A NOVEL FAMILY OF L-AMINO ACID-BASED
BIODEGRADABLE POLYMER-LIPID CONJUGATES FOR THE
DEVELOPMENT OF LONG-CIRCULATING LIPOSOMES WITH
EFFECTIVE DRUG TARGETING CAPACITY

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ABSTRACT

Purpose. The objective of this study was to develop biodegradable polypeptide-lipid conjugates for the design of polymer-coated long-circulating liposomes (LCL).

Methods. Lipid conjugates of poly(hydroxyalkyl L-asparagine/L-glutamine) were synthesized and incorporated into 0.15 µm dipalmitoyl phosphatidylcholine (DPPC) - cholesterol liposomes. Circulation times and biodistribution were assessed in rats using a radioactive lipid marker. Evaluation of the therapeutic activity of prednisolone phosphate loaded in 0.1 µm PHEA-DPPC-cholesterol liposomes in a rat experimental arthritis model was performed to demonstrate the drug-targeting potential of the polymer-coated liposomes.

Results. Coating of liposomes with poly(hydroxyethyl L-asparagine) (PHEA) and poly(hydroxyethyl L-glutamine) (PHEG) extended the circulation half-life to a similar extent as poly(ethylene glycol) (PEG), which is normally used for the preparation of LCL. Glutamine polymers with a hydroxypropyl or a hydroxybutyl group instead of hydroxyethyl group also yield prolonged circulation, however, not to the same extent as PHEA/G. The pharmacokinetic properties of PHEA-liposomes were independent of the lipid dose even at very low lipid doses of around 50 nmol per rat. PLP was successfully entrapped in PHEA-liposomes. These liposomes were shown to be stable in the circulation and equally effective in rat experimental arthritis as PLP encapsulated in PEG-liposomes.

Conclusions. PHEA and PHEG are attractive alternative polymers for the design of LCL: their performance is similar to that of PEG-liposomes but they have the advantage of being biodegradable.
INTRODUCTION

Liposomes are small spherical particles that consist of one or more lipid bilayers enclosing an aqueous interior (1). Liposomes are highly suitable as drug carriers as they are composed of natural lipids and offer the opportunity to incorporate a wide variety of therapeutic agents for the purpose of drug-targeting (2). Biodistribution studies in laboratory animals showed that intravenously (i.v.) injected liposomes predominantly home to cells of the mononuclear phagocyte system (MPS) that is present in liver, spleen and bone marrow. As a result, liposomes show generally a short circulation half-life after i.v. administration (3,4).

The development of bilayer surface modifications that were able to reduce rapid uptake by the MPS, represented a major step forwards to the successful clinical application of liposomes (5). Incorporation of a lipid conjugate of the water-soluble polymer poly(ethylene glycol) (PEG) results in a polymeric layer around the liposome, which reduces the adhesion of plasma proteins that would otherwise cause rapid recognition of the liposomes by MPS-phagocytes. With a PEG-coating, liposomes oppose rapid uptake by the MPS and acquire a prolonged circulation property. With these so-called ‘long-circulating liposomes’ (LCL) drug targeting to tissues other than liver, spleen and bone marrow became possible. Indeed, LCL were shown to selectively accumulate at sites of enhanced vascular permeability as found in tumors and inflamed areas in the body (6,7). This form of targeted drug delivery resulted in increased therapeutic efficacy and/or reduced toxicity of several cytostatic, antifungal and antibacterial drugs and led to the market approval of liposomal doxorubicin as the first commercially available liposomal cytostatic agent (8-11).

Besides PEG a few other polymers have also been shown to induce prolonged circulation behavior of liposomes upon attachment to their surface (12). Woodle and coworkers investigated oxazoline-derived polymers, Maruyama et al. designed poly(glycerol)-coated liposomes, while Torchilin and coworkers developed several water-soluble vinyl-based polymers for the creation of long-circulating liposomes. All groups reported circulation half-lives comparable to PEG-liposomes (13-18). Up to now PEG-liposomes are the only polymer-coated liposomes that have been approved for clinical use. However, despite the fact that low-molecular weight PEG has been shown to be non-toxic and to be readily excreted by the kidneys, the biological fate of (liposome associated) PEG after cellular uptake is not known (19,20). Since PEG is expected not to be easily intracellularly degraded, it cannot be excluded that PEG may affect cell functioning at the long term (21).

It was the objective of this study to design a biodegradable and biocompatible polymer-lipid conjugate for the development of LCL. To achieve minimal toxicity of the polymer as well as the compounds that are formed upon biodegradation, we selected natural L-amino acids as starting material for the synthesis of polymer-lipid conjugates in this study. Besides the advantage of being intracellularly degradable, such poly(L-amino acid)-lipid conjugates are expected to be degraded by proteolytic enzymes that are present at pathological target tissues such as tumors and sites of inflammation. Therefore, these
polymer-lipid conjugates may allow the incorporation of bilayer functionalities that have to remain shielded in the circulation until arrival at the pathological target sites where they can perform their specific function.

