Fluorescence imaging of oxidized phospholipid uptake into cultured macrophages

Master thesis

from

Bojana Stojčić

Graz University of Technology

Institute of Biochemistry

Examiners:

Hermetter, Albin, Ao.Univ.-Prof. Dr.phil.

Strauß, Juliane Gertrude, Mag.rer.nat.Dr.rer.nat. Univ.-Doz.

Trajanoski, Zlatko, Univ.-Prof.Dipl.-Ing.Dr.techn.

Supervised by

Hermetter, Albin, Ao.Univ.-Prof. Dr.phil.

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…… date

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Abstract

Atherosclerosis is an inflammatory disease of the arterial wall including the interactions of modified lipoproteins, monocyte-derived macrophages, T-cells and the cells of the arterial wall**.** The inflammatory progression leads to the development of plaques, which are causally related to acute clinical complications such as myocardial infarction and stroke. Lipoproteins are oxidized as a consequence of oxidative stress (enzymatic and nonenzymatic), which in the end can cause lipid and protein modifications of LDL particle. Lipid modifications can induce generation of oxidized phospholipids (oxPL) primarily from (poly)unsaturated diacyl- and alk(en)ylacyl glycerophospholipids including palmitoyloxovaleroyl-PC (POVPC) and palmitoylglutaroyl-PC (PGPC). Fluorescent analogs of POVPC and PGPC labeled with BODIPY (POVPE-BY and PGPE-BY, respectively) were used as tools for studying uptake and intracellular stability of these compounds in cultured macrophages. The aim of this study was to understand in more detail how oxPL are delivered to the cells and to analyze their metabolic fate inside the cells. The emphasis was on the role of the (supra)molecular carriers delivering oxPL to the cells including lipid micelles, albumin and (mm)LDL. The import of the fluorescent lipids from these donors into macrophages was studied using fluorescence microscopy. In addition, we analyzed the transfer of oxPL between albumin and LDL and the degradation products of PGPE-BY as well as the complexes of the chemically reactive POVPE-BY with proteins or lipids. From our results, it can be inferred that POVPC mainly elicits its biological effects as a conjugate with lipids and/or proteins. In order to understand the biological activites of PGPC, we have to take into account that they are mediated by various compounds including the intact oxPL and its metabolites.

List of Content

Abbreviations and Acronyms

ACAT-1	Cholesterol Acyltransferase-1
ApoB	Apoprotein B
APS	Ammonium persulfate
BSA	Bovine serum albumin
CoA	Coenzyme A
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
HDL	High Density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IDL	Intermediate Density Lipoprotein
LDL	Low Density Lipoprotein
M-CSF	Macrophage Colony Stimulating Factor
mmLDL	Minimal modified Low Density Lipoprotein
oxLDL	Oxidized Low Density Lipoprotein
oxPL	Oxidized Phospholipid
PAF	Platelet-Activating Factor.
PAPC	1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PGPC	1-Palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine
PGPE-BY	1-Palmitoyl-2-glutaroyl-sn-glycero-3-phosphoethanolamin – BODIPY
PI3K	Phosphoinositide-3-Kinase
POVPC	1-Palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine
POVPE-BY	1-Palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphoethanolamine – BODIPY
SDS-Page	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
TBE	Tris/Borate/EDTA

TEMED	Tetramethylethylendiamin
TLC	Thin Layer Chromatography
VLDL	Very Low Density Lipoprotein
VSMC	Vascular Smooth Muscle Cell

1 Introduction

1.1 Atherosclerosis

Atherosclerosis and its consequences are causally related to many deaths in the western civilized world. It is an inflammatory disease of the arterial wall and causally related to the effects of modified lipoproteins, monocyte-derived macrophages, T-cells and the cellular elements of the arterial wall. The inflammatory progression, in the end, results in the development of atherosclerotic lesions and plaques. Plaque rupture and thrombosis eventually lead to the acute clinical complications of myocardial infarction and stroke [12-14]. Main risk factors for the development of arthrosclerosis may be: factors with a genetic component (e.g. elevated levels of LDL and VLDL, low levels of HDL, hypertension, diabetes mellitus, male gender, insulin resistance, obesity, etc.) and/or environmental factors (e.g. smoking, lack of exercise, high fat diet, infectious agents). However, elevated serum cholesterol levels associated with plasma LDL seem to be the highest risk factor [11].

1.1.1 Lipoproteins

Lipoproteins transport cholesterol/cholesterolester, triglycerides and fat-soluble vitamins circulating in the bloodstream. Plasma lipoproteins are spherical particles. The surface layer contains phospholipids, with the fatty acid pointing to the interior and the hydrophilic phosphate groups localizing to the surface of the lipoproteins. The phospholipids are surrounded with proteins known as apolipoproteins. The inner core of the lipoproteins is filled with cholesteryl esters and triglyceride molecules. The plasma lipoproteins are categorized by their density, electrophoretic mobility, lipid and protein content into: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The chylomicrons are the largest and the “lightest” of all lipoproteins. Their diameter is 1 µm, which is still much smaller than the diameter of an erythrocyte. Other lipoproteins are smaller, contain more protein and are denser e.g. HDL is 100 times smaller than a chylomicron and has the highest density (1.20) compared to the other lipoproteins.

An important role in the transport of dietary fats is played by the chylomicrons, where the nutritional fats absorbed in the intestine are packaged. The chylomicrons are triglyceride rich particles which are secreted from the intestine into the lymph and eventually to the blood stream

where they undergo lipolysis of triglycerides catalyzed mainly by endothelial lipoproteinlipase. As a result, chylomicron remnants are formed which recruit Apolipoprotein E and cholesterol from HDL. The final trygliceride-rich particles are then recognized by the ApoE receptor in the liver, where they are taken up and metabolized. The liver can synthesize and release endogenous lipoprotein namely VLDL. In plasma, these cholesterolester-rich particles are degraded by lipoproteinlipase into IDL (VLDL-remnants) and finally into LDL (see Figure 1.1). LDL can also be taken up by the liver via the ApoE receptor (for LDL-Clearance) and is recognized by cells of other tissues by the ApoB-100 receptor [19, 20].

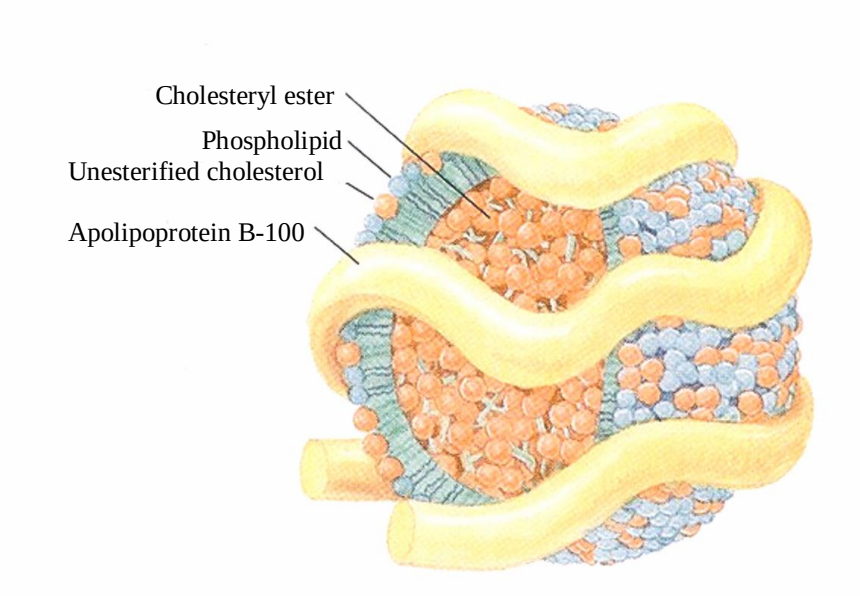

Figure 1.1 Low density Lipoprotein [http://walzemlab.tamu.edu/lipo1.jpg]

1.1.2 LDL Modifications and the onset of atherosclerosis

The formation of fatty streaks is a very early sign of atherosclerosis. They contain lipid-rich macrophages and T-lymphocytes within the intima (the innermost layer of artery wall, Figure 1.2) [15].

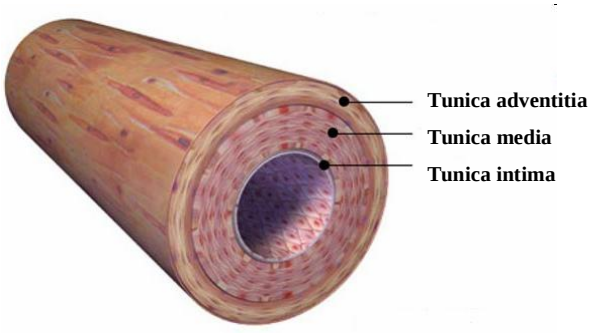

Figure 1.2 The structure of the arterial wall [http://i.livescience.com/images/060619_artery_anatomy_02.jpg]

The main causes of fatty streak formation are the recruitment of macrophages across a damaged endothelium into the intima and the uncontrolled uptake of oxLDL by these cells. Different studies suggest that modifications of ApoB-100 by oxidized lipids drive this process [12, 14]. Brown and Goldstein have shown that circulating LDL must go through a significant structural modification before it becomes fully proatherogenic [21]. They found out that the macrophages, which are precursors of the cholesterol-loaded foam cells, are incapable of loading the cell with native LDL and therefore with cholesterol. On the other hand, they indicated that patients completely lacking the native LDL receptor still accumulate large amounts of cholesterol in their macrophages. For that reason, they suggested that modifications of LDL must occur, and that modified LDL is recognized by receptors other than the classical LDL receptor, which they called “scavenger receptors” [22].

Oxidative modifications of native LDL can be induced *in vitro* (with iron and copper). Depending on the extent of modification, minimally modified (mmLDL) or extensively oxidized LDL (oxLDL) are formed. The mmLDL are still recognized by the LDL receptor [12]. OxLDL, in which ApoB is degraded or modified covalently by oxidized lipids [14], is recognized by “scavenger receptors” expressed on macrophages and vascular smooth muscle cells [11]. There is a number of potential oxidants, including the oxidizing enzymes myeloperoxidase, nitric oxide synthase and 15-lipoxygenase, which directly or indirectly target LDL lipids [14, 16]. While LDL is protected from oxidation in the plasma compartment by antioxidants it becomes more prone to enzymatic and nonenzymatic oxidation in the artery wall [17, 18].

1.1.3 Role of macrophages in atherosclerosis

The recruitment of monocytes to the arterial wall and their eventual differentiation into macrophages, which is induced by the macrophage colony stimulating factor (M-CSF), may originally serve the protective function of eliminating cytotoxic and proinflammatory oxLDL particles. However, the progressive accumulation of macrophages and their uptake of oxLDL may also result in the development of atherosclerotic lesions [11]. At the site of a pre-damaged endothelium, oxLDL can enter the intima and cause intern adherence of more cells and migration of monocytes and T lymphocytes into the inner parts of the intima [15].

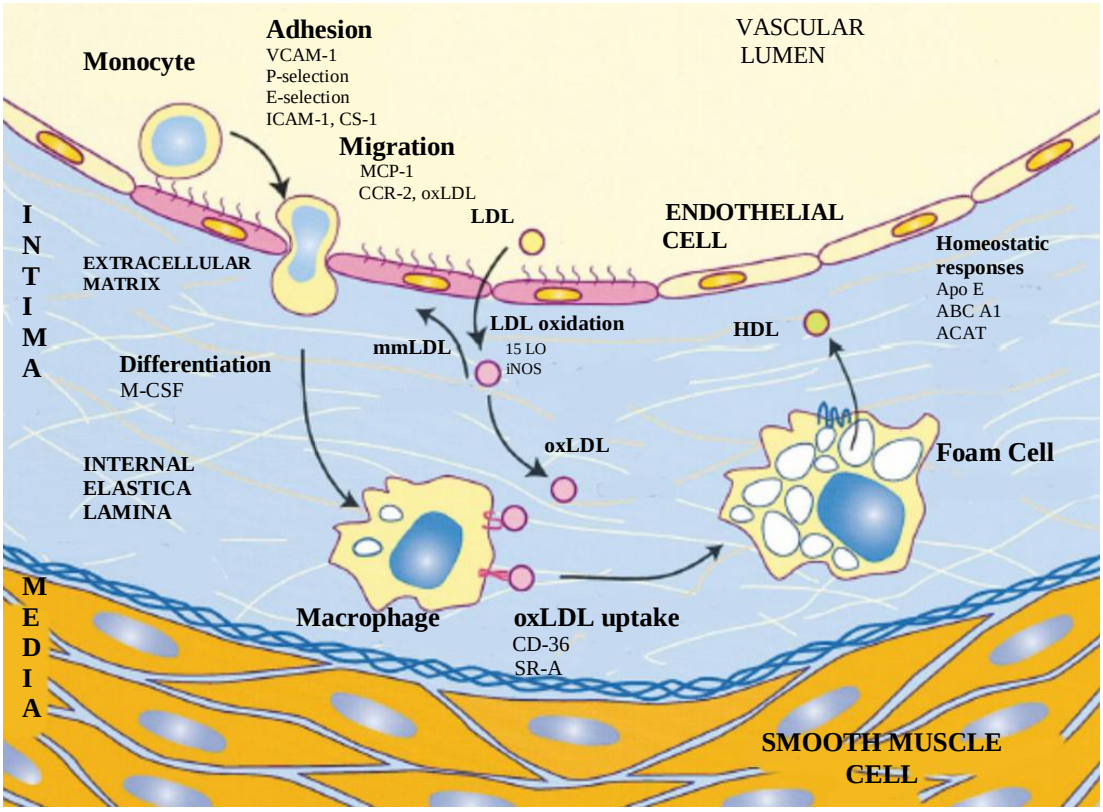

Figure 1.3 Initiating Events in the Development of a Fatty Streak Lesion [11]. LDL is oxidized minimally or extensively (mmLDL or oxLDL) in the sub-endothelial space of macrophages. Monocytes migrate to the extracellular matrix of the intima, as a consequence of stimulation by mmLDL and inflammatory cytokines. Adherent monocytes pass through the endothelium and differentiate into macrophages. Macrophages take up oxLDL via "scavenger receptors" (A and CD36), which leads to the formation of foam cells.

Macrophages take up oxLDL via scavenger receptor A and CD36. After internalization the oxLDL components are hydrolyzed in lysosomes. Free cholesterol is esterified inside the cells

by acyl CoA (cholesterol acyltransferase-1 (ACAT-1)) and then stored in lipid droplets giving rise to the formation of foam cells [11]. Macrophages possess two possible mechanisms for the disposal of excess cholesterol: enzymatic modification leading to more soluble forms (27-OH-cholesterol) and via membrane transporters, with HDL as the primary extracellular acceptor [11]. The early phases of atherogenesis are shown in Figure 1.3.

The change from a simple to a more complex fatty streak is due to the migration of smooth muscle cells from the tunica media to tunica intima. *"Intimal smooth muscle cells may proliferate and take up modified lipoproteins, contributing to foam cell formation, and synthesize extracellular matrix proteins that lead to the development of the fibrous cap"* [11]. Signaling molecules secreted by monocytes/macrophages and T cells lymphocytes influence smooth muscle cell proliferation and migration thereby activating lesion growth and causing a broad range of cellular and humoral responses (i.e. production of cytokines, proteases, free oxygen radicals and complement factors [23]) with features of the chronic inflammatory state which altogether indicates that immune activation is ongoing in atherosclerotic lesions [11].
Necrotized macrophages and smooth muscle cell-derived foam cells form a necrotic core and lead to the accumulation of extracellular cholesterol. The fibrous cap is weakened by the secretion of matrix metalloproteinases by the macrophage and neovascularization. Finally, plaque rapture initiates the coagulation cascade, platelet adherence and thrombosis, which in the end can lead to myocardial infarction and stroke [11].

