overnight (Fig. 1B) [7]. Separation of proteoliposomes from unmodified rKRKTNF was effected using a Sepharose 4B column (Fig. 2) preequilibrated with HBS pH 7.4.

2.3. Cytotoxicity assay

Liposome conjugated rKRKTNF cytotoxicity was determined in L929 mouse fibroblast using an MTT assay. Briefly, L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (200 U/ml) and streptomycin (0.1 mg/ml) onto 96 well plates at 2.5×10^4 cells/well and grown for 24 h at 37°C in a 5% CO_2/95% air humidified atmosphere. After washing the cells, 200 μl of medium containing 1 μg/ml actinomycin D and various concentrations (0.0002–200 ng/ml) of free or liposomal rKRKTNF was added. After 16 h of incubation, 20 μl of MTT (5 mg/ml) were added in each well and the cells were incubated for an additional 4 h. The supernatant was discarded and 100 μl DMSO was added to each well. The amount of the purple formazan derivative created by the cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of living cells was quantitated by measurement of the absorbance at 560 nm using a multiscan auto platereader (Carmichael et al., 1987).

2.4. Biodistribution study

Biodistribution studies were performed after tail vein injection of ^{111}In-labeled liposomes bearing ^{125}I-rKRKTNF in female C3H/Hej mice (6–8 weeks old) at a dose of 0.5 μg rKRKTNF or 0.17 mg lipid/mouse in 0.2 ml HBS, pH 7.46. At variable time intervals the mice were anesthetized and sacrificed by cervical dislocation. Blood, liver, spleen, kidney and lungs were collected. Biodistribution analysis of rKRKTNF and liposomes was

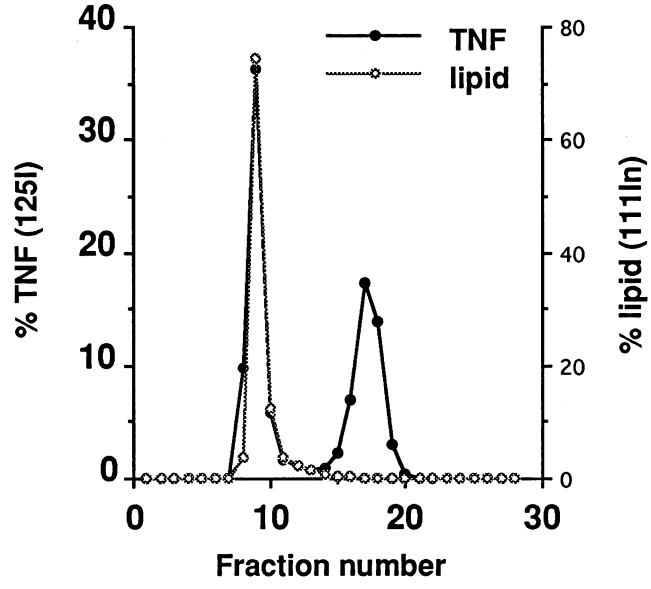

Fig. 2. Coupling efficiency of ^{125}I labeled-rKRKTNF-polyethyleneglycol (PEG)-coated-^{111}In labeled-liposomes. rKRKTNF-PEG-coated liposomes were eluted through a Sepharose 4B column and fractions were collected and analyzed for rKRKTNF and liposomes by ^{125}I and ^{111}In counting, respectively.

performed by assessing the ^{111}In and ^{125}I levels in the various organs utilizing a gamma counter. The levels of radioactivity in different organs were corrected to exclude intravascular counts. The blood volume of mice was taken to be 7.3% of the total mouse weight (Wu et al., 1981).

3. Results and discussion

In the present study, PEG bis carboxylic acid was first conjugated to DOPE. The resultant HOOC-PEG-DOPE conjugate was mixed with ePC and chol (7:62:31 mol/mol) in chloroform. After complete elimination of the organic solvent the dry lipid mixture was hydrated with HBS and extruded to give a homogeneous population of liposomes. Coupling of rKRKTNF to the liposome grafted PEG terminals was effected via an amide bond formation.

The proteoliposome size remained stable at 138 ± 11 nm during the reaction and after purification. No liposome aggregation was observed at any time. A total of 18.08 μg rKRKTNF was conjugated on liposomes of fraction 9 (fraction used for in vivo studies) corresponding to 33.4% of the rKRKTNF added (Fig. 2). The overall coupling efficiency was $\sim$55% (fraction 8, 9 and 10; Fig. 2). The percentage of conjugated cytokine to the liposome grafted PEG of after the coupling reaction was 0.13 and the average number of rKRKTNF per liposome was 22 (see Table 1).