The present study investigates the feasibility of polymers based on hydroxyalkyl derivatives of L-glutamine and L-asparagine as monomers. Poly(hydroxyethyl L-glutamine) (PHEG) was selected as a starting polymer as PHEG is known to be water-soluble, biocompatible and biodegradable by lysosomal peptidases from the papain family (22). Subsequently, structure-activity relationships were established by replacing the hydroxyethyl side group with other hydroxyalkyl groups and selecting L-asparagine as backbone monomer instead of glutamine. Different lipid molecules were evaluated to ensure stable anchoring of the polymer-lipid conjugates in the liposome bilayer. In addition, the effect of the polymer grafting density on the liposome surface and the effect of lipid dose on the circulation half-life were assessed. Finally, the feasibility of liposomal incorporation and targeting an anti-inflammatory glucocorticoid to inflamed sites in an experimental animal model of arthritis was evaluated.
MATERIALS AND METHODS

Synthesis and characterization of different PEG-lipid conjugates

PEG5000-lipid conjugates were synthesized from methoxy-PEG5000-isocyanate, commercially available from Shearwater Polymers, Huntsville Al, USA), and long-chain alkylamines: octadecyl amine (stearylamine) NH₂-C₁₈H₃₇ (ODA), having a single C₁₈-tail, dioctadecyl amine (distearylamine) NH-(C₁₇H₃₅)₂ (DODA), and 1-heptadecyl-octadecylamine NH₂-CH-(C₁₇H₃₅)₂ (HOA) both with a double alkyl tail. Isocyanate groups reacted with these primary and secondary alkyl amines rapidly at room temperature to form ureas of a general formula: mPEG-NH-CO-NH-R in case of a primary amine (ODA, R=C₁₈H₃₇ or HOA, R=CH(C₁₇H₃₅)₂) or mPEG-NH-CO-N-R₂ in case of a secondary amine (DODA , R being a C₁₈-tail).

All three PEG5000 derivatives were synthesized in exactly the same way. For the synthesis of the PEG5000-stearyl derivative a solution of 300 mg methoxyPEG-isocyanate (0.06 mmol, MW 5000, Shearwater Polymers) and 18 mg (0.07 mmol) ODA in 1.5 ml chloroform was stirred at room temperature for ca. 0.5-1 hour. The solution was then precipitated into 20-30 ml petroleum-ether. A fine white powder (220 mg, 75 %) was obtained after filtration and drying.

Characterization:

\[ ^1H\text{-NMR (CDCl}_3, \delta \text{ in ppm relative to TMS): } \]

PEG: 3.6 (CH₂-O)
Stearyl anchor: 1.2 (CH₂) & 0.8 (CH₃)

The ratio of PEG and stearyl peak integrals confirms the presence of one stearyl group per PEG moiety.

Synthesis and characterization of PHEA-DODASuc

Synthesis of poly(L-hydroxyethyl asparagine)-N-succinyl-dioctadecylamine (PHEA-DODASuc) is schematically represented in Figure 1 A. To a solution of 3 g β-benzyl L-aspartate N-carboxy anhydride (NCA) (synthesized as described by Fuller et al. (23)) dissolved in 9 ml dry dimethylformamide (DMF) (Aldrich-Chemie, Steinheim, Germany) was added 0.3 ml of a 2 M solution of methylamine in tetrahydrofuran (THF) (Aldrich) as initiator. The solution (initially clear, after ca. 2 hrs cloudy) was stirred for one day under a nitrogen atmosphere at room temperature and then precipitated into water (150 ml), collected by filtration and dried. Yield: 2 g poly(benzyl-L-aspartate) (PBLA).

Polymer-lipid coupling was performed to obtain the PBLA-DODASuc-conjugate. Briefly, a solution of 2 g PBLA in 5 ml chloroform containing ca. 150 ml triethylamine (Merck, Darmstadt, Germany) was added to a solution of 200 mg dicyclohexycarboxdiimide DCC (Acros Chimica, Geel, Belgium), 15 mg 4-(dimethylamino) pyridinium-4-toluene sulfonate (DPTS) (Acros) and 350 mg N-succinyl-dioctadecylamine (DODASuc; for synthesis
see (24)) in 6 ml chloroform, that had been stirred for 1 hour. The mixture was stirred for one day and then precipitated into methanol. The polymeric product was filtered off and dried in vacuo. Yield: 1.4 g PBLA-DODASuc. Aminolysis of 1.4 g PBLA-DODASuc was performed with ca. 4 ml ethanolamine (Aldrich), using 0.4 g 2-hydroxy pyridine (Aldrich) as a catalyst, in 10 ml DMF solution at 40 °C for 1 day yielding PHEA-DODASuc. Yield: 0.8 g after dialysis (molecular weight cut off 500) and freeze-drying.

