# A synthetic low density lipoprotein particle capable of supporting U937 proliferation in vitro

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**Abstract** **A synthetic LDL (sLDL) has been prepared by combining a lipid microemulsion with amphipathic peptides containing the apoprotein B receptor domain. The biological properties of sLDL have been investigated using the U937 in vitro cell proliferation assay. sLDL exhibits a concentration dependent and saturable stimulation of U937 proliferation. By utilizing different amphipathic peptides, variable proliferation is achieved, indicating a specific interaction between sLDL and the U937 LDL receptor are possible. U937 proliferation is reduced by the addition of an anti-LDL receptor antibody, indicating that sLDL is assimilated via the LDL receptor pathway. The behavior of sLDL mimics that of native LDL, and this approach represents a viable technique for the production of an sLDL particle on a large scale for research and general application.—** Baillie, G., M. D. Owens, and G. W. Halbert. **A synthetic low density lipoprotein particle capable of supporting U937 proliferation in vitro.** ***J. Lipid Res.*** **2002.** 43: **69–73.**

**Supplementary key words** U937 • low density lipoprotein • amphipathic peptide • apoB • synthetic LDL • phosphate-buffered saline • dimethyl sulfoxide

LDL is a natural plasma component involved in the transport of cholesterol in the form of cholesteryl esters around the body. LDL is a spherical particle (diameter 20–22 nm) composed of an internal core of cholesteryl esters containing small amounts of triglyceride, solubilized by a monolayer of phospholipid with small quantities of free cholesterol. Located in the monolayer is the receptor protein apoprotein B (apoB) which accounts for approximately 20% of the particle's weight (1). LDL is of interest in a variety of research areas, primarily atherosclerosis and lipid metabolism where the original studies defining receptor-dependent uptake were performed by Brown and Goldstein (2). It has also been investigated as a drug-targeting vector in cancer chemotherapy; however research has been limited to in vitro (3, 4) and tracer in vivo studies (5).

Currently, in vitro and in vivo LDL studies are impeded due to a requirement to isolate LDL from fresh plasma samples. The isolation procedure takes up to 48 h and, depending on the donor's plasma levels and technique employed, may provide only 50–100 mg of LDL (6). Once isolated, the material is difficult to manipulate and degrades due to lipid oxidation or physicochemical instability arising from apoB's amphipathic properties that lead to aggregation. Attempts to produce synthetic LDL have centered around producing microemulsions of similar size and lipid composition but which obviously lack receptor competency (7, 8). ApoB can be grafted on to the microemulsion after production, but this still requires a source of protein from fresh plasma (9).

ApoB is one of the largest mammalian proteins known and exists in two forms (apoB-100 and apoB-48, a fragment of apoB-100) that vary in molecular weight. Due to its chemical properties, classical sequence analysis proved difficult, and its primary structure was determined from complementary DNA analysis (10, 11). It consists of an amino acid sequence that contains amphipathic regions maintaining apoB's position in the LDL phospholipid monolayer, with current evidence suggesting that it surrounds the particle in an equatorial band (12). The putative receptor domains within apoB have been identified by comparison with related apoproteins and consist of a nine-amino-acid segment (11). Previous studies have demonstrated that synthetic amphipathic molecules will spontaneously interact with LDL and, in addition, can impart different receptor-binding status after insertion in the particle's surface layer (13). We hypothesize that by employing suitably modified amphipathic peptides containing the apoB receptor domain in combination with the lipid microemulsions described above, it may be possible to prepare sLDL particles, without the requirement to isolate from natural sources.

Cells obtain cholesterol for membrane production from

Abbreviations: apoB, apoprotein B; CO, cholesterol oleate; DFCS, delipidated fetal calf serum; FCS, fetal calf serum; PC, egg yolk phosphatidyl choline; PEPXsLDL, synthetic low density lipoprotein system containing PEPX, where X represents the peptide number; sLDL, synthetic low density lipoprotein; TO, triolein.