1.2 Apoptosis and Necrosis of macrophages due to oxidized LDL

During the initial step of LDL modification, oxidants preferably interact with the most oxidizable compounds, which are antioxidants, polyunsaturated fatty acids and cholesterol [24, 25]. Oxidized LDL (oxLDL) is very toxic and can cause cell death (apoptosis or necrosis) depending on concentration and the extent of oxidation [2].
After entering macrophages, oxLDL is transferred into lysosomes, where it is degraded into oxidized lipids. Apart from this internalization of the entire lipoprotein, its constituent oxidized lipid may also be transferred inside the cells by selective uptake (exchange between lipoprotein and cell surface). These toxic oxidized lipids including lipid peroxides, epoxyisoprostanes, oxidized phospholipids, oxysterols and aldehydes, are able to cause modifications of cell

proteins, induce oxidative stress and lipid peroxidation, and activate various signaling pathways leading to the expression of specific genes. They trigger an intense, delayed and sustained calcium peak which causes either apoptosis or necrosis. Apoptosis can be caused by the activation of mitochondrial and death receptors (Fas/FasL), which activate the classical caspase cascade (which can be prevented by caspase inhibitors). OxLDL can trigger a death process involving lysosomal proteases and lysosomes (which are independent of caspases). If apoptosis is blocked, for example by over-expression of Bcl-2, oxLDL can activate necrosis through a calcium-dependent pathway [2]. *"Apoptosis occurring in atherosclerotic areas is potentially involved in endothelial cell lining defects, necrotic core formation and plaque rupture or erosion which may trigger atherothrombotic events* [2]*"*. The toxic effects can be reduced by inhibitors of relevant signaling pathways (see above), by antioxidants, HDL and dihydropyridine calcium channel blockers. All these reagents prevent the generation of the "calcium peak" and can therefore be taken as proof for the assumption that calcium is a main trigger for apoptosis or necrosis [2].

1.2.1 Apoptosis

Apoptosis is programmed cell death during which cell integrity is maintained until the dying cells are removed by phagocytosing cells. This process is mediated by one of several specific intro-cellular signaling cascades. Typical signs of apoptosis are DNA fragmentation, alterations of nuclear morphology (chromatin condensation and nuclear fragmentation), and organelle reorganization release of apoptotic bodies without leakage of cytosolic macromolecules.
Among other numerous signaling components, the activation of different cellular proteases (including cysteine proteases termed caspases [26]) and some serine proteases [27] are specific proteases of the apoptotic process.

1.2.2 Necrosis

Necrosis is cell death associated with cellular swelling, rupture of the plasma membrane, organelle changes, and leakage of the cellular components [2]. The consequences of necrosis are dramatic since the release of intra-cellular enzymes into the environment leads to severe inflammation and ultimately to failure of vital function.

1.3 Oxidized LDL and macrophage cell death

Massive amounts of oxLDL are localized in atherosclerotic lesions [30-32]. This is supported by immunohistochemical examination of these lesions confirming oxidation-specific epitopes of oxLDL [33, 34]. It is known that macrophages undergo apoptosis through all stages of atherosclerosis [41]. Some *in vivo* studies imply that in early lesions macrophage death is a beneficial effect as the dead cells are removed by rapid phagocytic clearance thus reducing the macrophage burden and slowing lesion progression [35-37]. However in late lesions, macrophage death causes necrotic core formation, which is considered to support plaque rupture [37]. Therefore, this equilibrium between macrophage survival and death in atherosclerosis is an important determinant of lesion development and progression. It is thought that macrophages survive due to the "survival factors" (such as the anti-apoptotic short isoforms of caspase-2, which is upregulated in response to increased DNA damage) [41]. While most *in vitro* studies have confirmed the strong pro-apoptotic effects of fully oxidized LDL mimicked by copper-oxidized LDL, it has been shown that early forms of oxidized LDL (mimicked by mmLDL, generated by exposure of LDL to 15-lipoxygenase expressing cells) are able to activate PI3K/Akt in macrophages [38, 39], which is a pathway known to promote cell survival.

1.4 Oxidized Phospholipids

Oxidized lipids are generated during oxidation of LDL, as a consequence of oxidative stress (enzymatic or nonenzyimatic). They are mainly generated from (poly)unsaturated diacyl- and alk(en)ylacyl glycerophospholipids. The enormous diversity of reaction products depends on the degree of modification, hydrophobicity, chemical reactivity, physical properties and biological activity [9]. Phosphatidylcholine (PC) is one of the most abundant phospholipids in mammalian cells (40-50%). Its oxidation products are detected in many mammalian tissues [9]. In mammalian cells, the sn-1 position of glycerol in PC is either linked to an acyl residue via an ester bond or an alkyl residue via an ether bond. The sn-2 position mostly contains (poly)unsaturated acyl residues that are highly oxidizable [9]. Numerous studies have shown that different modified forms of lipids are involved in the development of atherosclerosis [8, 29]. In particular, the products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) seem to play an enormous role in atherogenesis. Their presence in atherosclerotic

plaques has been proven using antibodies against the oxidized forms of PAPC. Many other products of PAPC modification have so far been identified and investigated in biological systems. This study focuses on 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) [1, 4], shown in Figure 1.4. These compounds are found in minimally modified LDL, oxidized LDL and atherosclerotic lesions and evidence is accumulating that they are inducers of different cellular responses such as inflammation, proliferation, or cell death [28].

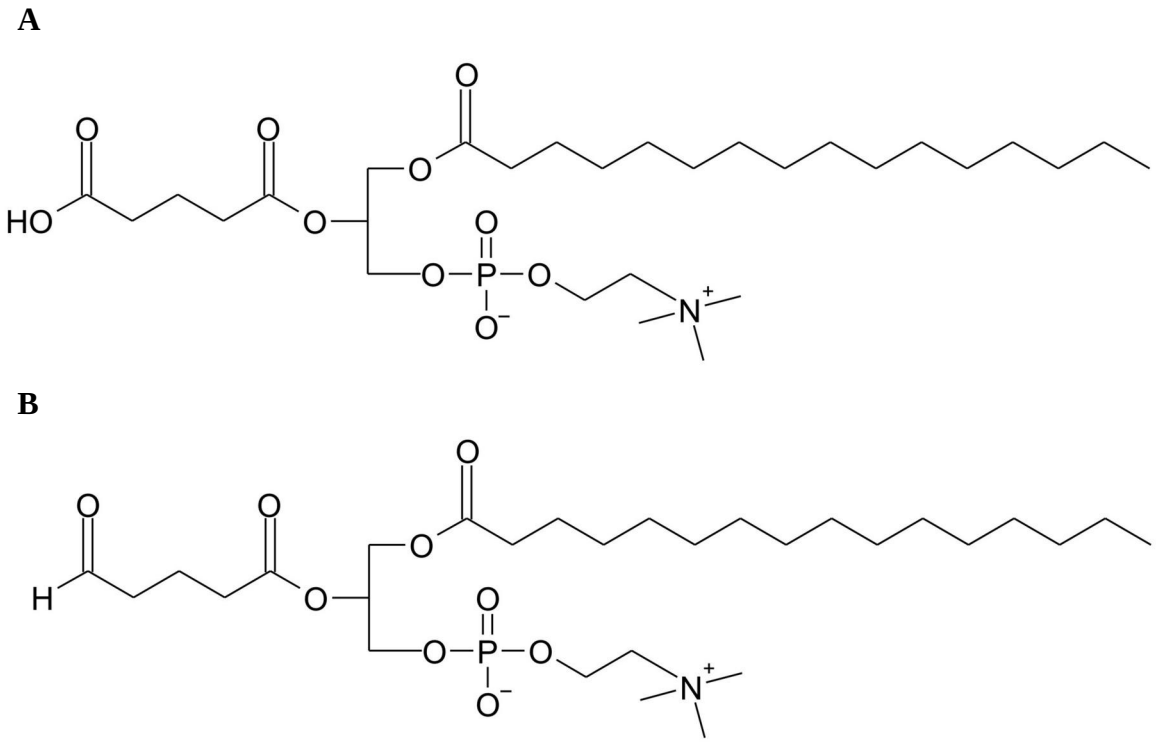

Figure 1.4 Molecular structures of truncated oxidized phospholipids. A: 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), **B:** 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC).

Fluorescent analogs of the oxidized phospholipids (POVPC and PGPC), labeled with BODIPY fluorophore (Figure 1.5), were used as tools for studying uptake, intracellular stability and distribution of these compounds in the RAW 264.7 murine cell line [4].

It has already been shown for VSMCs [4], that PGPC derivatives were rapidly transferred to lysosomes, while POVPC is retained in the plasma membrane. Despite their considerable

structural similarity (i.e. a single hydrophobic chain, the polar head group and the polar sn-2 acyl fragment), they show different cellular effects. The aldehydo lipid POVPC is chemically reactive and forms covalent Schiff bases with the amino groups of phospholipids and proteins [4]. Prior to internalization, PGPC becomes enriched in caveolae and clathrin-coated pits [40] followed by endocytosis.

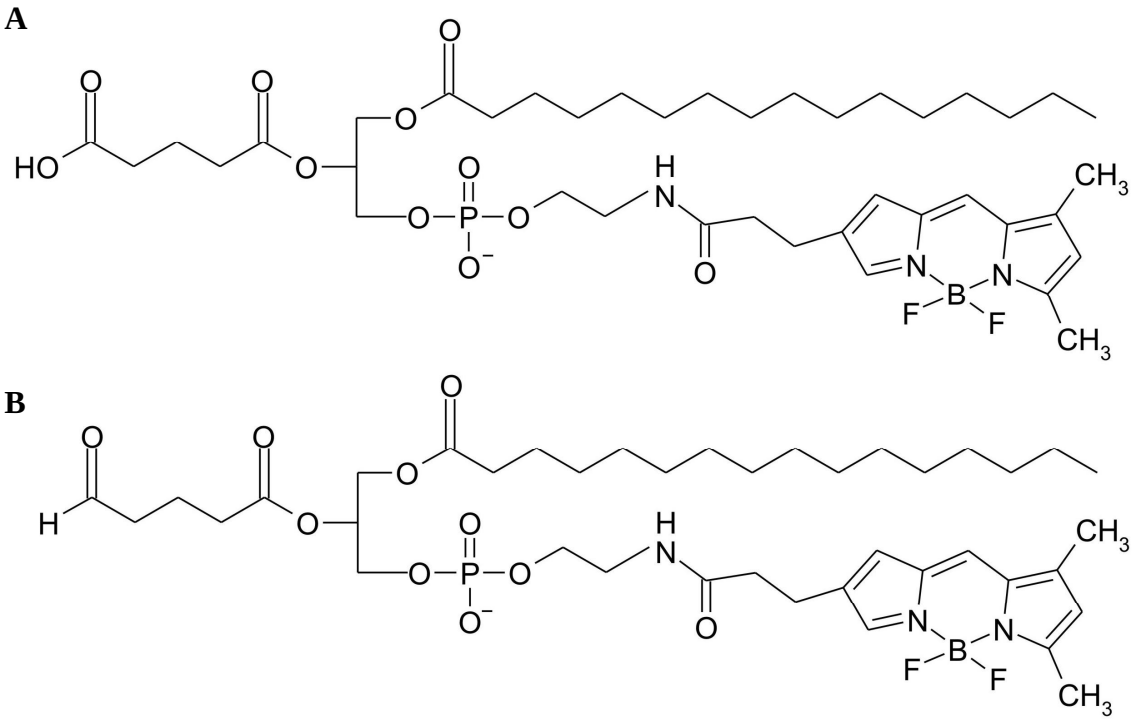

Figure 1.5 Bodipy–labeled analogs of oxidized phospholipids. A: Fluorescent analog of PGPC (PGPE-BY), **B:** Fluorescent analog of POVPC (POVPE-BY)

It was the aim of this study to understand in more details how the fluorescent oxPL analogs are delivered to the cells and to analyze their metabolic fate inside the cells. The emphasis was on the role of the (supra)molecular systems that are candidates as physiological carriers which deliver the oxPL to the cells. Therefore, we prepared three model donor systems consisting of lipid micelles, albumin or LDL and fluorescent PGPE-BY and POVPE-BY. The import of the fluorescent lipids from these donors into cultured macrophages was studied using fluorescence microscopy. In addition, we analyzed transfer of POVPE-BY between albumin and LDL as well as we prepared and analyzed lipid extracts from the cells after incubation with the lipid donors

(lipid micelles, LDL and mmLDL) to identify complexes of the chemically reactive POVPE-BY with lipids and find modification and degradation products due to phospholipase-induced hydrolysis of oxPL. We found a considerable complexity of oxPL modifications responsible for the formation of various products with potential biological activities. From our results, it can be concluded that the biological effects of oxPL in cultured cells are rather a combination of events triggered by various compounds rather than simple effects due to the intact oxPLs.

2 Materials and Methods

2.1 Materials and Reagents

Cell culture

- RAW 264.7 macrophages were obtained from ATCC Nr. TIB-71.
- Dulbecco's Modified Eagle Medium (DMEM Low Glucose, L-Glutamine, no HEPES, Phenol Red, Sodium Puryvate) and Fetal Calf Serum (FCS) were purchased from GIBCO, Invitrogen.
- Dulbecco's Phosphate Buffered Saline (PBS, without Calcium and Magnesium), Penicillin/Streptomycin (10000 U/ml Penicillin and 10 mg/ml Streptomycin in 0.9% NaCl) were obtained from PAA Laboratories GmbH (Pasching, Austria).
- Dimethylsulfoxid (DMSO) and Trypan blue were purchased from SIGMA-Aldrich (Steinheim, Switzerland).
- Tissue culture dishes and culture flasks were obtained from Sarstedt (Nürmbrecht, Germany; Newton, USA).

Chemicals

- Ethylenediaminetetraacetic Acid (EDTA), Disodium Salt Dihydrate ($Na_2EDTA \times 2H_2O$) was purchased from Carl Roth GmbH (Karlsruhe, Germany).
- Tris was purchased from Carl Roth GmbH (Karlsruhe, Germany).
- Borate Acid was purchased from Carl Roth GmbH (Karlsruhe, Germany).
- Potassium bromide (KBr) and Sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany).
- Sodium Dodecyl Sulfate (SDS) Natrium Sulfate was obtained from Carl Roth GmbH (Karlsruhe, Germany).
- Glycin was purchased from Carl Roth GmbH (Karlsruhe, Germany).
- 2- mercaptoethanol was purchased from Bio Rad Labartories.
- Tetramethylethylendiamin (TEMED) purchased from Carl Roth GmbH (Karlsruhe, Germany).
- Ammoniumperoxodisulfat (APS) purchased from Carl Roth GmbH (Karlsruhe, Germany).

- Rotiphorese® Gel 40 (37, 5:1) was purchased from Carl Roth GmbH (Karlsruhe, Germany).
- Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Merck (Darmstadt, Germany).
- Bio-Rad Protein Assay Dye Reagent Concentrate, used for determining protein concentration, was obtained from BioRad Labaratories (Hercules, California/USA).
- Bovine Serum Albumin (BSA), was purchased from SIGMA-Aldrich (Steinheim, Switzerland).
- SEA Kem LE Agarose- pure agarose powder derived from agar was purchased from Cambrex Bio Science Rockland.
- Brilliant Blue G 250 was purchased from SIGMA-Aldrich (Steinheim, Germany).
- Solvent chemicals were purchased from Carl Roth GmbH (Karlsruhe, Germany) and/or Merck (Darmstadt, Germany):
    - Ethanol (C_2H_5OH)
    - Chloroform ($CHCl_3$)
    - Methanol (CH_3OH)
    - 25 % Ammoniac (NH_4)
    - Acetic Acid
    - Magnesium chloride ($MgCl_2$)
    - Diethylether
    - HEPES
- Sodiumcyanoborhydride ($NaCNBH_3$) was obtained from Carl Roth GmbH (Karlsruhe, Germany).
- Sypro Ruby Stain Solution (100 µl of RuBPS (20 mM) per one liter with 20% Ethanol in ddH_2O).

Kits for cell culture

The following kits were obtained from Invitrogen (Carlsbad, CA, USA)

- MycoFluor™ Mycoplasma Detection Kit (M-7006)
    - Microscopic Optical Replicas for Fluorescence (MORFS) Stock suspension
    - 20 X concentrated Myco Fluor Reagent.
- Cell Mask Deep Red™ Plasma Membrane Stain, Catalog number C10046.

Buffers

- One liter of Na-EDTA-Buffer pH 7.4 (was adjusted with NaOH or HCl) contains:
  - 1 g Na_2EDTA x 2 H_2O
  - 1L ddH_2O.