The biological activity of rKRKTNF-PEG-coated liposomes as assessed in L929 cells was shown to be $\sim$18 times reduced compared to the unmodified one. Specifically, the IC_{50} of free unconjugated rKRKTNF was approximately 5 ng/ml compared to 88.9 ng/ml of conjugated one. Studies performed by Tsutsumi et al. (Tsutsumi et al., 1996) indicated that modification of TNF-α with a single PEG chain did not significantly harm its cytotoxic activity. In contrast, TNF-α biological activity was drastically decreased with an increase in the degree of PEG modification, irrespective of the PEG molecular weight. Thus, the observed reduction in the biological activity of the liposomal rKRKTNF could be due to: first, an extended chemical modification of the protein which took place during the coupling reaction and may have caused permanent alterations of the

proper conformation of the cytokine necessary for biological activity; second, a possible dissociation of the trimeric rKRKTNF into a monomeric form of reduced activity; and third, a possible crosslinking between PEG and rKRKTNF which took place during the coupling reaction.

Biodistribution data were expressed as the percentage of the total injected dose of rKRKTNF and liposomes in each organ. Protein-free liposomes showed high blood circulation times accompanied by a reduced accumulation in liver and spleen. After 4.5 h, ~73% of the dose was still in the blood (Fig. 3). In sharp contrast, in as little as 30 min, all proteoliposomes were cleared from the blood circulation (Fig. 4). The rapid plasma elimination rate was in agreement with liver and spleen rKRKTNF accumulation. Lungs and kidneys were practically devoid of any proteoliposome. It is really astonishing that the presence of 99.87% of unconjugated PEG grafted on the liposome surface was not able to protect the liposomes from macrophage recognition and subsequent clearance from the blood.

Table 1
Proteoliposome characteristics

	rKRKTNF-PEG-liposomes[a]
PEG content (mol% of total lipid)	7
Mean diameter (nm)	138 ± 11
Initial protein:lipid ratio (w/w)	1:62
Coupling efficiency (%)[b]	33.43 ± 3.86
Conjugated PEG (%)[c]	0.13 ± 0.01
Number of rKRKTNF molecules per liposome[d]	22 ± 1.41

[a] All data except the polyethyleneglycol (PEG) content (mol% of total lipid) and the initial protein:lipid ratio (w/w), refer to fraction 9. All calculations were based on the trimer rKRKTNF molecular weight (MW) 52 000. The results presented are the average of two independent experiments.

[b] The lipid concentration and the coupling efficiency of rKRKTNF were measured by ^{111}In and ^{125}I radioactivity counting, respectively.

[c] Percentage of grafted PEG-PE coupled to the rKRKTNF.

[d] The average number of lipid molecules per vesicle was calculated by the method of Enoch and Strittmatter (Enogh and Strittmatter, 1979). The presence of cholesterol (chol) molecules in the bilayer was not taken into account.

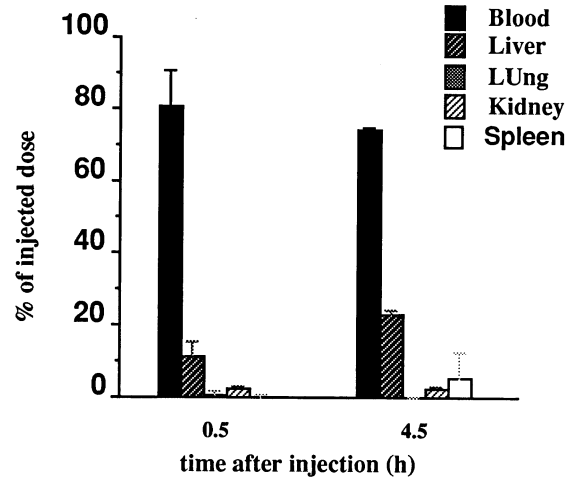

Fig. 3. Biodistribution of rKRKTNF-free liposomes with an average diameter of 148 ± 35 nm after tail vein injection into mice at a dose of 0.17 mg total lipid/mouse. Levels of liposomes in the blood and other collected organs were examined at the indicated time intervals after injections. Bars represent S.D.; $n = 3$.

