Plasma decay curves of radiolabeled LDL in rabbits

- Native LDL
- LDL reconstituted according to the present invention
- LDL reconstituted according to Krieger et al

Method for the preparation of a carrier loaded with lipophilic biologically active substance based on reconstituted LDL (Low Density Lipoprotein), wherein (1) LDL is lyophilized in the presence of a protective agent; (2) the lyophilized LDL is extracted with an organic solvent; (3) the biologically active substance solubilized in a solvent is incubated with extracted LDL; the solvent is evaporated and the reconstituted LDL solubilized in an aqueous buffer; the non-incorporated biologically active substance is separated from the LDL-complex, characterized in that in step (1) the protective agent is a monosaccharide, a disaccharide, a water-soluble polysaccharide, a sugar alcohol or a mixture of these and in step (3) optionally the extract obtained during the extraction of the lyophilized LDL is mixed with lipophilic biologically active substance solubilized in an organic solvent and this mixture is then incubated with the LDL. Preferred protective agents are sucrose, glucose and fructose. The reconstituted LDL obtained by the claimed process has properties similar to native LDL in vitro and in vivo.
<table>
<thead>
<tr>
<th>AT</th>
<th>Austria</th>
<th>GA</th>
<th>Gabon</th>
<th>MR</th>
<th>Mauritania</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU</td>
<td>Australia</td>
<td>GB</td>
<td>United Kingdom</td>
<td>MW</td>
<td>Malawi</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
<td>HU</td>
<td>Hungary</td>
<td>NL</td>
<td>Netherlands</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
<td>IT</td>
<td>Italy</td>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
<td>JP</td>
<td>Japan</td>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
<td>KP</td>
<td>Democratic People's Republic of Korea</td>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
<td>KR</td>
<td>Republic of Korea</td>
<td>SE</td>
<td>Sweden</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
<td>LI</td>
<td>Liechtenstein</td>
<td>SN</td>
<td>Senegal</td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
<td>LK</td>
<td>Sri Lanka</td>
<td>SU</td>
<td>Soviet Union</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
<td>LU</td>
<td>Luxembourg</td>
<td>TD</td>
<td>Chad</td>
</tr>
<tr>
<td>DE</td>
<td>Germany, Federal Republic of</td>
<td>MC</td>
<td>Monaco</td>
<td>TG</td>
<td>Togo</td>
</tr>
<tr>
<td>DK</td>
<td>Denmark</td>
<td>MG</td>
<td>Madagascar</td>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>FI</td>
<td>Finland</td>
<td>ML</td>
<td>Mali</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FR</td>
<td>France</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
METHOD FOR THE PREPARATION OF A MACROMOLECULAR CARRIER LOADED WITH A BIOLOGICALLY ACTIVE SUBSTANCE, PRODUCT THUS OBTAINED AND USE THEREOF

The present invention relates to a method for the preparation of a carrier for biologically active substance, below called drug, based on the reconstitution of LDL (Low Density Lipoprotein).

The term biologically active substance, or drug, is in the present paper used in its most wide definition and includes substances such as pharmaceutically active substances, enzymes, toxins, radiosensitizers, radioactive substances etc.

Many attempts have been made to increase the concentration of a biologically active substance, for example an antitumoral drug, in a certain organ or in certain target cells in order to increase the efficacy of the treatment and reduce the side effects.

One way to accomplish this is to link the drug to a carrier, for example a macromolecule. The rationale is that the macromolecule should have a high uptake by the target cell or that the linkage carrier/drug in other aspects would give a better efficacy than would be the case with the free drug.

A number of macromolecules have been investigated with respect to their use as carriers, such as DNA, liposomes, red blood ghost cells, lectines, different proteins such as antibodies, peptide hormones, and glucoproteins. Other molecules such as estrogens have as well been tried.

An ideal carrier linked to a drug should fulfil the following criteria:
1. It should permeate through the anatomical barriers separating the administering site and the target cell,

2. The linkage between the carrier and the drug should be sufficiently stable in the blood,

3. It should not be rapidly cleared from the blood circulation by the reticuloendothelial system in liver and spleen,

4. It should be unable to cross the cell membranes through diffusion in order to prevent unspecific uptake by non-target cells,

5. It should interact as selectively as possible with receptors on the target cell surface,

6. Substances which are inactively linked to the carrier but biologically active in the cell shall be carried into the cell by the carrier where the linkage should be cleaved,

7. It should be non-immunogenic.

Several proposed carriers, such as liposomes, do not fulfil the third criteria above, i.e. they are cleared rapidly from blood by the liver and spleen, and hence do not stay for a sufficient time to give the required effect.