- Tris buffers:
  - 1 M Tris, pH 6.8 was adjusted with HCl
  - 1 M Tris, pH 8.8 was adjusted with HCl.

2.2 Culturing of RAW 264.7 murine cell line

2.2.1 Cell maintenance and splitting

DMEM (10% FCS, 1% Penicillin/Streptomycin) was warmed to 37°C (water bath). Splitting into smaller or larger flasks depends on the cellular count requirement determined by surface area to be covered by the cells.

For example, 75 cm^2 of a cell layer can be divided into three aliquots and only one aliquot was transferred into a new 75 cm^2 flask.

At first, cells were scraped off and centrifuged for 2-3 minutes at 100 g. The medium is removed carefully. The cells are re-suspended in 6 ml of warm fresh medium:

75 cm^2……6 ml

25 cm^2……2 ml

2 ml of the resultant suspension is transferred to a 75 cm^2 culture flask. Before seeding the cells, the 75 cm^2 culture flask should be previously loaded with 10 ml of warm medium to make sure that cells can spread around and do not instantly adhere. Leave the cell cultures in incubator at 37°C until use.

2.2.2 Cell Freezing

For cell freezing a NALGENE^{TM} Cryo 1°C Freezing Container was used. The container is usually stored at room temperature. It allows a -1°C/minute cooling rate once it has been placed into a -70°C freezer.

The medium used for cell freezing contains 5% DMSO (11.4 ml of DMEM incl. 10 % FCS, 1% Penicillin/Streptomycin and 600 µl of DMSO).

Confluent cells from two 175 cm^2 culture flasks were scraped, the cell suspensions were centrifuged and the pellets were finally re-suspended in 5% DMSO medium (12 ml). Aliquots were pipetted into 12 ampoules (1 ml per ampoule). Thereafter the ampoules were stored in a Freezing container overnight at –70°C, followed by storage in liquid nitrogen.

2.2.3 Cell thawing

Cell ampoules were taken from liquid nitrogen and placed in a water bath at 37°C, making sure the core stays frozen (or let thawing at room temperature). Afterwards the cells were re-suspended in 12 ml warm (37°C) DMEM medium (10% FCS, 1% Penicillin/Streptomycin) and centrifuged. The medium is removed (to remove DMSO). The cells are re-suspended in 12 ml warm medium (37°C) and transferred into 75 cm^2 flask. After two or three days, cells should be confluent for further use.

2.2.4 Cell counting with Neubauer Counting Chamber

For cell counting we used a NEUBAUER Counting Chamber obtained from Brand (Wertheim, Germany).

The Neubauer counting chamber consists of a thick microscopic glass slide with rectangular indentations creating a chamber (see Figure 2.1). This chamber is imprinted with a laser-etched grid of perpendicular lines. The area and depth of the chamber is known (see Figure 2.1, B). It is therefore possible to count the number of cells in a specific volume of fluid, and thereby calculate the concentration of cells in the total suspension.

First, a cover glass was placed onto the chamber and pressed until Newtonion rings can be seen. Cells were scraped and transferred into a 25 ml plastic tube. 100 µl of scraped cells were transferred from the tube into an Eppendorf tube and mixed with 200 µl Trypan blue solution (dilution 1:3). After pipetting the solution up and down, a 10 µl aliquot was placed into the Neubauer chamber. The cells were than counted under a microscope. At appropriate dilution, cells in all subdivided 5x5 squares (Figure 2.1) can be counted. If there are too many cells, at least five of subdivided squares must be counted in order to achieve an accurate mean.

Cells/ml = (counted cells) / (surface of counted cells [cm^2]* chamber depth [cm]) * $f_{dilution}$

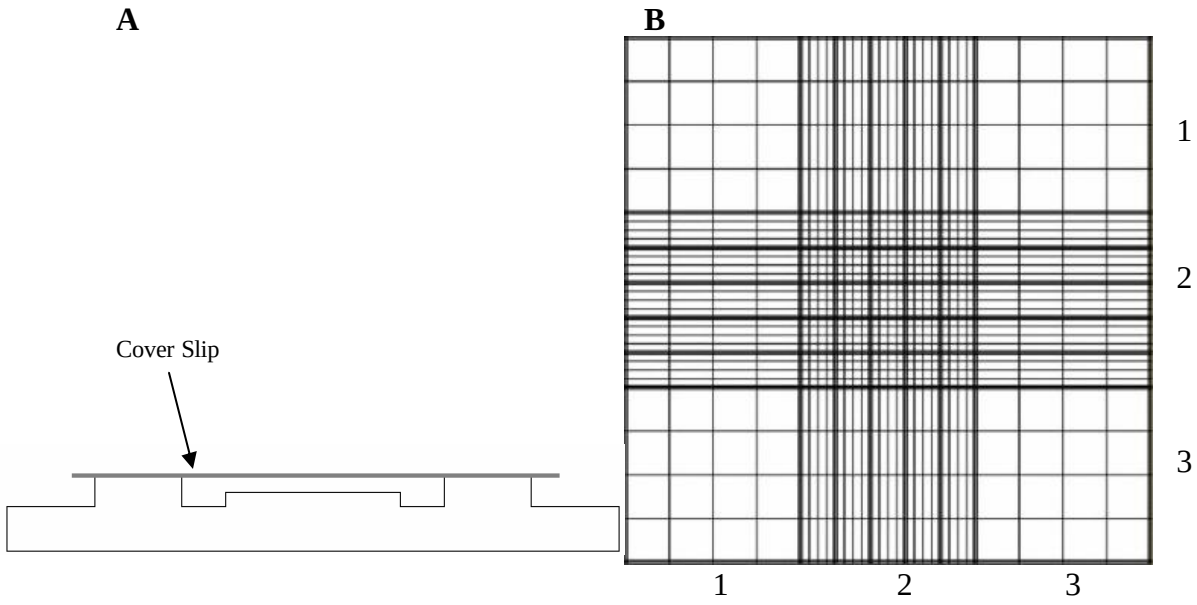

Figure 2.1 Counting Chamber. A: Side View. **B:** [©LO Laboroptik]Chamber consist of 3x3 large squares, each with 1 mm^2 surface area; the central square is subdivided into 5x5 squares each with 0.04 mm^2 surface area; 5x5 squares are further divided into 4x4 squares each with 0.0025 mm^2 surface area; depth of a chamber is 0.1 mm.

2.2.5 Cell integrity and purity

Plasma Membrane Staining

The CellMask™ Plasma Membrane Stains are amphipathic molecules containing a lipophilic moiety for membrane localization and negatively charged hydrophilic groups for fast membrane

uptake. For staining with CellMask we followed the instructions provided with the Invitrogen CellMask™ Plasma Membrane Stains (Catalog nos. C10046).

Mycoplasma Test

During our work with macrophages we noticed that their morphology changed and 70% or more of the cells died. Figure 2.2, panel A shows a light transmission image of such cells on a cover slip. Figure 2.2, panel B is a corresponding fluorescence image of the cells stained with a cell mask plasma membrane dye. The yellow arrows in both panels mark the altered cell, which is not successfully stained by the plasma membrane dye. We suppose that this effect was due to a bacterial infection or contamination by mycoplasma.

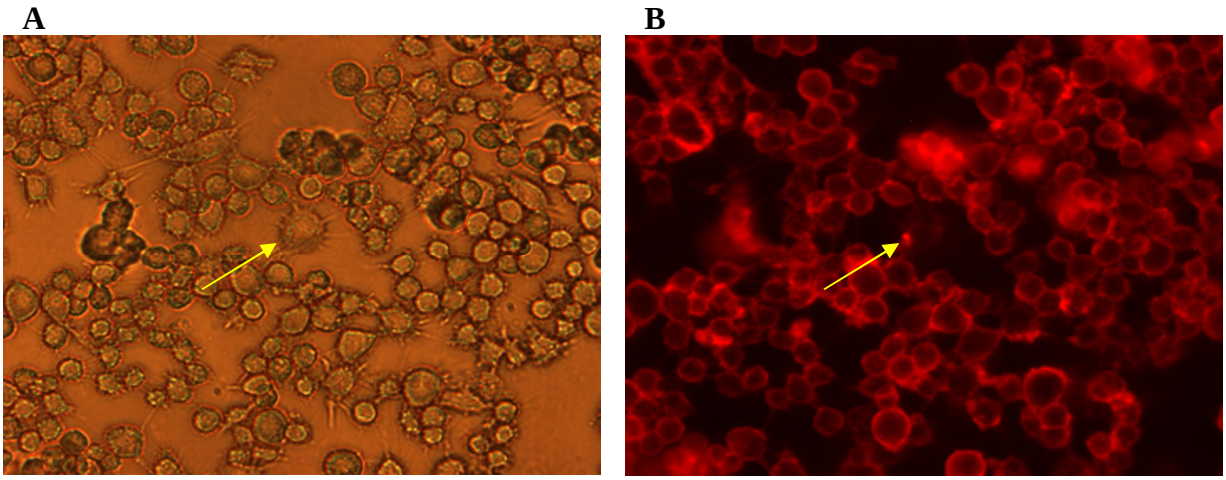

Figure 2.2 A: Transmitted light image. B: Fluorescence image of cells after staining with Cell mask (see Table 2.3)

We tested for Mycoplasma[1] species (using MycoFluor Mycoplasma Detection Kit (M-7006) from Molecular Probes). These microorganisms are common contaminants found in cell culture laboratories. Contamination can be caused by improper handling by individuals or infected cell culture media. Mycoplasma cells are small – less than 1 µm – and therefore they are difficult to detect with a conventional microscope. Mycoplasmas may induce cellular changes, including chromosome aberrations, changes in metabolism and cell growth.

[1]Mycoplasma is a genus of bacteria which lack a cell wall. Therefore, they are unaffected by many common antibiotics such as penicillin and streptomycin.

As no mycoplasma cell infection was detected (Figure 2.3) we increased our efforts to maintain a sterile work environment (new scrapers, cleaning incubator, etc.) which helped establish proper macrophage-cultures again.

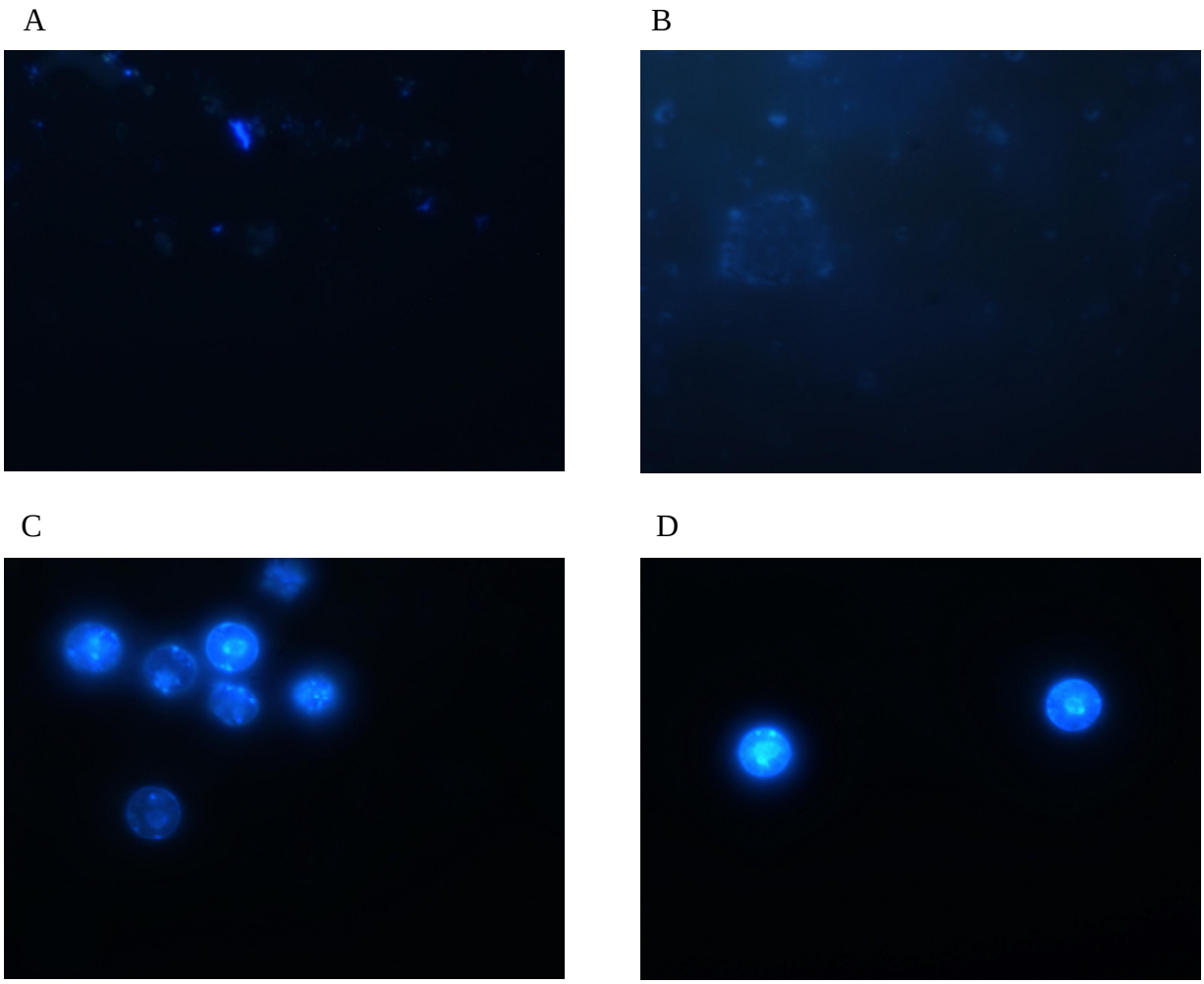

Figure 2.3 Mycoplasma detection with the fluorescence microscope (magnification 1000x, Ex at 365 nm and Em with bandpass filter at 450-490 nm, beam splitter at 510 nm and longpass at 520 nm). A: Control Slide: Microscopic Optical Replicas for Fluorescence (MORFS) assay with no cells. **B:** Positive Control: MORFS added to pre-stained cells with MycoFluor Reagent. **C and D:** Sample: live cells fixed on a cover slip and stained with reagent for Mycoplasma detection.

2.3 Isolation of Low-density lipoproteins (LDL) from human plasma

Ethylenediaminetetraacetic acid (EDTA) Human Plasma was obtained from Univ. Prof. Dr. Gholam Ali Khoschsorur, Clinical Institute of Medicine and Chemical Lab Artery Diagnostics (Medical University of Graz).

OptiSeal Tubes and Beckmann NVT65 Rotor, which were used for LDL isolation, were from Beckmann Coulter.

Sorvall^{R}Combi Plus Centrifuge was from Sorvall Thermo Scientific.

PD-10 Desalting Columns were purchased from GE Healthcare (Buckinghamshire, UK). Its use was based on Gravity Protocol from GE Healthcare Instructions 52-1308-00 BB.

2.3.1 Procedure

During LDL isolation it was very important to avoid vortexing or shaking the plasma otherwise the LDL would be destroyed. The whole procedure was performed at room temperature. The Na-EDTA Buffer pH value was adjusted with NaOH to pH 7.4 followed by 15 min deoxygenation with argon gas and storage at 4°C until use.

1. 12.211 g of Potassium bromide (KBr) was dissolved in 32 ml of EDTA- Human Plasma in a 50 ml Falcon tube. Subsequently, the tube was deoxygenated with argon gas. The Falcon tube was slowly rotated until the KBr was completely dissolved in the Plasma. The final density of KBr-loaded plasma was 1.24.
2. 8 ml of deoxygenated EDTA – Buffer are transferred with a 10 ml glass Pipette to each of 8 centrifuge tubes (OptiSeal Tubes).
3. 4 ml of EDTA-Plasma (density 1.24) is injected into each tube. During this process, excess buffer will overflow.
4. The samples are centrifuged in a Beckmann NVT65 rotor (60000 rpm) for 2 h at 4°C. Centrifuge was adjusted as follows: Temperature: 4-10°C; Run: 60000 rpm; Accelerate Rate: 5).