Studies performed by others (Beutler et al., 1985) have shown that the average half life of free TNF-α in the blood after i.v. injection in mice was 6–7 min. The main organs reported to accumu-

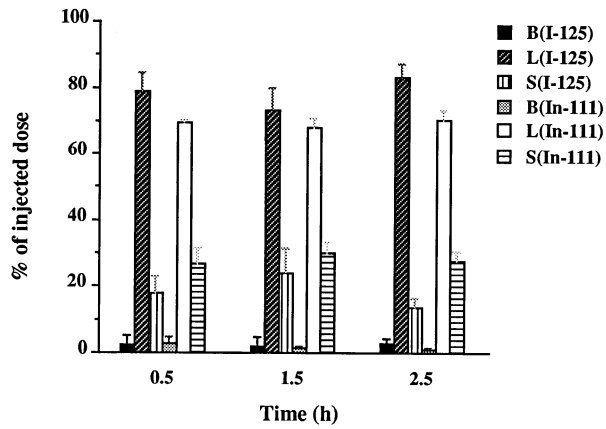

Fig. 4. Biodistribution of rKRKTNF liposomes with an average diameter of 138 ± 11 nm. ^{111}In labeled-liposomes bearing ^{125}I labeled-rKRKTNF were injected through tail vein at a dose of 0.5 μg rKRKTNF or 0.17 mg total lipid/mouse. Liposome levels in the blood, liver and spleen were determined at the indicated time intervals after injections. Bars represent S.D.; $n = 6$. B, blood; L, liver; S, spleen.

late TNF was kidney and liver. The protein was found to degrade rapidly in these tissues without formation of any intermediate products.

The pharmacokinetic property of conjugated-rKRKTNF long-circulating liposomes in this study is completely different. Conjugated rKRK-TNF was found almost exclusively in the liver and the radioactivity of ^{125}I-rKRKTNF in that organ remained constant for at least 4 h (not shown). The fact that elimination of the proteoliposomes (500 ng of rKRKTNF conjugate) from the blood was very fast and complete (100%) and that the protein remained undegraded in the tissues suggests that Kupffer cell recognition took place. This is probably related to the fact that Kupffer cells represent the largest population of macrophages in the mammalian body. They are strategically located at the venous return from the GI tract and have as a primary function the ingestion and degradation of particulate material in portal blood. Binding of TNF-α to the sinusoidal endothelial wall may have also contributed to the rapid elimination of proteoliposomes.

Previous experiments performed in the laboratory (Mori et al., 1996) have shown that it is possible to prolong the mean residence time of rHuTNF in the blood. The study, however, was conducted using a rHuTNF-NGPE conjugate (phospholipid conjugate) incorporated into liposomes composed of phosphatidylcholine, chol and PEG-phosphatidylethanolamine. It is known that although this type of design, where the ligand is completely shielded by the grafted PEG chains, is able to effectively shield the targeting ligand from the mononuclear phagocyte system, the steric hindrance generated by the polymeric chain motion of the PEG interferes with the receptor–ligand interaction, similar to what has been reported for antibody conjugated to liposomes (Klibanov et al., 1991; Torchilin et al., 1994).

In an attempt to improve the therapeutic efficacy of TNF-α, chemical coupling of a genetically modified TNF has been employed, to the distal end of liposome anchored PEG. Since the N-terminus modification was shown not to severely harm TNF's biological activity (Goh and Porter, 1991), the use of rKRKTNF was supposed to provide an easy, reproducible, and safe chemical coupling of the cytokine N-terminus to the distal end of the PEG chains. In view of the fact that the cytotoxic activity of TNF can be exerted upon binding of the protein to either of the two cell surface receptors, TNF-R55 or TNF-R75, chemical conjugation of rKRKTNF was attempted on the distal terminals of liposomal surface grafted PEG chains. Ideally, this type of design could be capable of providing direct interaction of the TNF with the tumor cells after passive accumulation in the tumor region, usually provided by the long-circulating liposomes as a result of a leaky tumor vasculature. Unfortunately, conjugation of such a small portion of TNF to the distal ends of liposome grafted PEG resulted in a rapid clearance of the formulation from the blood.

In conclusion, two of the thorny problems associated with targeted particulate drug delivery systems are RES recognition of the drug delivery system and/or the targeting ligand and the slow rate of extravasation to the target tissue. It was suggested in the past that direct conjugation of functional proteins and antibodies to the liposomal PEG terminals could provide high blood circulation times and tissue specific targeting. Although the coupling ratios were lower than the ones previously reported (Blume et al., 1993; Allen et al., 1995; Maruyama et al., 1995; Kirpotin et al., 1997), it was not possible to avoid the RES. It is believed that both non specific chemical modification of rKRKTNF and an inherent strong recognition of the TNF by the RES contributed to the fast elimination of the current delivery system from the blood stream. The results alerted the need for a more selective bioconjugate chemistry. Furthermore, our data suggest that not all the biological molecules are suitable as targeting ligands exposed to the grafted PEG extremities of long circulating liposomes.

Acknowledgements

This work was supported by the NIH grant CA 24553. We are grateful to Kathy Davis for editing the manuscript.

References

Allen, T.M., Agrawal, A.K., Ahmad, I., Hansen, C.B., Zalipsky, S., 1994. Antibody-mediated targeting of long-circulating (Stealth) liposomes. J. Liposome Res. 4, 1–25.