NMR (DMSO-d6) (δ in ppm relative to TMS):
distearyl: 0.8 (CH₃), 1.2 (CH₂), 1.4 (CH₂-N)
PHEA: 2.4-2.8 (β-CH₂), 3.2 & 3.4 (hydroxyethyl), 4.6 (α-CH + OH), 7.8-8.5 (NH)

From the ratio of integrals of the distearyl signals and the α-CH signal the molecular weight of the polymer part of the conjugate was calculated to be ca. 3000 g/mol.

Synthesis and characterization of poly(L-hydroxyalkyl glutamine)-DODASuc-conjugates

Synthesis of poly(hydroxyethyl glutamine) (PHEG)-lipid conjugates is schematically shown in Figure 1 B. To a solution of 3 g γ-benzyl-L-glutamate NCA (for synthesis see (23)) in 8 ml dry DMF was added a solution of 0.1 g N-BOC-1,4-diaminobutane (Fluka, Zwijndrecht, The Netherlands) in chloroform as initiator. This solution was stirred for 1 day under a nitrogen atmosphere at room temperature. After precipitation into ca. 100 ml methanol the polymer was filtered off and dried, yielding 2 g PBLG with a BOC-protected amino end group. To remove BOC, a solution of 1.7 g PBLG-diaminobutane-BOC in 12 ml 2 M HCl/dioxane was stirred for 4 hrs and then added dropwise to ca. 150 ml water in which appr. 10 g NaHCO₃ was dissolved. The product was filtered off, washed with water and dried in vacuo. Yield: 1.4 g PBLG-diaminobutane. Deprotection was complete as demonstrated by NMR analysis.

To obtain the PBLG-DODASuc-conjugate, 340 mg DODASuc, 180 mg DCC and 15 mg DPTS were dissolved in 5 ml chloroform. The solution was stirred for 1 hour at room temperature. Next, a solution of 1.4 g PBLG-diaminobutane and 120 mg triethylamine in 8 ml chloroform was added. After stirring overnight at room temperature the obtained solution was added dropwise to an excess of methanol (ca. 150 ml). The polymeric product was filtered off, washed and dried. Yield: 1.2 g PBLG-DODASuc.

¹H-NMR (CDCl₃) (δ in ppm relative to TMS):
disteary signals at 0.8-0.9 (CH₃) and 1.2-1.4 (methylene protons)
PBLG: 2.2 & 2.6 (β,γ-CH₂), 4.0 (α-CH), 5.0 (benzyl CH₂), 7.3 (phenyl)

Aminolysis with ethanolamine was performed to obtain PHEG-DODASuc: 1.2 g PBLG-DODASuc (see above) and 0.5 g 2-hydroxypyridine were dissolved in 10 ml DMF. Then ca. 4 ml ethanolamine was added dropwise. After stirring for 24 hrs at 40 °C under a nitrogen atmosphere the solution was precipitated into ca. 200 ml diethylether. The precipitate was dissolved in water, dialyzed (molecular weight cut off 500) and subsequently freeze-dried yielding 0.8 g PHEG-DODASuc conjugate.
Figure 1. Synthesis of poly(hydroxylalkyl L- asparagines/glutamine)-lipid conjugates. (A) Schematic representation of PHEA-DODASuc synthesis strategy. (B) Schematic representation of the synthesis of PHEG-DODASuc. Note that DODASuc is attached to PHEA at the amino end group (on the right) of the poly(amino acid) back bone, whereas to PHEG it is attached to the carboxylic end (on the left, using dianinobutane to connect the poly(amino acid) back bone to DODASuc).
1H-NMR (DMSO-d6) (δ relative to TMS):
disteary signals at 0.8-0.85 (CH₃) and 1.2-1.5 (methylene protons)
PHEG: 1.7-2.2 (β,γ-CH₂), 3.1 & 3.3 (hydroxyethyl), 4.2 (α-CH), 4.7 (OH), 7.8 & 8.2 (NH)
From the ratio of integrals of the disteary signals and the α-CH signal molecular weight of PHEG was calculated to be ca. 4000.
By varying the monomer/initiator ratio similar conjugates with different PHEG molecular weights (3000 and 8000) were synthesized.

Maldi-TOF confirms the molecular structure of the PHEG-DODASuc conjugate as depicted in Figure 1 A.
Na+-adduct: m/z 3064.5 (n=13), 3236.1 (n=14), 3408.7 (n=15), 3580.6 (n=16), 3752.9 (n=17), 3924.7 (n=18), 4096.7 (n=19), 4268.4 (n=20), 4441.1 (n=21), 4613.3 (n=22), 4785.1 (n=23), etc.

The same procedure of aminolysis but with 3-propanol amine instead of ethanolamine yielded PHPG-DODASuc:

NMR (DMSO-d6) (δ in ppm relative to TMS):
disteary signals at 0.8-0.85 (CH₃) and 1.2-1.5 (methylene protons)
PHPG: 1.7-2.2 (β,γ-CH₂), 1.5 & 3.1 & 3.3 (hydroxypropyl), 4.2 (α-CH), 4.6 (OH), 7.8 & 8.2 (NH)
Maldi-TOF:
Na+-adduct: m/z 3623 (n=15), 3810 (n=16), 3996 (n=17), 4182 (n=18), 4368 (n=19), 4555 (n=20), etc.