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two sources, intracellular de novo biochemical synthesis or extracellular sources such as receptor-mediated uptake of LDL (2). The U937 histiocytic lymphoma cell line (14) is unable to perform de novo cholesterol synthesis and can only overcome this deficiency via the LDL-receptor-mediated uptake pathway (15). Therefore, for U937 cells in tissue culture to proliferate or increase in cell number, there is an absolute requirement for an extracellular cholesterol source. Usually this is provided by the addition of fetal calf serum (FCS), and the increase in U937 cell number is related to the level of FCS supplementation (15). However, LDL will also perform this function and the increase in U937 cell number in cholesterol-free media induced by added LDL is dependent on the added LDL or cholesterol concentration. Because LDL uptake is receptor-mediated, this effect is also saturable (15). Increase in U937 cell number in serum free-media can therefore be employed to assess LDL cholesterol concentration or the ability of apoB to stimulate uptake via the LDL receptor pathway. This latter property has been employed to screen for familial defective apoB (16, 17) and illustrates that increases in U937 cell numbers may be utilized to study the apoB LDL receptor interaction. To maximize the effect, cells must first be starved of cholesterol to upregulate LDL receptors and deplete internal stores, which may allow continued cell division (18). Additionally, a cholesterol-free tissue culture medium is required to ensure that increases in U937 cell number are due exclusively to the added system under investigation.

Lipid-free conditions can be achieved either by omission of serum from the culture media (16) or by inclusion of delipidated serum (19). A study by Van den Broek et al. (17), utilizing delipidated serum instead of serum deficient media at an LDL cholesterol concentration of 9 μmol/l, found that the increase in cell number with the former was over twice that previously reported with the latter. Subsequent experiments suggested that serum components other than LDL were involved in U937 growth and proliferation, especially in the later stages of the 72-h incubation period. As oxidized LDL is much less effective in promoting U937 proliferation (15), it was postulated that naturally occurring antioxidants present in serum may further enhance increases in cell number (17). These findings indicate that delipidated serum should be included in U937 growth assays to maximize cellular response.

In this article we present a novel method for the production of sLDL particles capable of supporting the growth of U937 cells in vitro. The approach utilized combines previous research in the formation of lipid microemulsions (7, 8), the published apoB structure (10, 11) and the known ability of LDL to interact with amphipathic molecules (13). The apoB receptor domain has been grafted onto a preformed microemulsion system via synthetic peptides rendered amphipathic by the use of lipid anchors on the terminal amino acids. The ability of sLDL to support the proliferation of U937 under a variety of conditions similar to those employed for native LDL has been utilized to investigate the receptor-dependent uptake of sLDL.

## METHODS

### Preparation of sLDL particles

A 3:2:1 molar mixture of egg yolk phosphatidyl choline (PC), triolein (TO), and cholesterol oleate (CO) respectively was dissolved in chloroform-methanol 2:1 (v/v) and the solvent removed under a stream of $N_2$. The lipids were then redissolved and lyophilized from t-butanol for 24 hours (EF4 Modulyo Freeze Dryer, Edwards High Vacuum, Crawley, UK), then resuspended in 0.01 M Tris-HCl buffer (pH 8.0) to give a final concentration of 7% to 8% w/v. The lipid dispersions were sonicated under a stream of $N_2$ for 2 h using a 250W sonicator, centrifuged at 10,000 rpm for 60 min (MSE Superspeed 75 Ultracentrifuge, MSE Ltd, London, UK), then transferred to the extruder vessel (Lipex Biomembranes Inc., Vancouver, Canada) maintained at 50°C to 55°C. The lipid mixture was successively extruded through polycarbonate filters (Costar, UK) of pore size 0.1 and 0.05 μm using two stacked filters and at least four extrusions under 60 psig pressure provided by a nitrogen source (20). Samples of lipid microemulsion were diluted with Tris-HCl buffer to give a CO concentration of approximately 1 mmol/l, and heated to 55°C in a stirring water bath. Aliquots of peptide (**Table 1,** synthesized at Thistle Peptide Services Glasgow, UK, supplied at > 90% purity and used as received) dissolved in DMSO were added under the surface of the stirring microemulsion; control experiments were performed with DMSO alone. The volume of DMSO added in each case was kept below 20 μl/ml of microemulsion mixture. The peptide-microemulsion complex was incubated at 55°C for 15 min, then dialyzed overnight (membrane molecular weight cut off 15,000 Da) against sterile PBS (5 liters). The resulting sLDL preparations were filter-sterilized (0.2 μm) and stored at 4°C under $N_2$ before use. Preparations are expressed as synthetic low density lipoprotein system containing PEPX (PEPXsLDL), where X represents the peptide number (Table 1) employed in the system.