Allen, T.M., Ahmad, I., Lopes de Menezes, D.E., Moase, E.H., 1995. Immunoliposome-mediated targeting of anticancer drugs in vivo. Biochem. Soc. Trans. 23, 1073–1079.

Bally, B.M., Ansell, M.S., Tardi, G.P., Harasym, O.T., 1997. Liposome targeting following intravenous administration: defining expectations and a need for improved methodology. J. Liposome Res. 7, 331–361.

Beutler, A.B., 1992. Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. Raven Press, New York.

Beutler, A.B., Milsark, W.I., Cerami, A., 1985. Cachectin/tumor necrosis factor: production, distribution, and metabolic fate in vivo. J. Immunol. 135, 3972–3977.

Blume, G., Cevc, G., Crommelin, M.D.J.A., Bakker-Woudengerg, I.A.J.M., Kluft, C., Storm, G., 1993. Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of polymeric chains combines effective target binding with long circulation times. Biochim. Biophys. Acta 1149, 180–184.

Carmichael, J., DeGraff, G.W., Gazdar, F.A., Minna, D.J., Mitchell, B.J., 1987. Evaluation of a Tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res. 47, 936–942.

Enogh, G.H., Strittmatter, P., 1979. Formation and properties of 1000-Å-diameter, single-bilayer phospholipid. Proc. Natl. Acad. Sci. USA 76, 145–149.

Gase, K., Korobko, V.G., Wisniewski, H.G., Le, J., Dobrynin, V.N., Filippov, S.A., Gutsche, W., Maksimova, Y.N., Schlott, B., Shingarova, L.N., Vilcek, J., Behnek, D., 1990. Critical role of the C-terminus in the biological activities of human tumor necrosis factor-alpha. Immunology 71, 368–371.

Goh, R.C., Porter, G.A., 1991. Structural and functional domains in human tumor necrosis factors. Protein Eng. 4, 385–389.

Goren, D., Horowitz, A.T., Zalipsky, S., Woodle, M.C., Yarden, Y., Gabizon, A., 1996. Targeting of stealth liposomes to erbB-2(Her/2) receptor: in vitro and in vivo studies. Br. J. Cancer 74, 1749–1756.

Hnatowich, D.J., Friedman, B., Clancy, B., Novak, M., 1981. Labeling of preformed liposomes with Ga-67 and Tc-99m by chelation. J. Nucl. Med. 22, 810–814.

Kirpotin, B.D., Park, W.J., Hong, K., Shao, Y., Shalaby, R., Colbern, G., Benz, C.C., Papahadjopoulos, D., 1997. Targeting of liposomes to solid tumors: the case of sterically stabilized anti-HER2 immunoliposomes. J. Liposome Res. 7, 391–417.

Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P., Huang, L., 1991. Activity of amphipathic poly(ethyleneglycol)5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to the target. Biochim. Biophys. Acta 1062, 142–148.

Maruyama, K., 1997. Long-circulating immunoliposome targeting in animal models. J. Liposome Res. 7, 363–389.

Maruyama, K., Takizawa, T., Yuda, T., Kennel, J.S., Huang, L., Iwatsuru, M., 1995. Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. Biochim. Biophys. Acta 1234, 74–80.

Mori, A., Duda, E., Huang, L., 1996. Recombinant human tumor necrosis factor-a covalently conjugated to long-circulating liposomes. Int. J. Pharm. 131, 57–66.

Torchilin, P.V., Omelyanenko, V.G., Papisov, I.M., Bogdanov, A.A., Trubetskoy, S.V., Herron, N.J., Gentry, A.C., 1994. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer coated liposome longevity. Biochim. Biophys. Acta 1195, 11–20.

Tsutsumi, Y., Tsunida, S., Kamada, H., Kihira, T., Nakagawa, S., Kaneda, Y., Kanamori, T., Mayumi, T., 1996. Molecular design of hybrid tumour necrosis factor-α II: the molecular size of polyethylene glycol-modified tumour necrosis factor-α affects its anti-tumour potency. Br. J. Cancer 74, 1090–1095.

Wu, M.S., Robbins, J.C., Bugianesi, R.L., Ponpipom, M.V., Shen, T.Y., 1981. Modified in vivo behavior of liposomes containing synthetic glycolipids. Biochim. Biophys. Acta 674, 19–29.

Zalipsky, S., Puntambekar, B., Bolikas, P., Engers, C.M., Woodle, M.C., 1995. Peptide attachment to extremities of liposomal surface grafted PEG chains: Preparation of long-circulating form of laminin pentapeptide, YIGSR. Bioconjug. Chem. 6, 705–708.