LDL, Low Density Lipoprotein, is a macromolecule that has several attractive properties. This lipoprotein is a sphericle particle with a diameter of approximately 220 Å, a density between 1.019 and 1.063 and molecular weight of approximately 3 megadaltons, and stays in the circulation for 2-3 days in humans. Each LDL particle
contains a non-polar core composed of about 1500 cholesterol molecules esterified to long-chain fatty acids. The core is surrounded by a polar coat of cholesterol, phospholipid and apoprotein B, apo B. LDL is the major cholesterol transporter in human plasma, containing about 70% of plasma cholesterol. Mammalian cells have specific LDL receptors on the surface that recognises the apo B and binds the LDL-particle. The LDL particle is then endocytosed and transported to the lysosomes where it is degraded.

If a biologically active substance could be linked or incorporated to the LDL particle in such a way that the reconstituted LDL behaves like native LDL with respect to LDL-receptor mediated uptake and biological half-life in plasma, the substance could be targeted to cells expressing high levels of LDL-receptors. Due to the long half-life of LDL the system could as well act as a slow release system for appropriate substances.

Krieger et al (J. Biol. Chem. 253 (1978) 4093-4101 and J. Biol. Chem. 254 (1979) 3843-3853) have described a reconstitution procedure. Their method in brief is that LDL is lyophilized in the presence of unsoluble potato starch, and the neutral lipids are extracted with an organic solvent such as heptane. The substance to be incorporated is solubilized in an organic solvent and incubated with the lipid-depleted LDL. After evaporation of the organic solvent the reconstituted LDL is solubilized in an aqueous buffer.

Even if the reconstituted LDL particle according to the method of Krieger et al shows LDL receptor mediated uptake in vitro, it can not be used in vivo since the reconstituted particles are rapidly taken up by the cells of the reticuloendothelial system in liver and spleen.
We have unexpectedly found a modified reconstitution procedure that gives a reconstituted LDL-particle with almost the same properties as native LDL both in vitro and in vivo.

The present invention relates to a procedure for the preparation of a carrier loaded with a biologically active substance on the basis of reconstituted LDL, where (1) LDL is lyophilized in the presence of a protecting agent; (2) the lyophilized LDL is extracted with an organic solvent; (3) the biologically active substance is solubilized in an solvent and incubated with the extracted LDL; (4) the solvent is evaporated; (5) the reconstituted LDL is solubilized in an aqueous buffer, and (6) the non-incorporated substance is separated from the LDL-complex. In the procedure in step (1) the protective agent is a monosaccharide, a disaccharide, a watersoluble polysaccharide, a sugar alcohol or a mixture of these and in step (3) optionally the extract obtained during the extraction of the lyophilized LDL is mixed with the lipophilic biologically active substance solubilized in an organic solvent and this mixture is then incubated with the LDL.

The method according to the present invention is different from the method of Krieger et al mainly in the use of the protective substance. In step (1) Krieger is using potato starch as a protective agent. Potato starch is a water-insoluble polysaccharide with high molecular weight. In our method we are using as a protective agent a monosaccharide, a disaccharide, a water-soluble polysaccharide, a sugar alcohol or a mixture of these.

In order to investigate plasma clearance, human LDL was injected intravenously in mice (Balb/C) and the percentage of injected dose remaining in plasma was determined after 90 minutes. The percentage of native LDL remaining in plasma after 90 minutes is about 59. The percentage
of LDL reconstituted according to the method of Krieger et al. remaining in plasma was very low, less than 3. The percentage remaining in plasma according to the present invention have values of 57-58, which is a very surprising and technically important development.

The plasma clearance in rabbits was determined from plasma decay curves for radiolabeled LDL: for native LDL, reconstituted LDL according to the method of Krieger et al. and reconstituted LDL according to the method of the present invention. As can be seen from Figure 1, native LDL and the reconstituted LDL according to the present invention had the same clearance, while the reconstituted LDL according to Krieger et al. disappeared rapidly from plasma.