To obtain PHBG-DODASuc, the same procedure of aminolysis of PBLG-DODASuc was repeated with 4-butanolamine (Merck). Stirring was performed for 48 hrs instead of 24 hrs at 40 °C.

NMR (DMSO-d6) (δ in ppm relative to TMS):
disteary signals at 0.8-0.85 (CH₃) and 1.2-1.5 (methylene protons)
PHBG: 1.7-2.2 (β,γ-CH₂), 1.4 & 3.1 & 3.3 (hydroxybutyl), 4.2 (α-CH), 4.5 (OH), 7.8 & 8.2 (NH)

Preparation of radiolabeled liposomes for comparative pharmacokinetics
Liposomes were prepared as described previously (25). Briefly, a lipid mixture in ethanol with a molar ratio composition of 1.85:0.15:1.0 (DPPC:polymer-lipid conjugate:cholesterol) was prepared. Such molar ratio results in liposomes containing 7.5% polymer-lipid conjugate as a percentage of the total amount of phospholipid. To the mixture [³H]-cholesteryl oleylether was added as a non-degradable liposome lipid phase marker. A lipid film was created by rotary evaporation under reduced pressure. The lipid film was hydrated with phosphate buffered saline (PBS) at an initial total lipid concentration of 20 µmol/ml.
The liposomes were sized by multiple extrusion using a medium pressure extruder equipped with two stacked polycarbonate membrane filters, one with a pore size of 200 nm on top of one with 100 nm pores. Components that were not incorporated in liposomes were removed by gel filtration on a PD-10 column (Pharmacia, Uppsala, Sweden) eluted with PBS.

Characterization of liposome preparations

Radioactivity of the liposomal dispersions was assayed in an Ultima Gold liquid scintillation cocktail purchased from Hewlet Packard (Groningen, The Netherlands) and counted in a Philips PW 4700 liquid scintillation counter. Lipid content of the liposomal dispersion was determined by assessing the radioactivity of the liposomes before and after preparation. The mean particle size of the liposomes was determined by dynamic light scattering with a Malvern 4700 system (Malvern, UK). The mean size ranged between 140 and 160 nm. In addition to the mean particle size, the system reports a polydispersity index (a value between 0 and 1; 0 indicating that a complete monodisperse system is obtained, whereas 1 indicates maximal variation in particle size). All liposome preparations used had a polydispersity index of below 0.15. In Table I the composition and characteristics of the different liposome types are summarized. Liposome preparations were stored under nitrogen at 4 °C and used within one week after preparation.

Table I. Liposome composition and characteristics. Means of 3 measurements are shown.

<table>
<thead>
<tr>
<th>Grafting density (%)</th>
<th>Composition DPPC:Chol:PLC*</th>
<th>Mean diameter (nm)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARE (no polymer)</td>
<td>2:1</td>
<td>151 ± 2</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>PEG-DSPE</td>
<td>1.85:1.0:0.15</td>
<td>139 ± 2</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>PHEG-DODASuc 1</td>
<td>1.98:1.0:0.02</td>
<td>151 ± 2</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>PHEG-DODASuc 2.5</td>
<td>1.95:1.0:0.05</td>
<td>156 ± 2</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>PHEG-DODASuc 7.5</td>
<td>1.85:1.0:0.15</td>
<td>151 ± 2</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>PHEG-DODASuc 15</td>
<td>1.85:1.0:0.30</td>
<td>160 ± 1</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>PHEA-DODASuc 7.5</td>
<td>1.85:1.0:0.15</td>
<td>146 ± 2</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>PHPG-DODASuc 7.5</td>
<td>1.85:1.0:0.15</td>
<td>153 ± 1</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>PHBG-DODASuc 7.5</td>
<td>1.85:1.0:0.15</td>
<td>148 ± 2</td>
<td>0.07 ± 0.04</td>
</tr>
</tbody>
</table>

* PLC: Polymer-lipid conjugate
Comparative pharmacokinetics of polymer-coated $^3$H-labelled liposomes in rats