5. The LDL is isolated. The upper layer contains the chylomicrons and VLDL which are lighter and float at the surface. The lower layer contains LDL which can be collected with a curved needle.
6. LDL was stored in a vacuum tube to avoid oxidation. 10 ml of LDL can be maximally collected from 8 OptiSeal Tubes.
7. ApoB 100 protein concentration of LDL was determined with the Bradford Protein Assay. It varied between 0.6 and 0.95 mg/ml.
8. PD-10 Column was used for desalting EDTA-LDL with PBS, as EDTA can interfere with reagents necessary for sample preparation.

2.4 Determination of protein concentration

2.4.1 Principle

The Bradford Protein assay is a procedure for quantitative protein analysis. It is based on binding of an acidic dye to a protein leading to absorption at 595 nm, which can be measured using a microplate reader.
The maximum absorption wavelength for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm upon dye binding to a protein. Coomassie blue dye mainly binds to basic and aromatic amino acid residues, especially arginine.

2.4.2 Procedure

For the protein assay, ninety-six-well microtiter plates obtained from Greiner Bio-One GmbH (Kremsmünster, Austria) were used. Protein samples were diluted and pipetted according to a defined schema. 200 µl of Bio-Rad Protein Assay Dye Reagent Concentrate, diluted in ddH_2O (1:5 v/v) and filtered, were added to each well. Immediately after mixing, absorbance was measured at 595 nm with a microplate reader (Anthos 2010, microplate reader from Anthos Labtec Instruments GmbH (Salzburg, Austria)) using WinRead v2.3 Software (Version 1.7).

2.5 Oxidation of Low-density lipoprotein (LDL)

LDL was desalted with a PD-10 desalting column (3.5 ml) to the desired protein concentration (50 µg/ml if not stated otherwise). The desalted lipoproteins were oxidized by Cu^{2+} or by Fe^{2+} as described in Figure 2.4.

2.5.1 Preparation of oxidized low-density lipoproteins

1. Modification of LDL by Cu^{2+}. Preparation of oxLDL.

3.5 ml of desalted LDL was divided into two 1.75 ml aliquots which were then separately transferred to Falcon tubes. To reach a final concentration of 25 µM $CuSO_4$ 44 µl of 1 mM $CuSO_4$ were added per aliquot. To adjust 50 µM CuSO4 concentration 85 µl of 1 mM $CuSO_4$ were added per aliquot. The Cu^{2+} containing samples were incubated at 37°C for 48 h. See Figure 2.4, A.

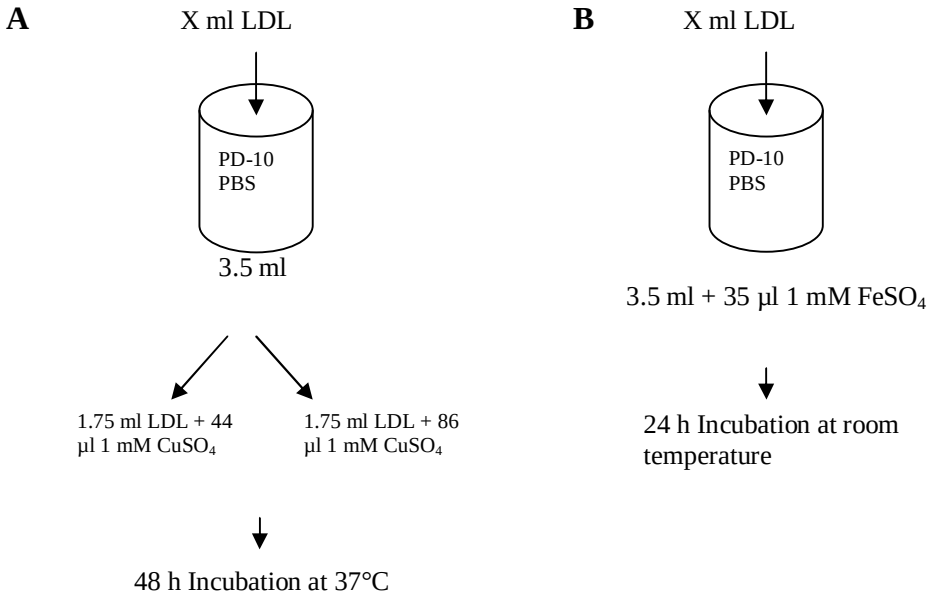

Figure 2.4 Preparation of mmLDL and oxLDL. The amount of LDL applied onto the column (X ml) depends on the desired protein concentration in the elute. **A:** oxLDL, **B:** mmLDL.

2. Modification of LDL by Fe^{2+}. Preparation of mmLDL.

3.5 ml of LDL were transferred into a round-bottom flask (10 ml). 35 µl of 1 mM $FeSO_4$ were added to reach a final concentration of 10 µM $FeSO_4$. The sample was incubated at room temperature for 24h.

2.6 Solubilization of fluorescent oxidized phospholipids

Oxidized phospholipids were solubilized in:

1. **Aqueous PBS buffer**

OxPL is dissolved in ethanol and the resultant solution is injected into 4 ml PBS buffer. The final oxPLs (POVPE-BY or PGPE-BY) concentrations are 5 or 10 µM. The amount of ethanol must not exceed 1% of total buffer volume.

2. **Albumin solution**

660 µg of BSA powder is dissolved in 2 ml PBS buffer. Final concentration of BSA dissolved in PBS was 5 µM. POVPE-BY is dissolved in ethanol and injected into 2 ml of 5 µM BSA solution. The final concentration of POVPE-BY was 5 µM. The amount of ethanol must not exceed 1% of BSA-PBS buffer volume. The sample was shortly agitated during injection and thereafter slightly shaken for 30 minutes.

3. **Low density lipoprotein**

LDL was desalted with a PD-10 column in PBS to reach a concentration of 50 µg protein/ml (if not stated otherwise). OxPL (POVPE-BY or PGPE-BY) is dissolved in ethanol and the resultant solution is injected into desalted LDL (4 ml). The final oxPL concentration is 5 µM. The amount of ethanol must not exceed 1% of total LDL buffer volume. POVPE-BY- labeled LDL is slightly shaken for 30 minutes. Lipoprotein phospholipid (LDL) / oxPL (label) ratio was 20/1 mol/mol.

4. Minimally modified low-density lipoprotein (mmLDL)

mmLDL was prepared from LDL by modification with Fe^{2+} (see above). After oxidation, mmLDL was desalted with a PD-10 column in PBS to reach concentration of 50 µg protein/ml (if not stated otherwise). OxPL (POVPE-BY or PGPE-BY) is dissolved in ethanol and the resultant solution is injected into desalted mmLDL (4 ml). The final oxPL concentration is 5 µM. The amount of ethanol must not exceed 1% of total mmLDL buffer volume. POVPE-BY-labeled mmLDL is slightly shaken for 30 minutes. Lipoprotein phospholipid (mmLDL) / oxPL (label) ratio was 20/1 mol/mol.

2.7 Agarose Gel electrophoresis

2.7.1 Procedure

The equipment and supplies necessary for agarose gel electrophoresis were purchased from BioRad (Hercules, CA, USA). They include an electrophoresis chamber, a power supply, gel casting trays and sample combs. Tris/Borate/EDTA Buffer (TBE) 1x for one liter: 100 ml TBE 10x (see Table 2.1) and 900 ml ddH_2O.

Tris/Borate/EDTA 10x	Tris	108 g
1 L in ddH_2O,	Borate	55 g
pH 8	0.5 M EDTA	186.12 g

Table 2.1 Tris/Borate/EDTA10x

Agarose powder was mixed with electrophoresis buffer (0.75 g agarose powder / 150 ml TBE1x see above), followed by heating in a microwave chamber until the agarose suspension was completely melted. After the solution reached about 60°C, it was poured into a casting tray. The sample comb was placed into the gel and the gel was allowed to solidify at room temperature for about an hour.

After the gel had solidified, the comb was removed. The gel, still in its plastic tray, was then covered with TBE1x buffer. Bromine Phenol Blue containing glycerol[2] was used as loading buffer and tracking dye.

66 µl (10 µg protein) of the samples (see Results) were pipetted into the sample wells (all containing glycerol), the power lead was attached to the apparatus, and a voltage was applied (5 min at 20 V and 2h at 80 V). After electrophoresis the fluorescent bands on the gel were visualized with transilluminating light using a Herolab camera.

After fluorescence imaging, proteins were stained with 0.02 % Brillant Blue (diluted in 10% acetic acid) on a shaker (8 rpm) for 10 to 15 minutes. The stained gel was washed with and incubated overnight with destaining Solution. Destaining Solution (10% acetic acid and 5% CH_3OH) for one liter:

- 100 ml acetic acid
- 50 ml CH_3OH
- 850 ml ddH_2O.

2.8 Uptake and stability of fluorescent oxPL in (lipo)proteins and cultured macrophages.

2.8.1 Labeling of LDL with POVPE-BY and PGPE-BY. Stability of oxPL in LDL and PBS

For analysis of oxPL stability in LDL, mmLDL and PBS the following samples were prepared.

1. LDL was desalted with PD-10 Column in PBS to reach a concentration of 50 µg protein/ml. The final sample volume was 3.5 ml. OxPL (POVPE-BY or PGPE-BY) was dissolved in ethanol and injected into a 1 ml of desalted LDL to reach a final concentration of 5 µM. The total amount of ethanol must not exceed 1 % of total sample volume. The labeled LDL was shaken at room temperature for 30 minutes. Lipoprotein PL (LDL) / oxPL (label) ratio was 20/1 mol/mol, lipids were extracted from 1 ml sample.

[2] Glycerol, a dense substance, enables the sample to “fall” into the sample wells.

2. For the preparation of a reference sample LDL was desalted in PBS leading to 50 µg protein/ml. Lipids were extracted from 1 ml of unlabeled desalted LDL.
3. Fluorescent analogs of oxPL (POVPE-BY and PGPE-BY) with a concentration of 10 µM were solubilized in 4 ml of PBS and incubated under gentle shaking for 5, 10, 30 and 60 minutes, respectively. Afterwards, lipids were extracted from each sample followed by one dimensional TLC analysis.

Corresponding samples (see above) were prepared with mmLDL. Therefore lipid extraction (see below LIPID EXTRACTION steps 8 to 12) was preformed followed by two dimensional TLC if not stated otherwise.

2.8.2 Uptake of oxPL into cells

For the individual experiments, macrophages were seeded into Petri dishes (diameter 9 cm) and grown in DMEM (1% Penicillin/Streptomycin, 10% FCS) medium to confluency at 37°C. The medium was removed and the cells were washed twice with an ice cold PBS. For analyzing the uptake of POVPE-BY and PGPE-BY from LDL carrier complexes into the macrophages, the cells were incubated with the fluorescent lipids as follows.

A) Uptake of lipids from LDL

1. LDL was desalted with PD-10 Columns in PBS leading to 50 µg protein/ml concentrations and total elution volume of 4 ml. 5 µM oxPL (POVPE-BY or PGPE-BY) were solubilized in this LDL sample as described, see 2.6. If LDL was labeled with POVPE-BY the sample was easily agitated for 30 minutes. If LDL was labeled with PGPE-BY the sample was used immediately after the fluorophore injection. Lipoprotein PL (LDL) / oxPL (label) ratio was 20/1 mol/mol. Cultured macrophages were incubated with the labeled LDL sample for 30 minutes followed by lipid extraction.
2. For the preparation of a reference macrophages were incubated with desalted unlabeled LDL for 30 minutes followed by lipid extraction.

B) Uptake of lipids from aqueous suspensions

3. 5 µM oxPL (POVPE-BY or PGPE-BY) were solubilized in 4 ml of PBS. Macrophages were incubated with these lipid suspensions for 30 minutes followed by lipid extraction.
4. For a preparation of a reference sample macrophages were incubated with 4 ml of PBS for 30 minutes followed by lipid extraction.

After incubation of the cells with the lipid samples, we collected the supernatant and scraped the cells into 10 ml cold PBS as a second fraction. Lipids were extracted from both fractions as described below.

LIPID EXTRACTION

1. After incubation of the cells with the fluorescent samples the supernatant were collected into Falcon tubes (supernatant fraction).
2. The remaining cells were washed twice with 10 ml of ice cold PBS.
3. Then the cells were scraped into 10 ml of PBS solution and transferred into Pyrex-tubes (cell fraction).
4. $NaCNBH_3$ (0.1 volume %) was added to both fractions (supernatant and cell) and samples were incubated for 10 minutes at room temperature.
5. 1 ml of each supernatant fraction was transferred to a Pyrex-tube.
6. The cell suspensions were centrifuged at 2400 rpm for 3 minutes.
7. The cell pellets were re-suspended in 3 ml of $CHCl_3$/ CH_3OH (2:1) and 20 µl of 10 mM $NaCNBH_3$. The samples were shaken (1700 rpm) for an hour at 4°C.
8. Alternatively, 1 ml of each supernatant fraction, was also mixed with 6 ml of $CHCl_3$/ CH_3OH (2:1) and 40 µl of 10 mM $NaCNBH_3$ and shaken (1700 rpm) for an hour at 4°C.
9. After agitation of the samples for an hour, 700 µl of $MgCl_2$-solution (0,036%) was added to cell samples. Alternatively 1 ml of $MgCl_2$-solution (0,036%) was added to 1 ml of each supernatant fraction. The respective biphasic systems were shaken (1700 rpm) again for 15 minutes at 4°C.
10. All samples were centrifuged at 2400 rpm for 2 minutes at room temperature. The lower chloroform phase was isolated and transferred into Pyrex tubes. They were left under N_2 until samples were dried out.

Reduction of Schiff-Bases between LDL and POVPE

11. 10 µl of 10 mM $NaCNBH_3$, 1 ml of Diethyl ether and 1 ml of alkaline Buffer (see Table 2.2) were added to the dehydrated samples, and left overnight at 4°C and shaken (1700 rpm).
12. The upper phase was transferred into vials on the next day. After water separation the samples were again dehydrated under N_2 and stored at -20°C until use for TLC analysis.

Alkaline Buffer, pH 8.5	NaCl	0.75 M
	HEPES	1 mM

Table 2.2 Alkaline Buffer

2.8.3 Thin Layer Chromatography (TLC)

2.8.3.1 *Procedure*

One and two dimensional TLC separation of lipid extracts was preformed using Silica plates 60 from Merck (Darmstadt, Germany).
Basic Solvent: $CHCl_3$/ CH_3OH /25%NH_3 (65:35:5 per volume).
Acidic Solvent: $CHCl_3$/Aceton/ CH_3OH /Acetic acid/ddH_2O (50:20:10:10:5 per volume).

Two dimensional TLC

1. The chambers (for the first (basic) and second (acidic) dimensions) were saturated with solvent vapor. For this purpose, filters were placed for 2h prior to use.
2. Silica plates were activated at 120°C for 10 minutes.
3. An aliquot (10 or 20 µl) of a lipid extracts (dissolved in 100 µl $CHCl_3$/MeOH (2:1 v/v)) were applied onto a silica plate.
4. Lipids were separated using the basic solvent (first dimension): $CHCl_3$/ CH_3OH /25%NH_3 (65:35:5 per volume). Afterwards the plate was rotated 90° counter clockwise and developed in an acidic solvent (second dimension): $CHCl_3$/Aceton/ CH_3OH / Acetic acid/Water (50:20:10:10:5 per volume). Before spot detection, the solvent was removed under an air stream.

5. Visualization of lipid spots was accomplished with iodine gas using an iodine chamber or with fluorescent detection using Herolab Camera (Ex 365 nm) from Herolab Labaratories (Germany) and Easy Win 32 Software.

One dimensional TLC

Several samples at the time were analyzed with one dimensional TLC. The lipid extracts were applied onto a silica plate and separated using the basic solvent.

2.9 Fluorescence Microscopy

2.9.1 Principle

An inverted fluorescence microscope Zeiss Axiovert 35 has been used for fluorescence imaging of lipids in RAW 267.4 macrophages. Macrophages were incubated with different Bodipy-labeled samples (see below 2.9.2). If not stated otherwise, cells have been imaged at 320x magnification using the filters indicated in Table 2.3. The software used for taking photo-acquisition was AxioVision.