### Analysis for cholesterol content

Microemulsions were assayed for cholesteryl ester content using the method of Allain (21). To 1 ml of cholesterol reagent at 30°C was added 0.01 ml of sample, blank (distilled water), or standard (Cholesterol Calibrator, 200 mg/100 ml). Reagent-sample mixtures were allowed to incubate at 37°C for 10 min. The absorbance of each sample was measured at 500 nm on a spectrophotometer (Cecil CE272 Ultraviolet Spectrophotometer). All readings were completed within 30 min after the end of the incubation period. CO content was calculated by reference to the cholesterol standard.

TABLE 1. Peptide structures used in the preparation of sLDL

| Peptide | N Terminal[a] | Peptide Sequence | C Terminal[b] |
|---|---|---|---|
| PEP1 | Retinoic acid- | Leu-**Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys**-Leu | -Cholesterol |
| PEP2 | Retinoic acid- | Gly-Thr-Thr-**Arg-Leu-Thr-Arg-Lys-Arg- Gly-Leu-Lys**-Leu | -OH |
| PEP3 | Retinoic acid- | Tyr-Lys-Leu-Glu-Gly-Thr-Thr-**Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys**-Leu-Ala-Thr-Ala-Leu-Ser | -Cholesterol |
| PEP4 | Retinoic acid- | Tyr-Lys-Leu-Glu-Gly-Thr-Thr-**Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys**-Leu-Ala-Thr-Ala-Leu-Ser | -OH |

Bold sequence commences at apoB residue 3359 (10).
[a] N-terminal linkage of retinoic acid via an amide bond.
[b] C terminal linkage of cholesterol via a C3 ester bond.

### Cell culture

U937 (European Collection of Animal Cell Cultures, UK) stock cultures were grown at 37°C in RPMI 1640 media supplemented with glutamine (10 mM), gentamicin (50 μg/ml), fungizone (0.5 μg/ml) and 10% (v/v) FCS (all from Life Technologies Ltd., UK). Cells were maintained in a humidified 5% $CO_2$ atmosphere between 1–8 × $10^5$ cells/ml and subcultured twice weekly. LDL was obtained from Sigma-Aldrich, Poole (Dorset, UK). Delipidated fetal calf serum (DFCS) was prepared from FCS by the method of Rothblat et al. (19). Monoclonal anti-LDL receptor antibody (clone C7, code number RPN.537) was purchased from Amersham International and reconstituted with sterile water just prior to use.

### Growth assay

All growth assays were conducted over 72 hours in 24 well plates unless otherwise indicated. One day before each growth experiment, cells were centrifuged at 1500 rpm for 5 min and resuspended in RPMI medium containing 5% (v/v) DFCS to initiate cholesterol starvation and upregulate LDL receptors. Cholesterol-starved cells were collected by centrifugation and seeded at 1 × $10^5$ cells/well. Appropriate amounts of cholesterol (either LDL, microemulsion or sLDL) were then added after filtration (0.2 μm) and the well volume made up to 1 ml with RPMI containing 5% (v/v) DFCS. After 72 h, an aliquot of each well was diluted with PBS, and the number of cells present determined in triplicate by Coulter Counter. Different concentrations were repeated four or eight times depending on the experiment. Results are expressed as the cell number present in the media (15). Three wells per experiment were checked at random before commencement of incubation to ensure that a uniform cell density was achieved during preparation (17). In all cases, the mean starting cell densities of control wells were between 0.95 and 1.05 × $10^5$ cells/ml. Statistical differences between results were determined using a non-parametric Mann Whitney test.