Male Wistar rats with an approximate body weight of 200 g were used (outbred, SPF-quality, Utrecht University, The Netherlands). Besides the different poly(hydroxyalkyl L-amino acid)-coated liposomes, non-polymer-coated (‘bare’) liposomes and liposomes coated with PEG2000 coupled to distearoyl phosphatidylethanolamine (PEG-DSPE) were prepared and evaluated as ‘negative’ and ‘positive’ controls respectively. Single-dose intravenous injections of liposomal preparations containing 5 µmol total lipid and approximately 50 kBq of radioactivity, were given in the tail vein. Blood samples of 100 µg were collected from the opposite tail vein of each rat at the following time points post-injection: 5 minutes and 1, 4, 8, 24 and 48 hours. Radioactivity in blood samples was determined by adding Solvable tissue solubilizer (NEN, Dreieich, Germany) and 35% hydrogen peroxide. After overnight incubation the samples were assayed in Ultima Gold scintillation cocktail (Packard BioScience B.V., Groningen, The Netherlands) and counted for radioactivity with a Philips PW 4700 liquid scintillation counter. At 48 hrs post-injection liver and spleen were dissected, homogenized and processed according to the same method as described for the blood samples. Besides tissue and blood samples also the radioactivity of the injected dose was counted. The % injected dose in the organs was calculated by dividing the total radioactivity of the organs by the injected dose. To obtain the % injected dose values in the circulation, the radioactivity of the blood samples was multiplied with a factor 10 times the total mass of the blood in grams (calculated as 7% of the total body weight) and divided by the injected dose. The results are presented as the mean ± standard deviation of the percentage of the injected dose of 4 rats.

Liposome preparation for assessing drug targeting potential

Liposomes containing the anti-inflammatory glucocorticoid prednisolone phosphate (PLP) were prepared as described previously (see Chapter 3). In brief, a lipid film containing 7.5% PHEA-DODASuc or PEG-DSPE was created by rotary evaporation and hydrated with a solution of 100 mg/ml prednisolone phosphate (PLP) (Bufa, Uitgeest, The Netherlands) in water at a initial lipid concentration of 100 µmol lipid per ml dispersion. Liposomes were sized to approximately 90 nm by multiple extrusion. A smaller size than the liposomes used in the comparative pharmacokinetic studies was chosen, as this may further increase target localization of the liposomes. PLP and was removed by repeated dialysis using Slide-A-Lyzer dialysis cassettes with a molecular weight cut off of 10.000 (Pierce, UK) against PBS. Phospholipid content was determined with a phosphate assay in the organic phase after extraction of the liposomal preparations with chloroform (26). The aqueous phase after extraction was used for determining the PLP content by HPLC. Each ml liposomal preparation contained around 5 mg PLP and approximately 60 µmol phospholipid.
Pharmacokinetics of PLP encapsulated in polymer-coated liposomes

For assessment of in vivo stability in the circulation and pharmacokinetic behavior of PLP in polymer-coated liposomes, the PLP plasma concentration was measured at the time of injection, at 24 and 48 hrs post-injection. The PLP-plasma concentration was compared with PLP in PEG-liposomes, as the latter has been shown to be completely stable in the circulation by comparing the plasma concentration of a radioactive liposome marker to the plasma concentration of encapsulated PLP (see Chapter 3). In the same study plasma levels of PLP could hardly be detected after i.v. injection of free PLP, which strongly suggests that all PLP measured in plasma after injection of liposomal PLP must be liposome-associated. To measure PLP in plasma samples, plasma was extracted according to a method reported by Derendorf et al. (27). The extracts were assayed with a reversed-phase HPLC method, using UV-absorption detection at 254 nm.

Therapeutic activity of PLP-containing polymer-coated liposomes in adjuvant arthritis

The Dutch Committee of Animal Experiments approved the animal studies. Male inbred Lewis rats between 7 and 9 weeks of age (170-200 g) were obtained from Maastricht University, Maastricht, The Netherlands. Adjuvant arthritis was induced according to Koga and Pearson (28). Briefly, incomplete Freund's adjuvant containing heat-inactivated Mycobacterium tuberculosis was subcutaneously injected at the base of the tail. Paw inflammation started around day 10 after the immunization, reached maximal severity around day 20, after which the inflammation process gradually resolved. The rats were daily scored for the visual signs of inflammation. All rats were treated on day 15 post-immunization, when the average score of all rats in the experiment is about half the maximal scores reached in these experiments. The effect of treatment on clinical scores and body weight was monitored up to 4 weeks post-treatment.

Statistical analysis

For statistically assessing and comparing therapeutic efficacy in different groups the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups regarding other parameters, one-way analysis of variance was used. P values of less than 0.05 were considered significant.
RESULTS

Synthesis of PHEA and PHEG-DODASuc-conjugates
Poly(hydroxyethyl L-asparagine) (PHEA) and poly(hydroxyethyl L-glutamine) (PHEG) were synthesized starting from benzyl L-aspartate NCA and benzyl L-glutamine NCA, respectively, as shown in Figure 1. Polymerization of these L-amino acid benzyl ester NCA monomers was followed by aminolysis with alkanolamines. Molecular weights in the range of 2000-5000 were obtained via a primary amine initiated polymerization of these side group-protected amino acid NCA monomers. The molecular weight of the polymer is controlled by the molar ratio of monomer/initiator. N-succinyl-dioctadecylamine (DODASuc) (lipid anchor) could be coupled to the polypeptide’s amino end group. Polymerization of benzyl-L-aspartate initiated by a primary amine (e.g. methylamine) yields a polypeptide (PBLA) with an amino end group. In contrast, polymerization of benzyl-glutamate NCA initiated by a primary amine results in the formation of a polypeptide (PBLG) without an amino end group. As could be concluded from Maldi-TOF, a 5-membered lactam end group is formed by an intramolecular reaction of the amino end group with the benzyl ester group. The terminal amino group is lost.