The particle sizes of various preparations of LDL re-solubilized in buffer have been determined by means of quasielectric light scattering. It was found that the apparent mean hydrodynamic radius of lyophilized LDL without any protective substance was about 2.9 times larger than that for native LDL, and LDL lyophilized in the presence of potato starch as protective substance had a radius of about 2.7 times, while LDL lyophilized in the presence of sucrose had a radius about 1.2 times that of native LDL. The drug-LDL complex according to the method of Krieger had an apparent mean hydrodynamic radius of about 3.8 times the value for native LDL, while the reconstituted drug-LDL complex according to the present invention had a radius of about 1.3 times that of native LDL.

The reconstitution method of Krieger et al. is based on a method developed by A. Gustafson (J. Lipid Res. 1965, 6, 512-517) to extract the cholesteryl esters from the core of LDL. The key step in this procedure is to use
potato starch to stabilize the lipid-depleted apo-
proteins during the lyophilization and heptaneextraction of the neutral lipids from the core of the LDL.

As shown above the method of Krieger et al will not give sufficient protection of the LDL. Important for the reconstitution of the LDL particle is to replace an appropriate amount of the neutral lipids in the core with the desired substance, or drug. If the drug-LDL complex should be used for targeting with the LDL receptor pathway and too much of the drug is inserted into the LDL particle there is a risk for the drug leaking out when the LDL particle is colliding with different cells in the blood and the blood vessels.

This gives an unspecific uptake of the drug and in this case the incorporated drug-LDL complex functions as a slow release system. A suitable protective substance should prevent aggregation of the LDL particles or other damages such as the falling apart of the particle and prevent too high a replacement of the lipids from the LDL particle.

If the amount of protective substance used is too small, then the resulting reconstituted LDL particle will have a rapid clearance from plasma; this effect is not linear, and the amount of protective substance should be at least 1 mg/mg LDL, where LDL is measured as protein. On the other hand, if the amount of protective substance is too high then the amount of drug incorporated will be too low; this means that there exists a certain range for the amount of protective substance used.

Protective substances fulfilling the above criteria are water-soluble saccharides such as monosaccharides, disaccharides, trisaccharides, watersoluble polysaccharides, sugar alcohols or mixtures of these.
Examples of monosaccharides, that can be used according to the present invention are: glucose, mannose, glyceraldehyde, xylose, lyxose, talose, sorbose, ribulose, xylulose, galactose and fructose. Examples of disaccharides are sucrose, trehalose, lactose and maltose. Examples of trisaccharides are raffinose. Among watersoluble polysaccharides certain watersoluble starches and celluloses can be mentioned. Examples of sugar alcohols are glycerol.

Preferred protective agents in the present invention is sucrose, glucose or fructose which gives a LDL complex behaving like native LDL.

The organic solvent used in step (2) for the extraction of the lyophilized LDL is preferably a non-polar solvent such as heptane or carbon tetrachloride. Heptane is the preferred solvent.

The biologically active substance that is incorporated into the lyophilized LDL shall be a lipophilic substance. Examples of such biologically active substances that can be incorporated into the LDL particle according to the present invention are anti-tumoral drugs, drugs affection the cholesterol synthesis, as well as radio-sensitizers, enzymes, enzyme inhibitors, radioactiv substances, toxins, etc.

The separation of the non-incorporated biologically active substance from the complex consisting of the LDL and the biologically active substance is done after solubilization in aqueous buffer by known procedures, such as centrifugation, filtration etc.

The invention is illustrated in the following examples, where the temperature is given in centigrades.

SUBSTITUTE SHEET
The LDL used in the examples was human LDL (density 1.019 - 1.063), obtained by ultracentrifugation of plasma from healthy human volunteers. All LDL amounts refer to protein according to the method of Lowry et al, J. Biol. Chem. 193 (1951) 265-275 where bovine serum albumin is used as standard. The biologically active substance used is the anti-tumoral drug AD-32, N-trifluoroacetyl adriamycin-14-valerate. The in vivo tests were performed on Balb/C mice.