## RESULTS

The results presented in **Fig. 1** demonstrate that the proliferation of U937 was supported by FCS containing media and to a lesser extent by LDL but not by media supplemented with DFCS. A microemulsion with a similar lipid composition to LDL but with no amphipathic peptide containing the apoB receptor domain was similar to DFCS. The increase in cell number from 1 × $10^5$/ml to 1 × $10^6$/ml in FCS-supplemented media over 96 h is consistent with published results (18). In media without cholesterol supplementation cell numbers slowly decrease as intracellular cholesterol stores are utilized and no additional external cholesterol source is available.

To determine if sLDL could also support increases in U937 cell numbers, the cells were incubated with PEP1sLDL at a cholesterol concentration (80 μmol/l) equivalent to FCS supplementation. The results presented in **Fig. 2** demonstrate that FCS, LDL, and PEP1sLDL produce increases in U937 cell numbers in vitro. The FCS increase is broadly comparable to literature values obtained employing similar conditions (15). The PEP1sLDL induced increase was significantly greater ($P < 0.05$) than that produced by control microemulsion, an identical system but with no amphipathic peptide. In addition the PEP1sLDL result was not statistically different ($P > 0.05$) from that of native LDL, producing a mean increase that is 87% of LDL. However, based on literature experiments, the noted LDL-induced increase appears slightly lower than published results [1.45 × $10^5$ cells/ml vs. 3 × $10^5$ cells/ml (16)].

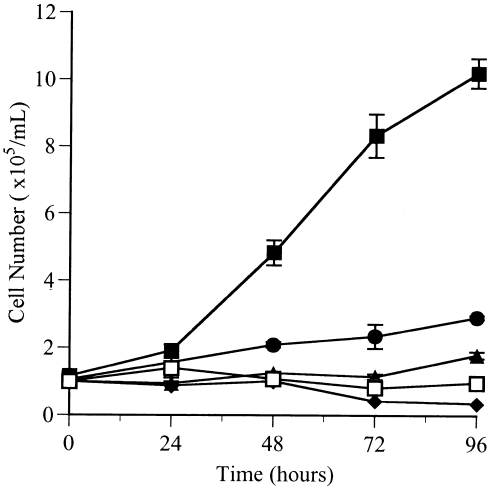


**Fig. 1.** Growth of U937 cells in RPMI supplemented with different cholesterol sources. Mean ± SD (n = 3). Cells were seeded at 1 × $10^5$ in 75 $cm^2$ flask containing 25 ml of RPMI 1640 supplemented with: serum 10% v/v (closed square), delipidated fetal calf serum 5% v/v (open square), no serum or lipid additions (closed diamond) LDL (closed circle), control microemulsion (closed triangle). LDL and control microemulsion were added at an equivalent cholesterol concentration (CO) (80 μmol/l) to fetal calf serum (FCS). At 24 h intervals, 0.5 ml of cell suspension was removed and counted. No cholesterol starvation was employed prior to the experiment.

To determine the effect of amphipathic peptide configuration on sLDL-induced increases in U937, cell number assays were conducted using four different sLDL systems

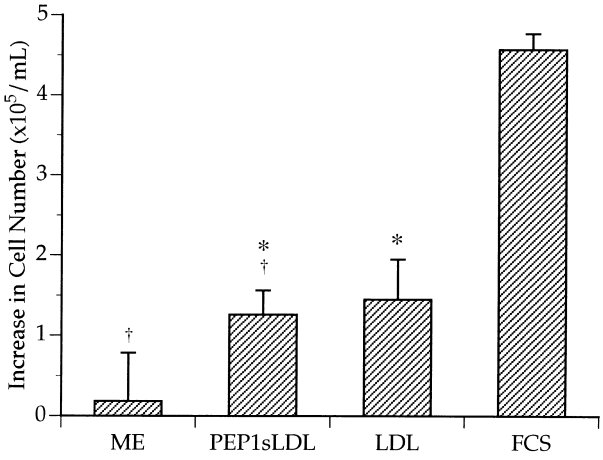


**Fig. 2.** Proliferation of U937 induced by different cholesterol sources. Mean ± standard deviation (n = 3). Result expressed as cell number after 72 h incubation. No DFCS supplementation was employed during the experiment. Cholesterol concentration 80 μmol/l; ME, microemulsion without amphipathic receptor peptide but containing cholesterol; PEP1sLDL, peptide concentration 0.03 mol/mol cholesterol oleate (CO); LDL added to cholesterol concentration of 80 μmol/l; FCS at 10% v/v. * No significant difference between PEP1sLDL and LDL ($P > 0.05$). † Significant difference between ME control and PEP1sLDL ($P < 0.05$).