Fluorophore/Stain	Wavelength [nm]		Fluorescence Microscope Filter [nm]			Light Source
	Excitation Maxima	Emission Maxima	Excitation Filter	Beam Splitter	Emission Filter	
BODIPY SE	505	510	BP 450-490	510	LP 520	HBO Mercury Gas
CellMask Deep Red	649	666	BP 575-625	645	BP 660-710	Xenon XBO
SyproRuby	460	617				

Table 2.3 Excitation/emission maxima of fluorophore/stains, and optical instruments settings.

2.9.2 Sample preparation and microscopy

The Lab-Tek™Chamber Slide System, used for microscopic visualization was purchased from Nunc. It consists of eight chambers (V = 1 ml per chamber). The cells were seeded into chamber slide prior to use. Before incubation with the lipid samples, cells were washed two times with cold PBS and incubated with the following reagents for 30 minutes.

1. 5 µM concentration of POVPE-BY or PGPE-BY in 1 ml PBS (see solubilization of oxPL in aqueous solutions). Lipid concentration was 10 µM if cells were treated with 0.1% Triton X-100.
2. 1 ml of desalted LDL and 1 ml of desalted mmLDL (50 µg Protein/ml) labeled with 5 µM POVPE-B or PGPE-BY. Lipoprotein PL (LDL) / oxPL (label) was 20/1 mol/mol.
3. 1 ml of LDL and 1 ml of mmLDL (50 µg Protein/ml).
4. 1 ml PBS.

Supernatants were removed after incubation followed by washing twice with PBS. Finally 500 µl of PBS was pipetted in each chamber and cells were examined under the microscope (see Table 2.3)

0.1% Triton wash

Cells were incubated with the lipid samples as described above. After incubation supernatant was removed and cells were washed twice with PBS which were than incubated with 500 µl of 0.1% Triton X-100 (in PBS) for 5 seconds. Triton was removed and cells were washed twice with cold PBS. Again 500 µl of PBS was pipetted onto the cells which were examined under the microscope.

2.9.2.1 Microscopy of cells on cover slips

10 µl of culture medium was pipetted into a 30 cm^2 Petri dish in which cover slips were carefully placed by pressing them until they were attached to the Petri dish. Cell suspensions were then carefully added directly onto the cover slips. The Petri dish was then placed in the incubator and left until use at 37°C.
Immediately before use, cells were washed two times with PBS followed by staining with Cell mask according to the procedure provided by manufacturer (Invitrogen).

2.10 Exchange of oxidized phospholipid between LDL and albumin

LDL protein concentration was 0.95 µg/ml. 50 µM Stock Solution of BSA contained 33 mg BSA in 10 ml PBS. Lipoprotein PL (LDL) / POVPE-BY ratio was 20/1 mol/mol.
$M_{ApoB100}$ = 550 000 g/mol, M_{BSA} = 66 000 g/mol.

2.10.1 Preparation of unlabeled and POVPE-BY labeled LDL and albumin

A) Labeled LDL

710 µl of LDL was incubated with 15 µM POVPE-BY (45 nmol lipid/ 3 ml) for 30 minutes, under slight stirring. Afterwards, POVPE-labeled LDL was desalted in PBS with a PD-10 Column. 3 ml of the sample were collected which were divided into three different aliquots and transferred into Pyrex tubes each containing.

B) Labeled albumin

10 nmol of POVPE-BY were injected into a 2 ml of 5 µM BSA solution in PBS (330 µg protein/ml). The final lipid concentration was 5 µM..

C) Unlabeled LDL

710 µl (≡ 674.5 µg) of LDL was desalted in PBS with PD-10 and 3 ml were collected.

D) Unlabeled albumin

2 ml of 5 µM BSA (330 µg/ml).

2.10.2 Exchange of oxPL between LDL and albumin

1. 1 ml of POVPE-BY labeled LDL (15 µM label) was prepared as described above and incubated with different volumes of 5 µM albumin solution (330µg BSA Protein/ ml). Molar ratios of POVPE-labeled LDL protein/BSA protein in the final mixture were 1/1, 1/5 and 1/10.

2. Alternatively, we incubated POVPE-BY-labeled LDL (3 ml), see above, with 50 µM $NaCNBH_3$ (adjusted by addition of 15 µl 10 mM $NaCNBH_3$) for 3 h. Again different volumes of 5 µM BSA were added depending on molar ratios of POVPE-BY-labeled LDL protein/BSA protein in the sample (1/1, 1/5 and 1/10).

After incubation proteins were precipitated according to Wessel-Fluegge and separated by SDS-Page followed by fluorescent analysis.

2.10.3 Protein precipitation with Wessel-Fluegge Method [42]

600 µl of methanol and 150 µl of Chloroform were added to 150 µl of an LDL/albumin sample (see above), and vortexed. 450 µl of double distilled water were added and the sample was vortexed thoroughly again.

The sample was centrifuged in an Eppendorf Centrifuge 5417R, from Eppendorf (Hamburg, Germany), at 10000 rpm at room temperature for 2 minutes. The upper aqueous phase was carefully removed and discarded without disturbing the interphase (which contains the proteins!). 450 µl of methanol were added to the sub-phase and the mixture was vortexed thoroughly.

The sample was centrifuged at 10000 rpm at room temperature for 5 minutes.

The supernatant was removed and discarded. The pellet was air dried (for 10 min) and the sample buffer[3] (see Table 2.4) was added (usually 40 µl as the samples were pipetted twice 20 µl per gel).

The sample was incubated at 95°C for 5 minutes. Because of condensation of water on the vessel wall, all samples were spun for a minute before application onto the SDS gels.

[3] The sample buffer solubilizes the proteins. It also contains mercaptoethanol, which cleaves disulfide bonds. The dissociation buffer, which is part of the sample buffer, contains the tracking dye which allows monitoring of the leading front.

2.11 SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

2.11.1 Principle

SDS-PAGE is a technique used to separate proteins according to their electrophoretic mobility and molecular weight. The SDS is an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures and applies a negative charge to each protein in proportion to its mass. Disulfide bonds between cystein residues were cleaved by reduction of mercaptoethanol. Polymerization of the gels is due to the 10% ammoniumpersulfate (APS) and the catalyst tetramethylethylenediamine (TEMED).

2.11.2 Procedure

First buffers described in Table 2.4 were prepared as. For SDS gel electrophoresis we used 1x running buffer which for 3 L contained: 600 ml of 5x Running Buffer and 2.4 L of ddH_2O.

5x Running Buffer, in ddH_2O	Tris	15 g/l
	EDTA	1.67 g/l
	Glycin	71 g/l
	SDS	2.5 g/l
Fixing Solution, in ddH_2O	7% Acetic Acid	70 ml/l
	10% Ethanol	100 ml/l
Dissociation Buffer in ddH_2O, pH 6.8	KH_2PO_4	20 mM
	EDTA	6 mM
	SDS	6%
	Glycin	10%
	Brom Phenol Blue	0.05%
Sample Buffer	Dissociation Buffer	780 µl
	Tris/HCl Buffer, pH 8.8	200 µl
	Mercaptoethanol	20 µl

Table 2.4 Buffers used for SDS Gel electrophoresis

Solutions of stacking and resolving gels are mixed as indicated in Table 2.5 and Table 2.6.

	Stacking gel	4.5% Acrylamid				
	2	**4**	**6**	**8**	**10**	**12**
AA/Bis 40	0.8 ml	1.5 ml	2.3 ml	3.0 ml	3.8 ml	4.5 ml
1M Tris pH 6.8	0.8 ml	1.7 ml	2.5 ml	3.3 ml	4.2 ml	5.0 ml
20% SDS	33 µl	67 µl	100 µl	133 µl	166.7 µl	200 µl
H_2O bidest.	4.9 ml	9.8 ml	14.8 ml	19.7 ml	24.6 ml	29.5 ml
10% APS	33 µl	67 µl	100 µl	133 µl	166.7 µl	200 µl
TEMED	7 µl	13 µl	20 µl	27 µl	33.3 µl	40 µl
End Volume	6.6 ml	13.2 ml	19.7 ml	26.3 ml	32.9 ml	39.5 ml

Table 2.5 Stacking Gel

	Resolving gel	10% Acrylamid				
	2	**4**	**6**	**8**	**10**	**12**
AA/Bis 40	8.6 ml	10.9 ml	13.1 ml	15.4 ml	17.7 ml	20.0 ml
1M Tris pH 8.8	12.9 ml	16.3 ml	19.7 ml	23.1 ml	26.6 ml	30.0 ml
20% SDS	171 µl	217 µl	263 µl	309 µl	354 µl	400 µl
H_2O bidest.	12.4 ml	15.7 ml	19.1 ml	22.4 ml	25.7 ml	29.0 ml
10% APS	165 µl	208 µl	252 µl	296 µl	340 µl	384 µl
TEMED	34 µl	43 µl	53 µl	62 µl	71 µl	80 µl
End Volume	34.2 ml	43.3 ml	52.5 ml	61.6 ml	70.7ml	79.9 ml

Table 2.6 Resolving Gel

First, the resolving gel was poured into the casting tray (stacked with gel cassettes). The resolving gel was then covered with water-saturated 1-butanol to maintain a smooth surface between the resolving and stacking gels. After polymerization of the resolving gel, the butanol was removed and the gel was washed with distilled water. Gel cassettes were then filled with the stacking gel and sample combs were inserted. After polymerization of the stacking gel (ca. ninety minutes) 20 µl of protein solutions were pipetted per sample well. Electrophoresis was preformed in 1x running buffer as follows:

1st phase: 15 minutes long at constant voltage and a current of 0.01A per gel.

2nd phase: ninety minutes at constant voltage and a current of 0.02A per gel.

The gels were removed and placed in a plastic bowl filled with fixing solution. Proteins were fixed 2 h on a shaker at 8 rpm. This prevented the proteins from diffusing in the gel. Fluorescently labeled proteins were visualized using BioRad Moleculare Imager FX Pro Plus (Table 2.7). Proteins were stained with a SyproRuby solution (100 µl of RuBPS (20 mM) per one liter with 20% Ethanol in ddH_2O) for an hour. Gels were then kept in a fixing solution for at least 2 h and scanned again with the BioRad Moleculare Imager FX Pro Plus.

Fluorescence imager		Wavelength [nm]	
		Excitation	Emission
Herolab Camera UVT-20/S/M/L		365	
BioRad Moleculare Imager FX Pro Plus	BODIPY	488	BP 530/30
	Sypro Ruby	488	LP 555

Table 2.7 Fluorescence Imagers

3 Results

3.1 Modification and Oxidation of LDL

For studies on LDL-macrophage interactions, LDL isolated from human plasma was modified by oxidation with Fe^{2+} ions (minimally modified LDL-mmLDL), Cu^{2+} ions (oxidized LDL-oxLDL) or by incubation with (fluorescent) oxidized phospholipids.

mmLDL is supposed to contain only (mainly) lipid modifications. In oxLDL, both the lipids and the proteins are extensively modified. As a consequence a dramatic shift towards negative net charges can be expected. The presence of oxPL in LDL gives rise to different modifications depending on chemical reactivity of the compound. POVPC forms covalent Schiff bases with the free amino groups of aminophospholipids and proteins thus rendering the lipoprotein net charge more negative. PGPC addition also leads to a more negative LDL, but the modification in this case is non-covalent.

To investigate effect of oxidation on integrity and net charge of LDL agarose gel electrophoresis was used. Compared to native LDL, mmLDL it showed almost the same electrophoretic mobility. Obviously, lipid oxidation by Fe^{2+} hardly affected the free NH_2 group of the particle (Figure 3.1, B). If LDL and mmLDL were loaded with fluorescent POVPE-BY (5µM) the electrophoretic mobility become faster (Figure 3.1, B). Figure 3.1 panel A confirms labeling of the particle with POVPE-BY (lanes 1 and 3 show fluorescent bands).

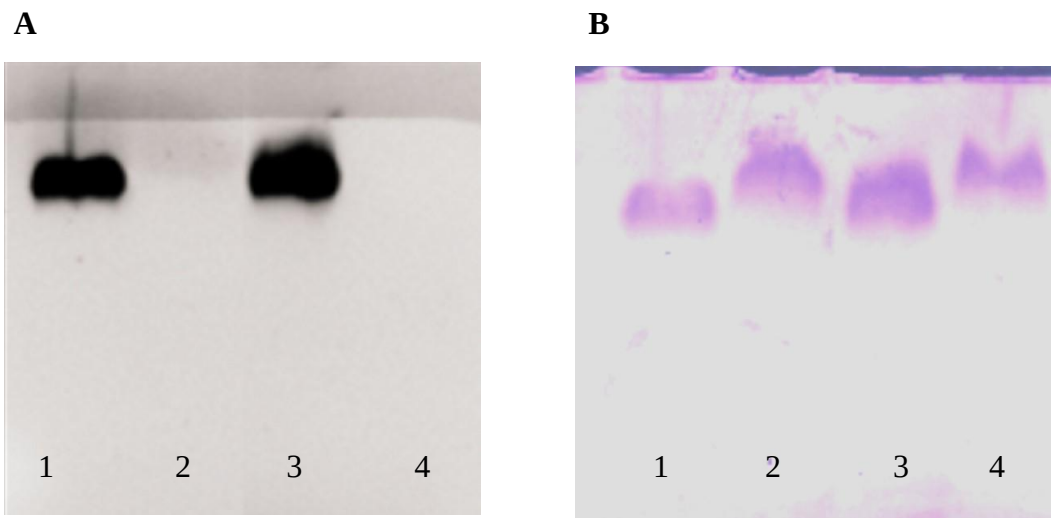

Figure 3.1 Agarose gel electrophoresis of POVPE-BY-labeled and unmodified LDL. Panels A and B: Lane 1: POVPE-BY -labeled mmLDL, lane 2: mmLDL, lane 3: POVPE-BY-labeled LDL, lane 4: LDL. **A:** Fluorescence image (Herolab imager, ex wavelength was 365). **B:** Comassie blue stain. Protein amount per lane was 7.5 µg. The fluorophore to phospholipid ratio was 20/ 1 (mol/ mol).

The protein modifications induced by $CuSO_4$ in LDL caused the particle to be more negatively charged and migrate faster in gel (Figure 3.2). This effect depended on the extent of modification and thus, on Cu^{2+} concentration.

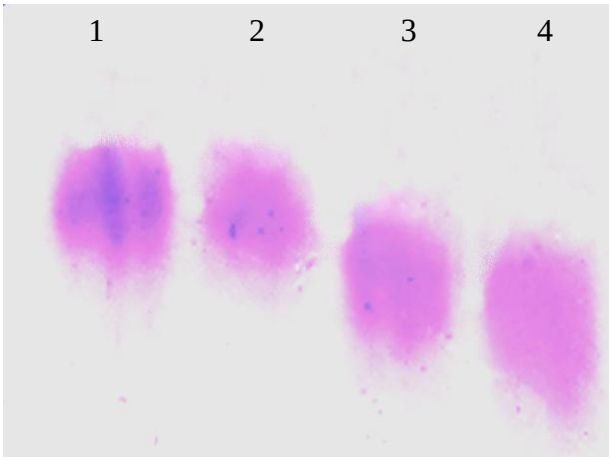

Figure 3.2 Agarose gel electrophoresis of (minimally and extensively) modified and unmodified LDL. Coomassie blue stain; Lane 1: LDL, lane 2: mmLDL, lane 3: oxLDL1 (oxidized with 25 µM CuSO4), lane 4: oxLDL2 (oxidized with 50 µM CuSO4). Protein amount per lane 10 µg.

3.2 POVPE-BY

3.2.1 Stability of POVPE-BY in phosphate buffer and (mm)LDL

Figure 3.3, panel A shows the analysis of a pure POVPE-BY reference sample by two dimensional TLC.

In order to analyze lipid stability in PBS, POVPE-BY (10µM) was incubated in this buffer (4 ml) for different time periods (5, 10, 30 and 60 minutes, respectively) followed by lipid extraction. TLC analysis (Figure 3.3, C) demonstrates that POVPE-BY was stable in PBS under these conditions.