Example 1
2 mg LDL (400 microliter of liquid) were after dialysis against 0,3 mM Na EDTA, transferred to a siliconized glass tube and 100 mg sucrose was added. The solution was rapidly frozen at -50 C in ethanol and lyophilized for 5-6 hours. The dried preparation was extracted three times with 5 ml of heptane. The heptane phases were pooled and evaporated. 2 mg of AD-32 in 0.5 ml of anhydrous diethyl ether were then added to the dried extracts. The solution was mixed with the extracted LDL by gentle agitation, and incubated at 4 C for 15 minutes. The solvent was evaporated under nitrogen, the drug-LDL complex was solubilized by the addition of 1 ml 10 mM Tricine, pH 8.4. Insoluble, non-incorporated drug was separated by centrifugation for 5 minutes in a Beckman microfuge. This gave a reconstituted LDL particle containing 110-130 molecules of AD-32 per LDL particle. The percentage remaining in plasma in Balb/C mice was about 58 after 90 minutes.

Example 2
Like example 1, but the lyophilized LDL was not extracted by heptane, instead AD-32 solubilized in ether was added directly. This gave about 35 - 43 molecules AD-32 per LDL particle and percentage remaining in plasma of about 55.
Example 3
Like example 1, but instead of sucrose as a protective agent 80 microliters of glycerol was used. This gave about 50 molecules AD-32 per LDL and the percentage remaining in plasma was about 55.

Example 4 - Comparative test I
Like example 1, but instead of sucrose 25 mg potato starch was used. The extract obtained after the extraction of LDL was discarded and AD-32 in anhydrous ether was added directly to the extractet LDL. (Method of Krieger et al). This gave about 400 molecules of AD-32 per LDL, but the percentage of injected dose remaining in plasma was below 3.

Example 5 - Comparative test II
Like example 1 but 25 mg of potato starch and 80 microliters of glycerol was used instead of sucrose. This gave about 50 molecules AD-32 per LDL particle and the percentage remaining in plasma was about 50.

Therapeutic test 1
In this test the drug AD-32 was used. The amount AD-32 administrated in this test was well below the toxic level for the mice and the result is presented here to give an example of the targetting effect of the drug-LDL complex compared to free drug. Balb/C mice were injected intraperitonally (i.p.) with 1.0 million WEHI-3B cells, a murine monomyelocytic cell line. No difference (2%) was found in survival (ILS, Increased Life Span) between the controls and the group treated with 1.9 mgAD-32/kg/day for five days. The AD-32 was administrered i.p. dissolved in a mixture of ethanol and castor oil. The mice that obtained the same dose of AD-32 incorporated into LDL according to the procedure in Example 1 administrated i.p. for five days had a 26% increase in life span.

SUBSTITUTE SHEET
Therapeutic test 2
Balb/C mice were injected i.p. with 1.0 million WEHI-3B cells and an alkylating agent, 4-amino N,N-bis (chloroethyl)-2-methyl-naphtalene, was incorporated according to the present method. The animals were treated for four consecutive days i.p. and the result is shown in Figure 2. The control group died during days 12-18, while in the treated group they died from day 25, with 50% still living on day 42 and 40% still surviving (on day 50).
CLAIMS

1. Method for the preparation of a carrier loaded with lipophilic biologically active substance based on reconstituted LDL, wherein (1) LDL is lyophilized in the presence of a protective agent; (2) the lyophilized LDL is extracted with an organic solvent; (3) the biologically active substance solubilized in a solvent is incubated with extracted LDL; (4) the solvent is evaporated and the reconstituted LDL solubilized in an aqueous buffer; (5) the non-incorporated biologically active substance is separated from the LDL-complex, characterized in that in step (1) the protective agent is a monosaccharide, a disaccharid, a water-soluble polysaccharide, a sugar alcohol or a mixture of these and that in step (3) optionally the extract obtained during the extraction of the lyophilized LDL is mixed with the lipophilic biologically active substance and this mixture is then incubated with the LDL.

2. Method according to claim 1, wherein the protective agent is glyceraldehyde, xylose, lyxose, raffinose, glucose, mannose, galactose, talose, ribulose, xylulose, trehalose, fructose, sorbose, maltose, lactose, sucrose, glycerol or a mixture of any of these.