Conjugation of DODASuc to PBLG, therefore, requires a slightly modified approach: first, benzyl-L-glutamate NCA is polymerized using BOC-butanediame (one amino group is BOC (tertiary butoxycarbonyl)-protected; the other amino group is not) as an initiator. The resulting PBLG containing a protected amino group is deprotected and then coupled to DODASuc using dicyclohexyl carbodiimide (DCC). Amphiphilic conjugates resulted after the polypeptides were made water-soluble by the aminolysis reaction of the benzyl ester side groups with alkanolamines (e.g., ethanolamine).

Preparation and characterization of polymer-coated liposomes
In Table I the characteristics of the polymer-coated liposomes are presented. The resulting mean diameter and polydispersity of PHEG-, PHPG-, PHBG- and PHEA-coated liposomes are comparable to those of PEG-liposomes. All preparations appeared to be physically stable upon storage and no signs of aggregation were found.

Selection of suitable anchor molecules
Figure 2 A shows the plasma concentration-time profiles of liposomes coated with different PEG5000-conjugates after i.v. injection. For the evaluation of the anchor molecules PEG5000 was chosen instead of PEG2000 as a molecular mass of PEG5000 more closely resembles the molecular mass of most of the polymers tested in this study. We coupled PEG5000 to a series of lipid anchor molecules and compared liposomes coated with these conjugates to liposomes without PEG (‘bare’ liposomes). Clearly, octadecyl amine (ODA) as a lipid anchor results in little prolongation of circulation time, suggesting that one single C_{18}-tail is not sufficient for stable grafting. Dioctadecyl amine (DODA) and heptadecyl octadecyl amine
L-amino acid-based biodegradable polymer-lipid conjugates for development of long-circulating liposomes

(HOA) yield improved prolongation of circulation behavior, similar to the phospholipid anchor distearyl phosphatidylethanolamine (DSPE), which is generally used for stable grafting of PEG on the liposome bilayer. Apparently, two alkyl tails are required for sufficient grafting stability. We selected DODA as the standard anchor molecule for synthesis of PHEA- and the different poly(hydroxyalkyl L-glutamine)-lipid conjugates.

Figure 2 B shows the tissue distribution to the MPS organs. The DODA- and HOA-PEG conjugates significantly reduce the hepatosplenic uptake of liposomes. As hepatosplenic uptake is the main cause of liposome elimination from the circulation, these data are in agreement with plasma concentration-time profiles shown in Figure 2 A.

![Graph A](image1.png)  
**Figure 2.** Pharmacokinetics and distribution to the MPS of PEG5000 conjugated with different anchor molecules incorporated with a grafting density of 7.5% in 150 nm DPPC-cholesterol liposomes. (A) %-injected dose in blood-curves of PEG5000–ODA (closed diamonds), PEG5000–DODA (closed squares), PEG5000–HOA (closed triangles), PEG5000–DSPE (closed circles), and bare liposomes without polymer-lipid conjugate (open circles). (B) Distribution to spleen (open bars), liver (gray bars) and total distribution to the MPS (liver and spleen) (black bars). Results are expressed as the mean percentage of the injected dose of 4 rats ± SD.

Successful prolongation of circulation half-life with PHEG- and PHEA-DODASuc

In Figure 3 A it is shown that coating liposomes with 7.5% PHEG4000 or PHEA3000 and PHEA5000 all coupled to N-succinyl-DODA (DODASuc) results in almost similar circulation behavior as compared to 7.5% PEG2000–DSPE, the conjugate which is most often used for the preparation of LCL. These results are in line with the tissue distribution data shown in Figure 3 B, which shows that all four polymer-lipid conjugates significantly reduce MPS uptake from the circulation.
Figure 3. Pharmacokinetics and distribution to the MPS of PHEA and PHEG versus PEG incorporated with a grafting density of 7.5% in 150 nm DPPC-cholesterol liposomes. (A) % injected dose in blood-curves of PEG2000–DSPE (closed circles), PHEG4000–DODASuc (gray diamonds), PHEA5000–DODASuc (gray triangles), PHEA3000–DODASuc (gray squares, and bare liposomes without polymer-lipid conjugate (open circles). (B) Distribution to spleen (open bars), liver (gray bars) and total distribution to the MPS (liver and spleen) (black bars). Results are expressed as the mean percentage of the injected dose of 4 rats ± SD.

Selection of optimal grafting density with PHEG-DODASuc

In Table II the effect of different grafting densities of PHEG4000 on circulation behavior and hepatosplenic uptake is shown. Although plasma concentration-time profiles show little differences at 4 and 24 hrs, hepatosplenic uptake at 48 hrs indicates that decreasing the grafting density to 2.5% and 1% or increasing the density to 15% leads to enhanced removal of liposomes by the MPS.