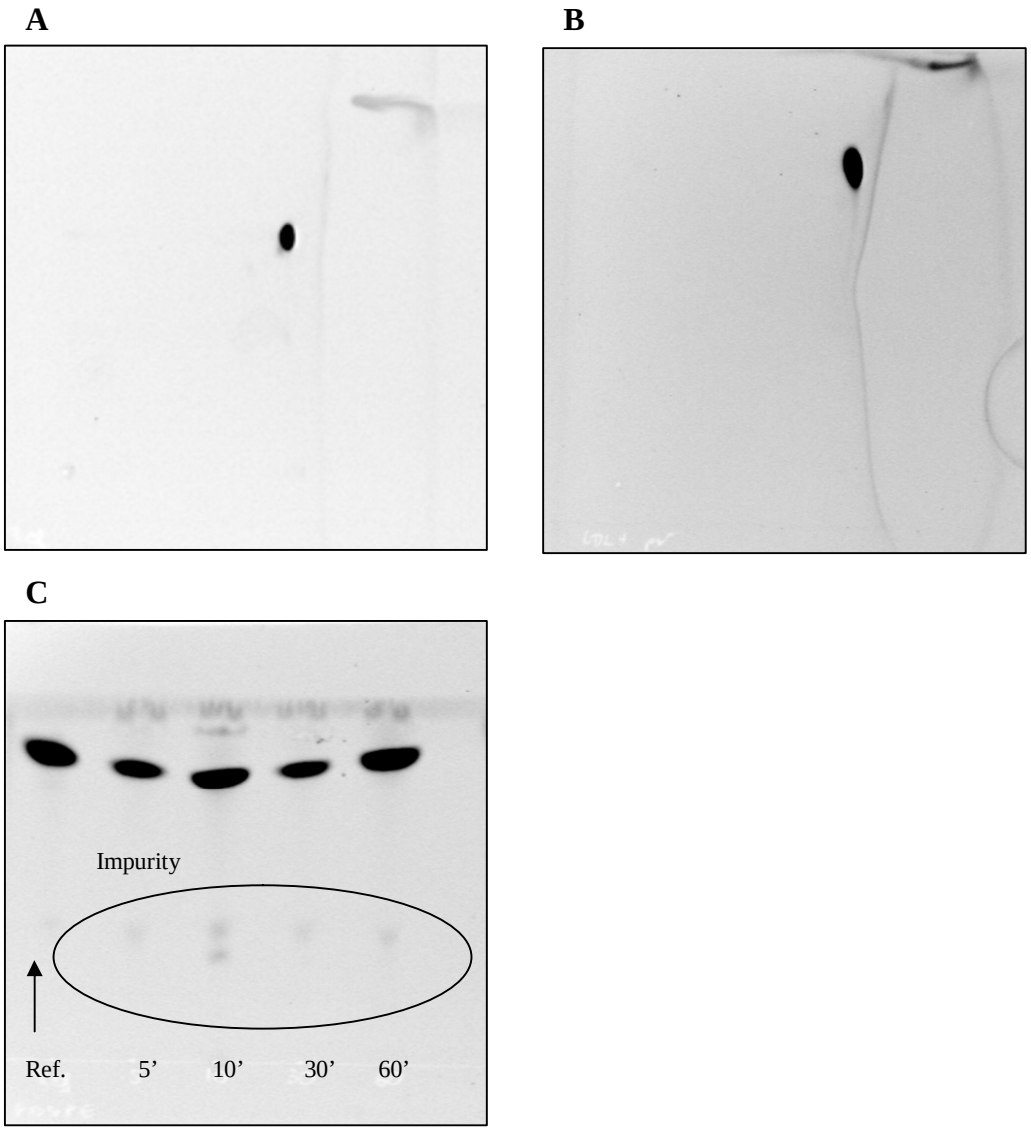

Figure 3.3 Purity and stability of POVPE-BY in PBS and in LDL. A: Labeled phospholipid solubilized in CHCl3-MeOH 2/1 (v/v) was applied onto a silica plate for 2-D TLC. **B**: POVPE-BY- labeled LDL was incubated in PBS for 30 minutes followed by lipid extraction. Lipid extracts solubilized in CHCl3-MeOH 2/1 (v/v) were applied onto a silica plate for 2-D TLC. **C:** POVPE-BY (10 µM) was dispersed in PBS under stirring followed by incubation for different times. Lipid was extracted and applied onto silica plate for1-D TLC. TLC analysis of the POVPE-BY was visualized with a fluorescence imager (Herolab, UV Light 365 nm). For solvents, see Materials and Methods.

Furthermore, LDL (50 µg/ml) was labeled with POVPE-BY (5 µM) for 30 minutes followed by lipid extraction and TLC analysis. Results (Figure 3.3, B) confirm the stability of POVPE-BY LDL under these conditions.

The same procedure was carried out with mmLDL as a carrier for oxPL (data not shown) showing the same stability of the fluorescent aldehydolipids.

It is remarkable that most of the POVPE-BY can be re-extracted from labeled (mm)LDL despite the fact that it can form conjugates with aminolipids and proteins. This effect might be due on the one hand to the large (excess) amount of labeled POVPE-BY and on the other hand to the high instability of the imines bonds around pH 7 (lipid removal from equilibrium).

3.2.2 Uptake of POVPE-BY from lipid micelles and LDL into RAW 264.7 cells

To study the uptake of POVPE-BY from lipid micelles, LDL and mmLDL in RAW 264.7 macrophages, the cells were incubated with the lipid donors followed by solvent extraction of free lipids from the cells and the incubation medium.
Figure 3.4 shows that after 30 minutes POVPE-BY formed lipid-lipid adducts in mmLDL. Panel B also shows the presence of such adducts in the lipid extract of the cells. Figure 3.4, panel A shows that the lipid extract from the supernatant contains most of the POVPE-BY (not taken up by the cells) and that only one of the adducts was released by the cells.

Figure 3.4, panel C.1 shows a fluorescent image of the cellular lipid fraction after incubation with mmLDL. The iodine stain (panel C.2) shows the total cellular lipids in the spot.

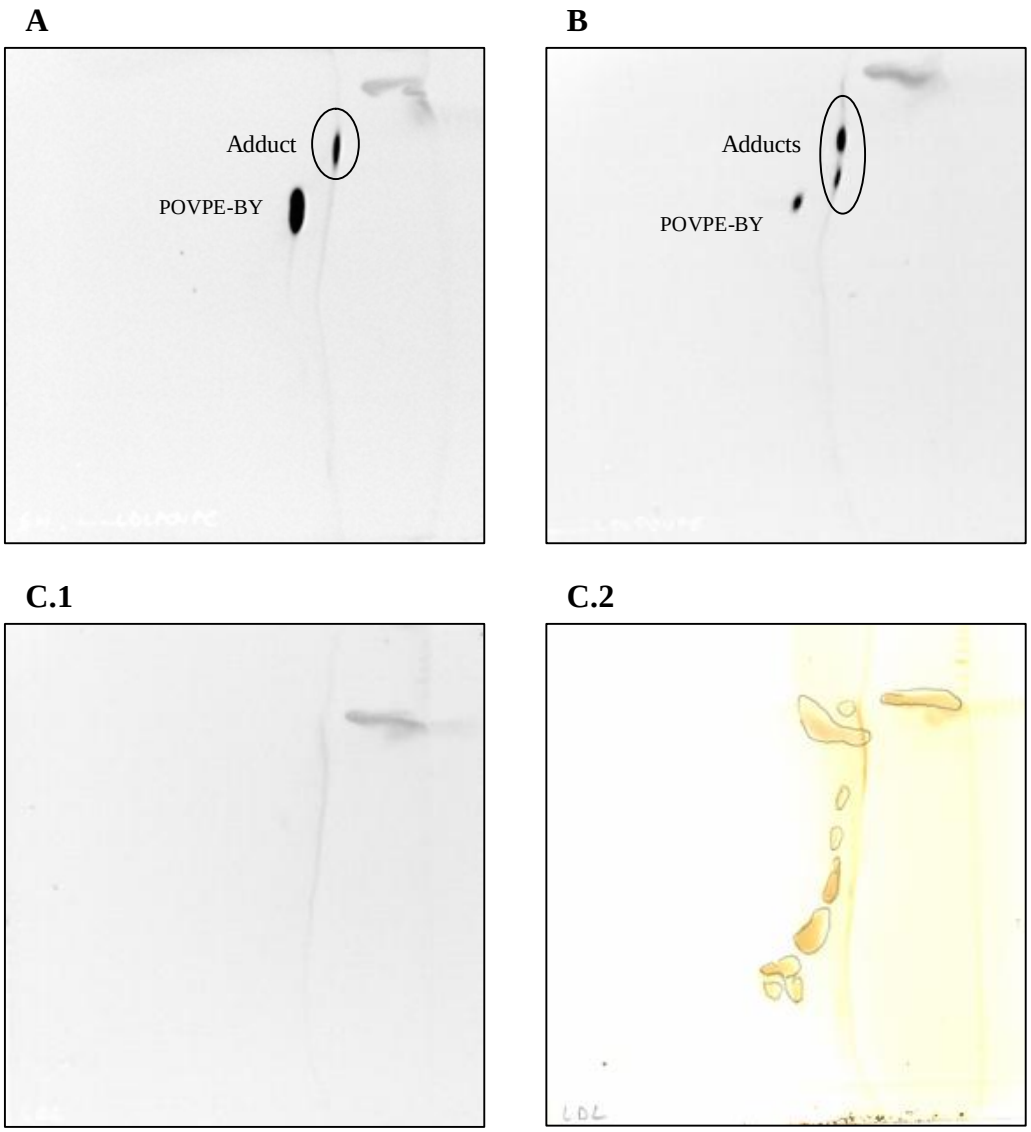

Figure 3.4 Uptake of POVPE-BY from mmLDL into macrophages. TLC analysis of organic lipid extracts. Fluorescence analysis of lipid components. mmLDL (50 µg protein/ml) was loaded with POVPE-BY (5 µM) followed by incubation with cultured cells for 30 minutes. The supernatant was separated from the cells and lipid was extracted from both the supernatant and the cells. Both lipid extracts were separated by 2-D TLC and fluorescence labeled lipids were visualized with a Herolab (UV Light 365) fluorescence scanner. **A:** Fluorescent lipid from supernatant, **B:** Fluorescent lipid from cells. Negative controls: lipids extract from cells preincubated with unlabeled mmLDL. **C.1:** Fluorescence image. **C.2:** Lipid staining by iodine.

We used fluorescence microscopy to find out whether the carrier of POVPE-BY influences lipid uptake by RAW macrophages. The cells were incubated with the following donor systems for 30 minutes: lipid micelles POVPE-BY (Figure 3.5, B), POVPE-BY-labeled mmLDL (Figure 3.5, C) and POVPE-BY-labeled LDL (Figure 3.5, D).

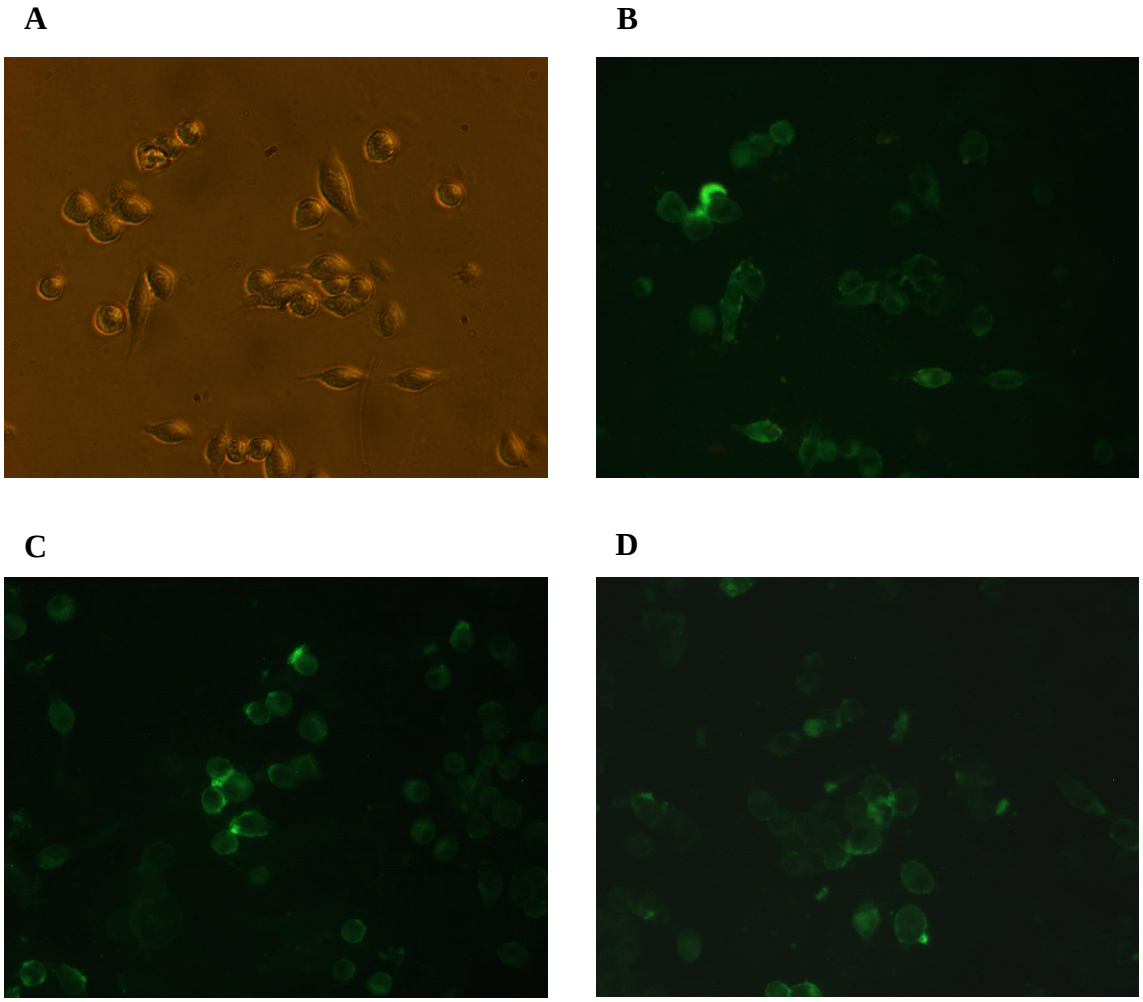

Figure 3.5 Uptake of fluorescent POVPE-BY from lipid micelles and LDL into RAW 264.7 macrophages. Fluorescence images of RAW 264.7 macrophages (Zeiss Axiovert 35). **A:** Light transmission image of cultured cells. **B-D:** Fluorescence micrographs of cells preincubated with POVPE-BY solubilized by different carriers (B, micelles), (C, mmLDL and D, LDL) in PBS for 30 minutes. Fluorophore concentration was 5 µM. Phospholipid to label ratio in LDL was 20/ 1 (mol/ mol). Ex and Em wavelengths were 450-490 nm and 520 nm, respectively.

Cultured macrophages basically showed very similar fluorescence patterns, irrespective of the donor, indicating that POVPE-BY mainly localizes to the plasma membrane. The most intense staining was seen, when the cells were incubated with pure lipid suspensions because the entire amount of lipid is a available for transfer to the cell surface.