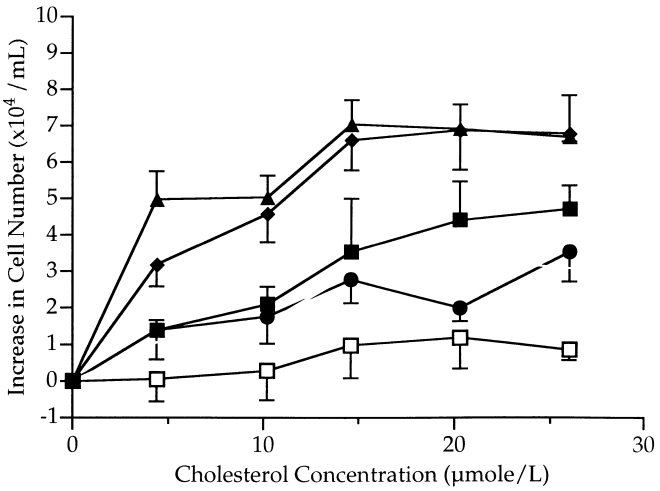


**Fig. 3.** Growth of U937 cells induced by different synthetic low density lipoprotein (sLDL) preparations. Mean ± standard deviation (n = 8). Result expressed as cell number after 72 h incubation. Peptide concentration, 0.03 mol/mol CO. Microemulsion control (open square), PEP1sLDL (closed circle), PEP2sLDL (closed triangle), PEP3sLDL (closed diamond), PEP4sLDL (closed square). All supplemented with DFCS at 5% v/v.

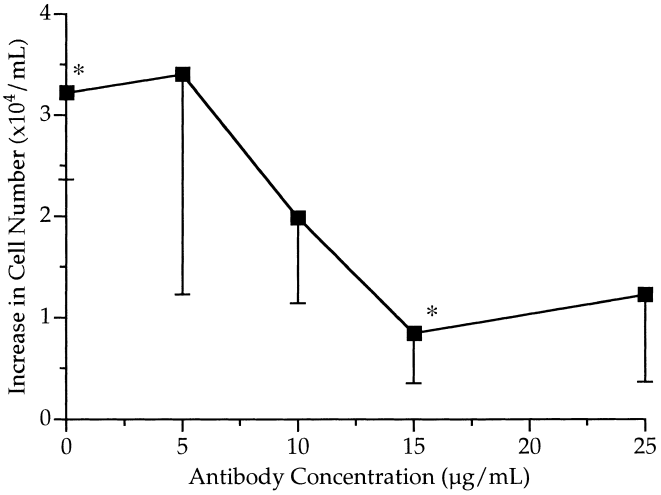


**Fig. 4.** Inhibition of PEP2sLDL-induced U937 proliferation by an anti-LDL receptor antibody. Mean ± standard deviation (n = 4). Result expressed as cell number after 72 h incubation. Monoclonal anti-LDL receptor antibody was added to cells after seeding and 60 minutes before sLDL materials. All supplemented with DFCS 5% v/v. Cholesterol concentration 33 μmol/l, peptide concentration 0.03 mol/mol CO. * Significant difference between control with no antibody and antibody at 15 μg/ml ($P < 0.05$).

(Table 1). All the systems over the range of cholesterol concentrations tested produced significant increases ($P < 0.05$) in U937 cell numbers **(Fig. 3)** when compared with control microemulsion without amphipathic peptide. The small proliferation supported by control microemulsion at high concentrations may be due to the weak nonspecific phagocytotic activity of U937 cells (14). This would be exacerbated at high concentrations due to the large numbers of microemulsion particles present.

To determine if the sLDL-induced U937 cell number increases are due to receptor-mediated uptake, cells were incubated with PEP2sLDL and a commercially obtained human anti-LDL receptor antibody at a range of concentrations. PEP2sLDL is the most effective sLDL in promoting U937 cell number increases suggesting a maximal receptor interaction. Low antibody concentrations (5 μg/ml) had no effect **(Fig. 4).** However, higher concentrations produced a dose-dependent inhibition. Even at antibody saturation (15 μg/ml), increases in U937 cell numbers are significantly more ($P < 0.05$) than cholesterol free control wells.