Table II. Percentage of injected dose of PHEG-liposomes in the circulation at 4 and 24 hrs post-injection and uptake by MPS organs at 48 hrs. Mean ± SD of 4 rats per group

<table>
<thead>
<tr>
<th>PHEG Grafting density</th>
<th>Blood circulation</th>
<th>Liver uptake</th>
<th>Spleen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>1%</td>
<td>55 ± 2</td>
<td>22 ± 1</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>2.5%</td>
<td>56 ± 8</td>
<td>22 ± 1</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>7.5%</td>
<td>61 ± 6</td>
<td>23 ± 4</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>15%</td>
<td>49 ± 4</td>
<td>17 ± 3</td>
<td>32 ± 5</td>
</tr>
</tbody>
</table>
L-amino acid-based biodegradable polymer-lipid conjugates for development of long-circulating liposomes

Effect of the side group

Introduction of longer hydroxyalkyl side groups to the poly(L-glutamine) back bone, such as hydroxypropyl and hydroxybutyl instead of hydroxyethyl, results in reduced prolongation of circulation time and enhanced MPS uptake to the level of ‘bare’ liposomes (Figure 4). The hydroxyethyl side group appears to be the optimal side group for the poly(hydroxyalkyl L-glutamine)-lipid conjugates.

Figure 4. Pharmacokinetics and distribution to the MPS of different poly(hydroxyalkyl L-glutamine)s incorporated with a grafting density of 7.5% in 150 nm DPPC-cholesterol liposomes. (A) %-injected dose in blood-curves of PEG2000–DSPE (closed circles), PHEG4000–DODASuc (gray squares), PHPG5000–DODASuc (gray diamonds), PHBG5000–DODASuc (gray triangles), and bare liposomes without polymer-lipid conjugate (open circles). (B) Distribution to spleen (open bars), liver (gray bars) and total distribution to the MPS (liver and spleen) (black bars). Results are expressed as the mean percentage of the injected dose of 4 rats ± SD.

Table III. Percentage of the injected dose of liposomes in circulation at 4 and 24 hrs post-injection. Mean ± SD of 4 rats per group.

<table>
<thead>
<tr>
<th>Lipid dose</th>
<th>PEG-liposomes</th>
<th>PHEA-liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td>5 µmol</td>
<td>66 ± 7</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>0.5 µmol</td>
<td>71 ± 10</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>0.05 µmol</td>
<td>46 ± 7</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>0.005 µmol</td>
<td>5 ± 3</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>
Effect of lipid dose

Table III shows the % injected dose of liposomes still present in the circulation at 4 and 24 hrs post-injection of PEG-coated liposomes and PHEA-coated liposomes, both at 4 different dose levels ranging from 0.005 to 5 µmol lipid per rat. At a dose of 0.05 µmol and especially at 0.005 µmol PHEA-liposomes show improved circulation behavior as compared to PEG-liposomes. PEG-liposomes are very rapidly eliminated at the 5 nmol dose level, they are hardly present in the circulation at 24 hrs post-injection, whereas still 9% of the injected PHEA-liposomes circulates at 24 hrs at the same dose level.

Drug targeting potential

Prednisolone phosphate (PLP) was encapsulated in both PEG- and PHEA-liposomes and injected in adjuvant arthritis rats. In this experimental arthritis model we previously showed that the therapeutic activity of PLP was dramatically increased by encapsulation in PEG-liposomes. Figure 5 A shows the plasma concentration of liposomally encapsulated PLP. The % fractions of the injected dose of PLP in PHEA-liposomes in the circulation at 24 and 48 hrs equals PLP in PEG-liposomes. As PLP-PEG-liposomes composed of DPPC and cholesterol have been shown not to leak PLP in the circulation, such may therefore also be assumed for PLP-PHEA-liposomes. The circulation half-life of these liposomes is somewhat longer than reported with the radionabeled liposomes in this study. However, this is most likely a result of a relatively small diameter of approximately 90 nm as compared to the radionabeled liposomes (between 140 and 160 nm). Figure 5 B shows that 10 mg/kg PLP-PHEA-liposomes induces a similar reversal of the inflammation reaction as 10 mg/kg PLP-PEG-liposomes. Both preparations induce a strong anti-inflammatory effect lasting for two weeks.