3.3 PGPE-BY

3.3.1 Stability of PGPE-BY in phosphate buffer and (mm)LDL

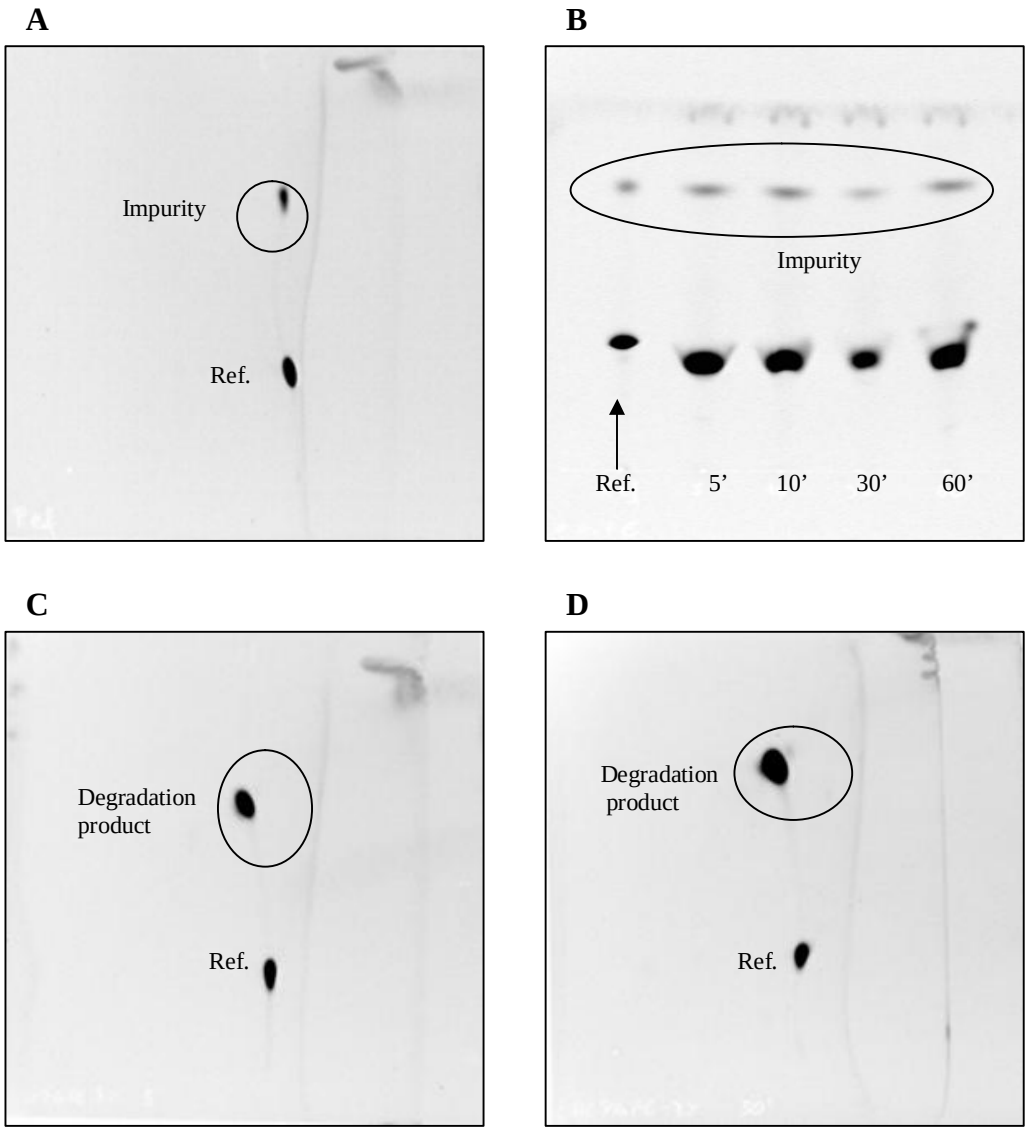

Figure 3.6 Stability of PGPE-BY in PBS and LDL (desalted with PD-10 Column in PBS). A: Labeled phospholipid (PGPE-BY) solubilized in $CHCl_3$-MeOH 2/1 (v/v) was applied onto a silica plate for 2-D TLC. **B:** PGPE-BY (10 µM) was dispersed in PBS under stirring followed by incubation for different times. Lipid was extracted (see Materials and Methods: Lipid Extraction) and applied onto silica plate for 1-D TLC. PGPE-BY- was added to LDL followed by incubation in PBS for 5 minutes (panel C) and 30 minutes (panel D) followed by lipid extraction (see Materials and Methods). Lipid extracts in CHCl3-MeOH 2/1 (v/v) were applied onto a silica plate for 2-D. Lipid spots on the TLC plate (for details see Materials and Methods) was visualized with fluorescence imager (Herolab, UV Light 365 nm).

Figure 3.6, panel B shows the stability of PGPE-BY in PBS. Figure 3.6, panel A shows a two dimensional TLC analysis of pure PGPE-BY reference sample.
LDL was loaded with PGPE-BY for 5 minutes (Figure 3.6, C) and 30 minutes (Figure 3.6, D) respectively, followed by lipid extraction and TLC analysis. PGPE-BY was degraded under these conditions (see degradation Products Figure 3.6, C and D) depending on incubation time. The same degradation was observed after incubation of PGPE-BY with mmLDL.

3.3.2 Uptake of PGPE-BY from lipid micelles and LDL into RAW 264.7

Macrophages were incubated with PGPE-BY (5µM) micelles in PBS for 30 minutes. After incubation, the supernatant was separated from the cells which were scraped into 10 ml of PBS. Lipid were extracted from both fractions with organic solvent followed by two dimensional TLC analysis (Figure 3.7, A and B). Both fractions showed similar degradation products. Three degradation products of PGPE-BY found in the cell extract. According to the spot intensities in panel B (cellular lipid) and panel A (supernatant lipid), more than 50 % of PGPE-BY was taken up by the cells.

In a separate experiment, LDL was loaded with PGPE-BY and the cells were incubated with this donor system for 30 minutes. After incubation, the supernatant and the cells were again separated and lipid was extracted from both fractions followed by two dimensional TLC analysis. More degradation products were found in the cellular fraction than in the supernatant fraction (Figure 3.7, C and D). Obviously, PGPC is particularly unstable as it was degraded both within the carrier (LDL) and inside the cells. A comparison of the fluorescence intensities in A/D and C/D shows that less PGPE-BY is released from LDL than from micelles into the cells during the 30 minutes incubation. However, it has to be emphasized that the LDL-associated PGPE-BY has already been degraded to a large extent before uptake. Therefore, we conclude that LDL rather delivers free the oxPL degradation products than the intact oxPL to the cell.

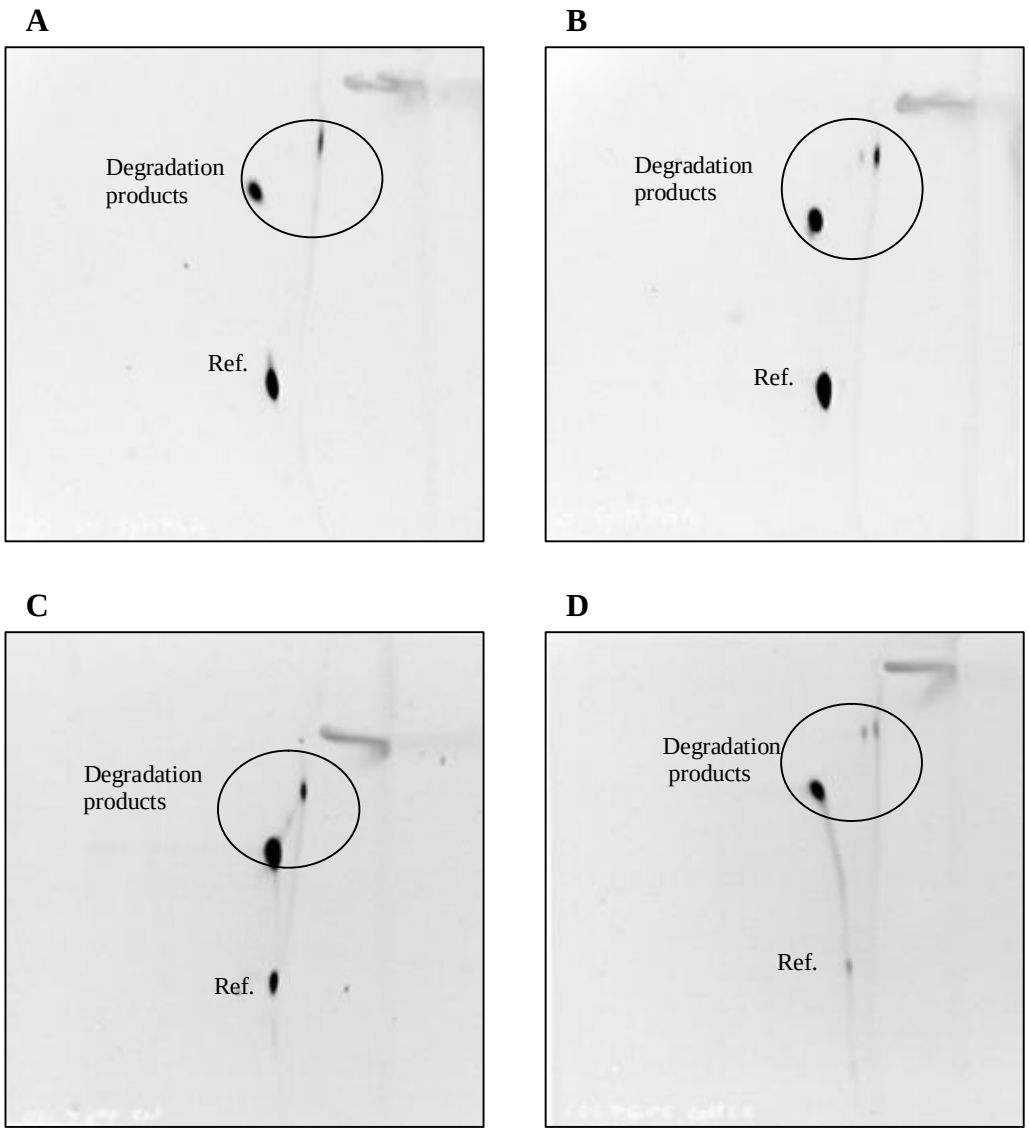

Figure 3.7 Uptake of PGPE-BY from lipid micelles and LDL into macrophages. Fluorescence analysis of lipid components. PGPE-BY (5 µM) was dispersed in PBS incubated with macrophages for 30 minutes (A and B). LDL (50 µg protein/ml) was loaded with PGPE-BY (5 µM) for 30 minutes followed by incubation with cultured cells (C and D). The supernatants and the cells were separated and lipid was extracted (see Materials and Methods, Lipid Extraction) from both fractions. Both lipid extracts were separated by 2-D TLC (see Materials and Methods) and fluorescently labeled lipids were visualized with a Herolab fluorescence imager (UV Light 365). **A and C:** Fluorescence lipid from supernatants. **B and D:** Fluorescence lipid from cells.

Fluorescence microscopy was used to monitor the uptake of PGPE-BY by live cells. Figure 3.8, panel A shows the incorporation of PGPE-BY from lipid micelles. Figure 3.8, panels B and C show the uptake of PGPE-BY from (mm)LDL. In all cases, fluorescent lipid was detected inside the cells as well as in the plasma membrane. Again, the amount of PGPE-BY released into the cells from LDL is lower than from lipid micelles.

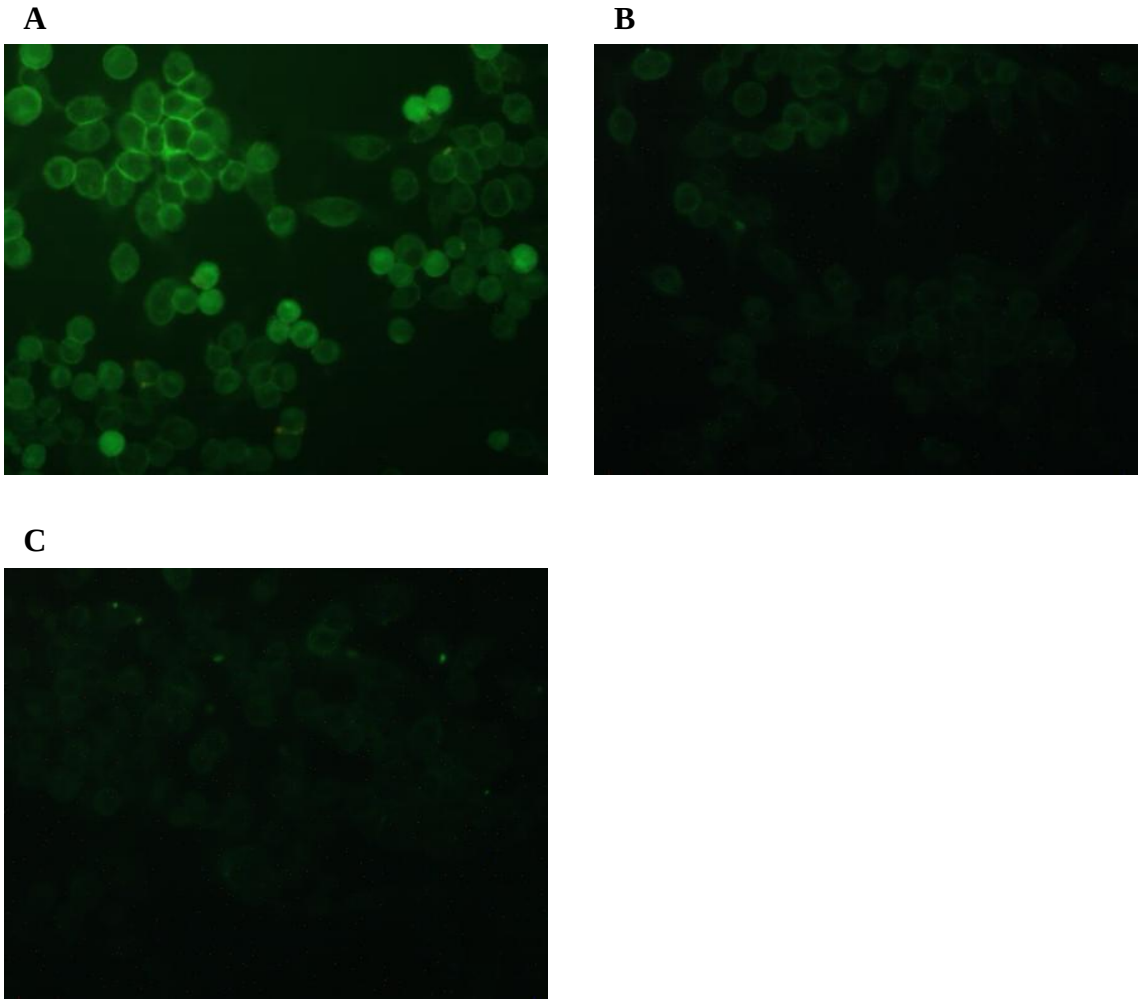

Figure 3.8 Uptake of fluorescent PGPE-BY from lipid micelles, mmLDL and LDL into RAW 264.7 macrophages. Fluorescence Images of RAW 264.7 macrophages (Zeiss Axiovert 35). **A-C:** Fluorescence micrographs of cells preincubated with PGPE-BY solubilized by different carriers (A, micelles), (B, mmLDL and C, LDL) in PBS for 30 minutes. Fluorophore concentration was 5 µM. Phospholipid to label ratio in LDL was 20/ 1 (mol/ mol). Ex and Em wavelengths were 450-490 nm and 520 nm, respectively.

3.4 Effects of Triton X-100 on fluorescence staining of cultured cells with POVPE-BY and PGPE-BY.

To investigate the localization of oxidized phospholipid after uptake into macrophages we made an attempt to re-extract the compounds from the cultured cells using 0.1% Triton X-100 diluted in PBS (ph 7.4). For this purpose, the cells were preincubated with 10 µM POVPE-BY and PGPE-BY for 5 and 30 minutes, respectively, followed by washing with PBS and an additional

incubation with Triton X-100 for 5 seconds. Afterwards, the cells were rinsed with PBS and immediately observed using a fluorescence microscope.

3.4.1 POVPE-BY

Figure 3.9 panel A shows a fluorescence micrograph of the cells after incubation with POVPE-BY for 30 minutes. Panel B shows the same cells after a Triton wash (fluorescence photographs were taken at the same illumination intensity). Since the fluorescence was much weaker after washing with Triton, we conclude that Triton extracted most of the POVPE-BY from the plasma membrane (see previous chapter).

Figure 3.9 shows transmission light images of cells before and after the Triton wash (panels C and D, respectively). The cells dramatically changed their appearance under the influence of the detergent. It is likely that they are destabilized under these conditions and lipid accessibility becomes higher (POVPE-BY extraction see also previous chapter). The same effects were seen after incubation of the cells with POVPE-BY for 5 minutes followed by washing with Triton X-100 (data not shown).