3. Method according to claims 1 and 2, wherein the protective agent is sucrose.

4. Method according to claims 1 and 2, wherein the protective agent is glucose.

5. Method according to claims 1 and 2, wherein the protective agent is fructose.

6. Method according to any of claims 1 to 5 wherein the organic solvent used in step (2) is n-heptane.
7. Method according to any of the claims 1 - 6 wherein the solvent used in step (3) for the solubulization of the biologically active substance is ethyl ether, heptane, carbontetrachloride or benzene.

8. Method according to claims 1 to 7 wherein the amount of protective agent is in the range of 0.5 to 100 mg/mg LDL, where LDL is measured as protein.

9. Use of the product obtained according to claims 1 to 8 for diagnostic and therapeutical purposes in humans and animals, preferably for cancer treatment in humans.

10. Product obtained by the process of claims 1 to 8.
Survival of Balb/C mice injected with $1.0 \times 10^6$ WEHI-3B i.p.

- - - untreated

---

treated day 1, 2, 3, and 4 with 0.4 mg x 2 / day of drug-LDL complex, were weight is expressed as LDL protein and with about 500 molecules drug/LDL particle. The drug is 4-amino N,N. bis (chloroethyl)-2-methyl-naphthalene
I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

A 61 K 37/02, 37/22, 47/00, 31/00, C 07 K 15/16

II. FIELDS SEARCHED

<table>
<thead>
<tr>
<th>Classification System</th>
<th>Minimum Documentation Searched</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPC 2, 3, 4</td>
<td>A 61 K 31/00, 37/00, /22, 47/00; C 07 G 7/00;</td>
</tr>
<tr>
<td>IPC 1</td>
<td>A 61 K 17/00</td>
</tr>
</tbody>
</table>

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>US, A, 4 356 117 (U S GOVT., DEPT. OF HEALTH &amp; HUMAN SERVICES) 26 October 1982 See inter alia col. 1, first paragraph</td>
<td>1-8, 10</td>
</tr>
<tr>
<td>Y</td>
<td>Journal of Biological Chemistry, Vol. 253, No. 12, issued 1978 June (Baltimore), M. Krieger et al, &quot;Replacement of endogenous cholesteryl esters of low density lipoprotein with exogenous cholesteryl linoleate&quot;, see pages 4093-4101, especially p. 4094-4095 (Experimental procedures)</td>
<td>1-8, 10</td>
</tr>
<tr>
<td>Y</td>
<td>Journal of Biological Chemistry, Vol. 254, No. 10, issued 1979 May (Baltimore), M. Krieger et al, &quot;Replacement of neutral lipids of low density lipoprotein with esters of long chain unsaturated fatty acids&quot;, see pages 3845-3853, especially p. 3845, first paragraph, and p 3846 (Experimental procedures)</td>
<td>1-8, 10</td>
</tr>
</tbody>
</table>

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier document but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search: 1986-09-01

International Searching Authority: Swedish Patent Office

Date of Mailing of this International Search Report: 1986-09-03

Signature of Authorized Officer: Martin Hjälm Dahl

Form PCT/ISA/210 (second sheet) (January 1985)
### FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

<table>
<thead>
<tr>
<th>II</th>
<th>Fields searched (cont)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>US Cl 260:112; 424:177; 514:2, 21</td>
</tr>
</tbody>
</table>

### V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers, because they relate to subject matter not required to be searched by this Authority, namely:

   Methods for treatment of the human or animal body by therapy, as well as diagnostic methods. (PCT, Rule 39 (iv))

2. Claim numbers, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim numbers, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

### VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest:
- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>B Blombäck &amp; L Å Hanson, &quot;Plasma-proteine&quot;, published 1976, by AB KABI, AWE/GEBERS (Stockholm), see page 89 (3.LDL (C-lipoproteiner))</td>
<td>1-8, 10</td>
</tr>
<tr>
<td>A,Y</td>
<td>Cancer Research, Vol. 43, No. 10, issued 1983 October (Baltimore), M.J. Rudling et al, &quot;Delivery of aclacinomycin A to human glioma cells in vitro by the low-density lipoprotein pathway&quot;, see pages 4600-4605; especially p. 4600 (Abstract)</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>Läkartidningen, Vol. 79, No. 38, issued 1982 (Stockholm), C Peterson, &quot;Bärar-styrda läkemedel- 'skräddarsyd' terapi&quot;, see pages 3275-3277, especially p. 3276 (Lipoproteiner)</td>
<td>1</td>
</tr>
</tbody>
</table>