Figure 5. Pharmacokinetics and therapeutic activity of PHEA- versus PEG-liposomal PLP in rat experimental arthritis. A) Plasma concentration at 0, 24, and 48 hrs post-injection of 10 mg/kg PLP in PHEA-liposomes (open bars) and PEG-liposomes (closed bars). Results are expressed as the mean concentration (µg/ml) of 5 rats ± SD. B) Daily arthritis score after induction of adjuvant arthritis at day 0 and treatment at day 15 (arrow) with 10 mg/kg PLP (120 µmol/kg phospholipid) in PHEA-liposomes (closed circles), 10 mg/kg in PEG-liposomes (closed squares), and saline (open diamonds). Results are expressed as the mean of 5 rats ± SEM.
DISCUSSION

In this paper biodegradable polymer-lipid conjugates based on amino acids are evaluated regarding their capacity to confer a long-circulation property to liposomes upon attachment to their lipid bilayer surface. Besides the polymer itself, also the choice of the lipid bilayer anchor deserves attention, as stable grafting of a polymer to the liposome bilayer is essential for achieving long-circulating behavior. Therefore, in this study the suitability of different lipid anchor molecules was evaluated with PEG5000 as a model polymer (Figure 2). The results show that successful prolongation of liposome circulation time can only be realized with anchor molecules with two stearyl chains. PEG coupled to DODA and HOA resulted in similar prolongation of circulation behavior as PEG-DSPE, which is at present most often used in liposome technology. In agreement with the findings of Webb et al., coupling of the polymer to a single alkyl chain yielded inferior results, most likely due to insufficient grafting stability (29). As DODA was easier to obtain from commercial sources than HOA, all polypeptides were tested in subsequent experiments with DODA as liposome anchor molecule.

The approach of L-amino acid-based polymer-lipid conjugates proved to be successful with PHEG4000, PHEA3000 and -5000, all coupled to DODASuc. The plasma concentration-time profiles as well as uptake by liver and spleen showed that these polymer-lipid conjugates prolonged the circulation time and reduced the uptake by the MPS to the same extent as PEG2000-DSPE (Figure 3). As has been shown in literature for PEG, lowering the grafting density of PHEG to 1 mol % resulted in increased uptake by the MPS (30). Interestingly, also a grafting density of 15 mol % PHEG increased MPS-uptake. However, the circulation times of liposomes grafted with 1, 2.5, 7.5, and 15 mol % PHEG did not differ significantly.

The results in this study show that coating the liposomes with poly(L-glutamine) with longer hydroxyalkyl side chains (PHPG and PHBG) instead of PHEG resulted in decreased circulation half-lives (Figure 4). An explanation for this observation may be found in the conformation of the polymers when dissolved in water. It has been suggested that suitable stealth polymers should be flexible and adopt a random conformation (31). E.g. dextran has a lower chain flexibility than PEG due to its carbohydrate backbone. When grafted on liposomes dextran is not able to prolong circulation times (32). Altschuler et al. showed that for poly(hydroxyalkyl-L-glutamine)s the degree of conformational freedom is optimal with hydroxyethyl as a side group and conformational freedom decreases for longer hydroxyalkyl groups. The use of hydroxypropyl and, even more so, hydroxybutyl resulted in the formation of α-helices in the polymer (33). The decrease in flexibility of these polymers correlates with the observed decrease of circulation half-life.

An important advantage of polymer-coated LCL over LCL without polymer-lipid conjugates is that incorporation of PEG leads to dose-independent pharmacokinetics over a broad dose range (34). To evaluate whether this phenomenon also applies to liposomes coated with poly(hydroxyalkyl L-amino acid)-lipid conjugates, pharmacokinetics of PHEA-liposomes were compared with PEG-liposomes at four different lipid doses ranging from...
0.005 to 5 µmol total lipid per rat. The lowest dose level at which the PEG-liposomes appeared to show dose-independent kinetics was around 500 nmol/rat (Table III) which corresponds with earlier reported data (35). Interestingly, with PHEA-liposomes dose-dependency of the pharmacokinetics was less pronounced even at a 10-fold lower dose. PHEA-liposomes may therefore offer an additional advantage over PEG-liposomes in cases when very low lipid doses are preferred, such as in scintigraphic detection of sites of pathology (7).

The final aim of this study was to show that successful drug targeting is possible, with liposomes exposing biodegradable polypeptide-lipid conjugates. Within the scope of this thesis a PEG-liposomal formulation of prednisolone phosphate (PLP) was developed that was shown to efficiently contain the drug in the circulation and to highly effectively deliver the encapsulated drug to inflamed joints in experimental arthritis models (Chapter 3 and 4). Incorporation of PEG increased the circulation half-life of the liposomal formulation from 6 hrs to 18 hrs, which was shown to be necessary to achieve sufficient local delivery for complete reversal of the development of joint inflammation in these models (Chapter 3). The present study shows that the use of PHEA-liposomes as PLP carrier results in a similar pharmacokinetic profile of encapsulated PLP. Also, the therapeutic activity of 10 mg/kg PLP-PHEA-liposomes in rat adjuvant arthritis was equal to that of 10 mg/kg PLP-PEG-liposomes. In Chapter 3 and 4 we report that non-encapsulated PLP was inactive at this dose level. The observation that a dramatic improvement of the therapeutic effect of PLP can be achieved with PHEA-liposomes, clearly points to the drug targeting potential of this formulation.

In conclusion, we present a novel sterically stabilized liposome formulation performing similarly to PEG-liposomes regarding in vivo long-circulation behavior and drug targeting potential. These novel polymer-coated liposomes are the first that are sterically stabilized with a biodegradable polymer coating.
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REFERENCES