## DISCUSSION

The results in Fig. 1 are consistent with published studies which demonstrate U937 has no ability to synthesize intracellular cholesterol and overcomes this by receptor-mediated uptake of LDL (15). Cholesterol addition to the media is required and FCS supports increases in cell numbers, whereas DFCS supplementation or serum free media does not. Although the control microemulsion without amphipathic peptide adds cholesterol to the media, this does not stimulate an increase in U937 cell numbers. These results are further validated by Fig. 2 where there is no statistical difference between LDL and sLDL, but a difference between sLDL and a control microemulsion without the amphipathic peptide. Overall the results strongly suggest sLDL uptake by U937 in a manner similar to LDL, and which also permits cellular proliferation. This result is because of the presence of amphipathic peptide on the particle as control microemulsions do not exhibit this property and literature data indicate that liposomes containing cholesterol at similar concentrations do not stimulate proliferation (15).

The experiment in Fig. 3 is comparable to the utilization of U937 proliferation to detect single amino acid changes in familial defective apoB (22) and will determine the amphipathic peptide configuration providing the best receptor interaction. The increases in cell numbers supported by the four different sLDL systems again suggest that amphipathic peptide inclusion on the particle enhanced uptake. In addition the data obtained is concentration-dependent and saturable similar to the native LDL interaction. The variation between systems indicates that amphipathic peptide sequence and the presence of a C-terminal lipophilic anchor influence the U937 interaction. The least effective system, peptide 1, contains the shortest chain length with both C and N terminal lipid anchors. Systems containing peptides 2, 3, and 4 exhibit the greatest increase and these contain either no C-terminal lipid anchor (peptides 2 and 4) or the receptor sequence embedded in a large 22 amino acid chain (peptide 4). The results imply interaction with a specific receptor and the possibility of exercising control via suitable peptide configurations. sLDL systems may therefore be capable of providing a suitable system with variable properties for research into LDL receptor function.

A further indication that the U937 sLDL interaction is via the LDL receptor is the inhibition of response by an

anti-LDL rececceptor antibody (Fig. 4). However, this does not completely abolish proliferation and may be caused by the mechanism, which allows small levels of cellular proliferation with control microemulsion. This result reinforces the explanation that this effect may be mediated via routes other than LDL receptor interactions, or that the antibody lacks the ability to completely block the U937 LDL receptor. Although the antibody has been shown to react with human receptors, it was raised against a partially purified LDL receptor isolated from bovine adrenal cortex tissue. The importance of antibody specificity to block LDL receptor/ligand binding was illustrated by Milne and coworkers (23) who produced 22 monoclonal antibodies against putative binding regions of apoB 100. Depending on the epitope employed, they isolated antibodies that could block 95% of specific LDL binding at antibody concentrations of less than 10 μg/ml, antibodies which could only partially block binding at these concentrations and ones which had almost no effect at 72 μg/ml. However, the large inhibition of cell growth (74%, $P < 0.05$) observed in this experiment at antibody saturation (15 μg/ml) further reinforces the supposition that sLDL-induced increases in U937 cell numbers occur via uptake by the LDL receptor.

The sLDL particles presented in this study exhibit a concentration-dependent and saturable ability to induce cell number increases in U937 cells in vitro. A comparison of sLDL systems also demonstrates that control of receptor interaction is possible by alteration of the amphipathic peptide sequence and by varying the number and location of the lipid anchors. The cell number increases induced by sLDL is inhibited but not abolished by the presence of an anti-LDL receptor antibody. These results illustrate that sLDL mimics the effects of native LDL in the U937 proliferation assay and indicate that sLDL exhibits a degree of receptor competency for the LDL receptor. sLDL represents an entirely synthetic low density lipoprotein particle capable of large-scale production and utilization in a range of research areas from tissue culture to drug targeting. Further research will be required to maximize uptake by variation in the amphipathic peptide and constituent lipids.

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