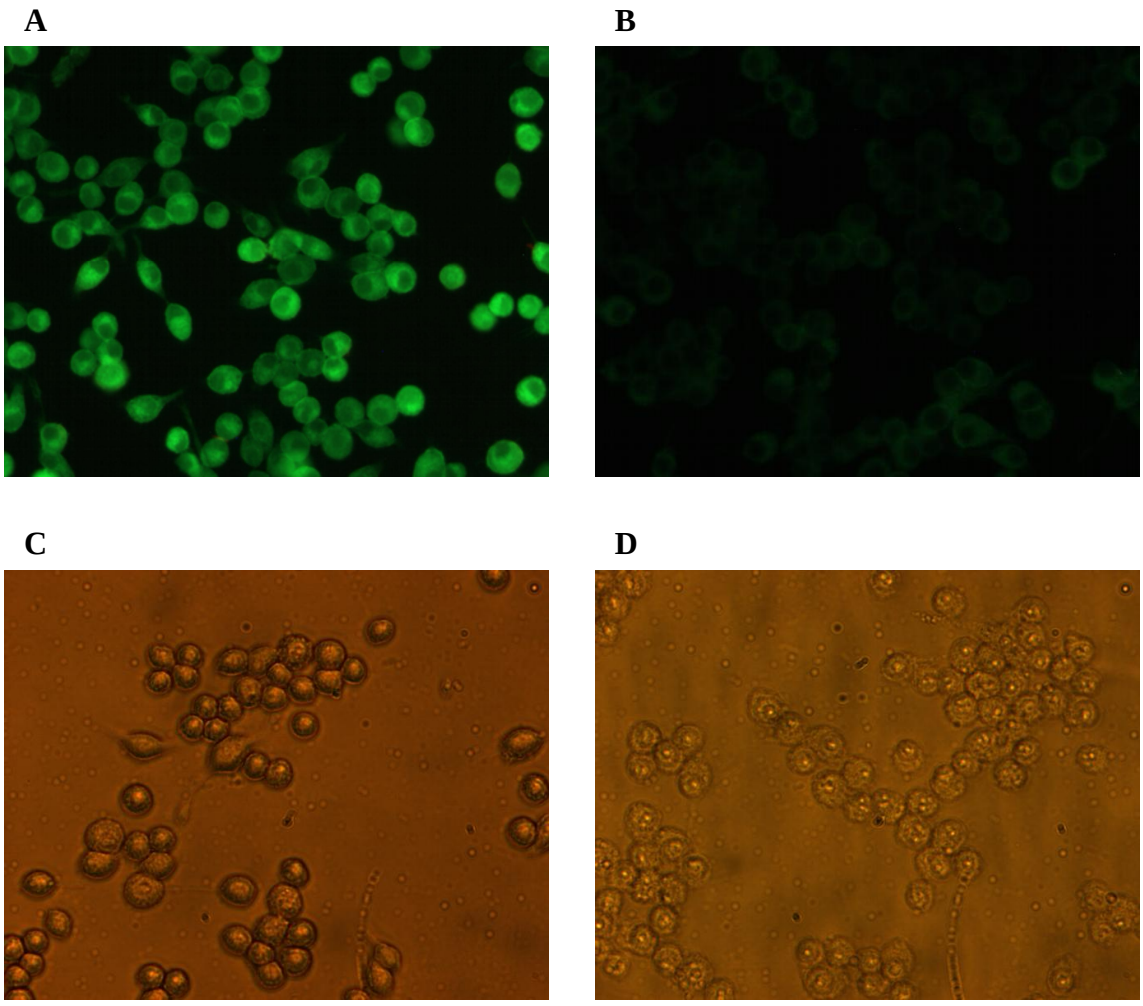

Figure 3.9 Effect of Triton X-100 on fluorescence staining of cultured macrophages with POVPE-BY. Fluorescence micrographs (A, B) and light transmission images (C, D) of POVPE-BY- labeled cells before and after washing with 0.1% Triton X-100. Cells were incubated with 500 µl of 10 µM POVPE-BY for 30 minutes (A and C) followed by washing with Triton X-100 two times (B and D). Fluorescence Ex and Em wavelengths were 450-490 nm and 520 nm, respectively.

3.4.2 PGPE-BY

Figure 3.10 panels A and B show fluorescence micrographs of cells before (5 minutes preincubated with PGPE-BY) and after a Triton wash respectively. Again, there is much weaker fluorescence in the cells after washing with Triton. The fluorescence in Figure 3.10 panel B only represents internalized PGPE-BY. The surface-bound fluorescence is no longer seen. Very similar results were observed after pre-incubation of the cells with PGPE-BY for 30 minutes followed by washing with Triton X-100 (Figure 3.10, C and D).

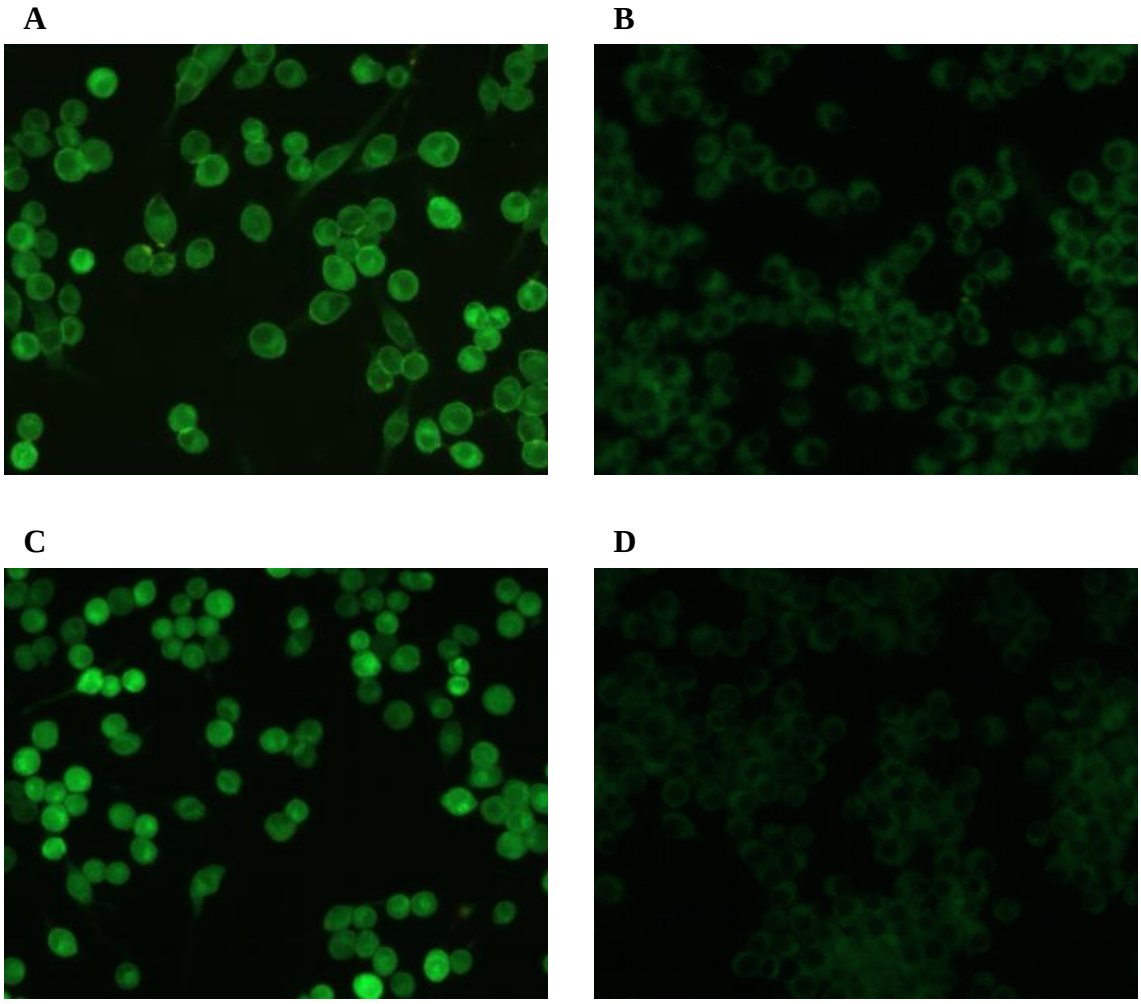

Figure 3.10 Effect of Triton X-100 on fluorescence staining of cultured macrophages with PGPE-BY. Fluorescence micrographs of PGPE-BY-labeled cells before and after washing with 0.1% Triton X-100. Cells were incubated with 500 µl of 10 µM PGPE-BY for 5 minutes (A and B) and for 30 minutes (C and D) followed by washing with Triton X-100 (B and D). Fluorescence Ex and Em wavelengths were 450-490 nm and 520 nm, respectively.

3.5 Lipid-protein interactions. Lipid exchangeability.

To find out whether POVPE-BY can be transferred from LDL to the other proteins, we labeled the lipoprotein with the fluorescent lipid and incubated the fluorescent sample with various amounts of albumin as an acceptor. POVPE-BY was transferred from LDL to albumin in an albumin concentration-dependent manner. The fluorescence protein complexes were detectable before and after stabilization of the Schiff bases with 50 µM $NaCNBH_3$.

Figure 3.11, panel A lane 9 shows the formation of a fluorescent BSA-POVPE-BY complex. Its protein molecular weight is 66 kDa (Figure 3.11, B, Sypro Ruby stain). Figure 3.11, A further demonstrates that the fluorescent protein band increases at higher BSA/LDL ratios. Obviously, higher concentrations of BSA bound more POVPE-BY, supporting the conclusion that POVPE-BY is exchangeable between LDL and BSA.

We also studied lipid exchange between LDL and albumin after stabilizing POVPE-BY Schiff bases in the LDL donor with 50µM $NaCNBH_3$ (incubated for 3h). Experiments were preformed at different molar albumin/LDL ratios. Under these conditions, less POVPE-BY was transported to the albumin (see lanes 5, 6 and 7 (Figure 3.11, panel A)). Nevertheless, it is striking that some POVPE-BY is still released after LDL treatment with $NaCNBH_4$. Obviously, there was no quantitative reduction of the Schiff bases under the reaction conditions used.

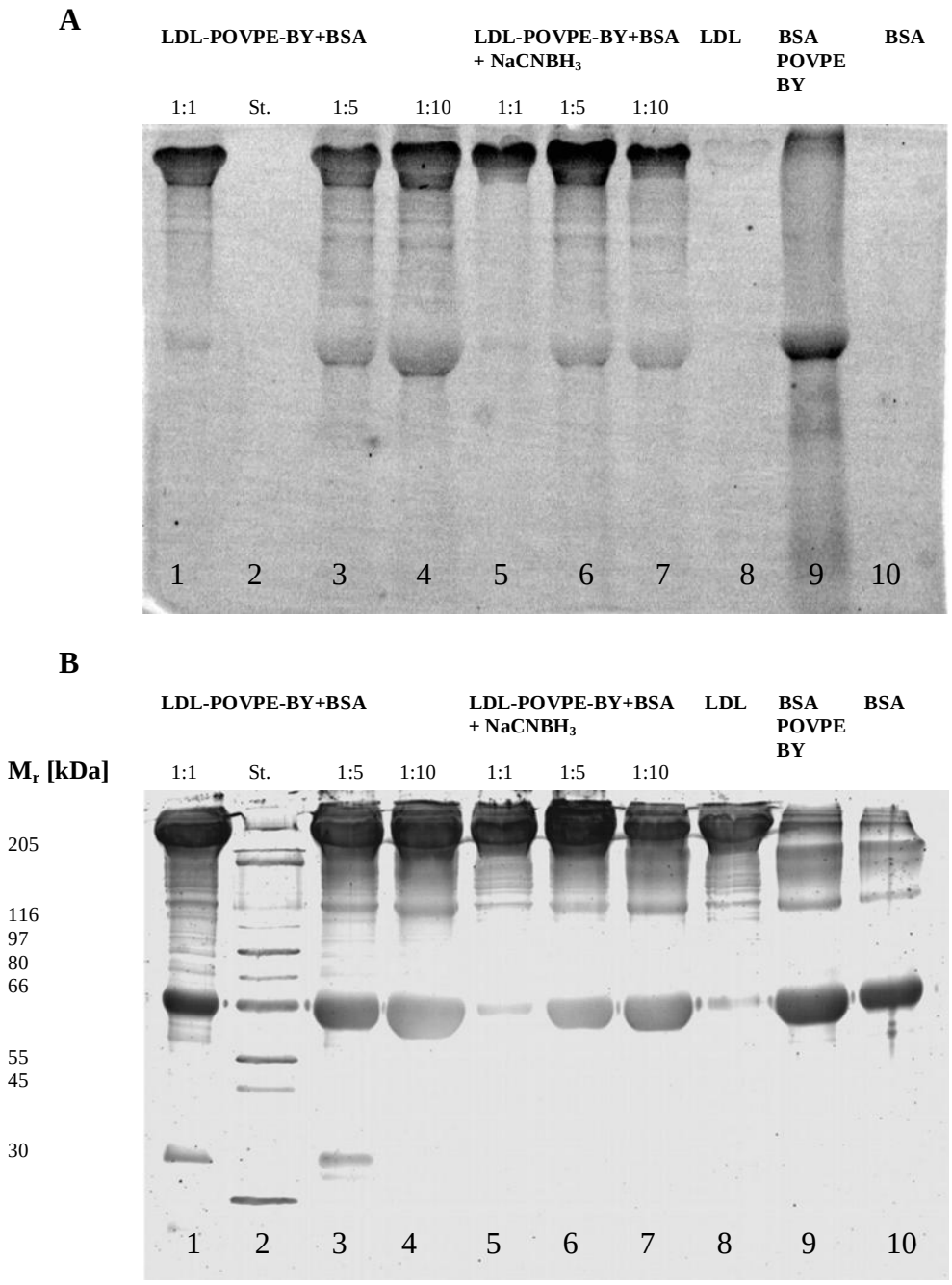

Figure 3.11 Transfer of POVPE-BY from LDL to albumin [Data from a joined experiment by U. Stemmer and B. Stojčić]. LDL was loaded with POVPE-BY (20/1, mol phospholipid/mol label) followed by incubation with different amounts of albumin in PBS at room temperature for 30 minutes. Proteins were precipitated with methanol/chloroform according to Wessel and Fluegge and separated by SDS gel electrophoresis (20 µg/ lane). Lipid transport from LDL to albumin was determined before and after stabilization of Schiff bases in LDL with 50 µM $NaCNBH_4$ for 3 h. Protein bands were visualized by fluorescence detection: **A:** Lipid stain (POVPE-BY). **B:** Protein stain (SyproRuby). The LDL (apoB) concentration in the incubation mixture was 0.95 µg/ml. Ratios of LDL (apoB) to albumin (mol/ mol) were as follows: Lane 2: reference proteins; Lane 1: 1/1; Lane 3: 1/5 ; Lane 4: 1/10 ; Lane 5: 1/1 LDL stabilized with NaCNBH3; Lane 6: 1/5 LDL stabilized with NaCNBH3; Lane 7: 1/10 LDL stabilized with NaCNBH3; Lane 8: LDL; Lane 9: BSA/POVPE-BY complex 1/1 (mol/mol); Lane 10: BSA. For Ex and Em of Bodipy and SyproRuby, see **Table 2.3**.

4 Discussion

The onset of atherosclerosis is associated with the increase of modified LDL in the vessel wall followed by the innate response of macrophages [10]. Macrophages take up modified LDL and form foam cells. Modified LDL was also shown to stimulate the inflammatory activity of macrophages [1]. Modified lipids which are components of modified LDL are involved in the development of atherosclerosis. The oxidation products of PAPC such as POVPC and PGPC are among the most important biologically active lipids. Both oxidized phospholipids accumulate in atherosclerotic lesions [8] and are responsible for the pathophysiologic actions of oxLDL, triggering immune response, inflammation, apoptosis and the underlying signaling pathways [9].

The aim of our study was to show how these oxidized phospholipids are delivered to the cells by different carriers including modified LDL, which can be isolated from atherosclerotic plaques or can be prepared by oxidation *in vitro.*

Therefore, we investigated the uptake of oxidized phospholipids from lipid micelles and phospholipid-labeled LDL by cultured murine macrophages. For this purpose oxidized phospholipids labeled with Bodipy TM were used as fluorescent lipid analogs. The Bodipy TM fluorophore was chosen for labeling of oxPL because of its high fluorescence yield and photostability [4]. POVPE-BY and PGPE-BY were used as fluorescent POVPC and PGPC analogs, respectively, at very low concentrations to avoid self-quenching effects.

POVPE-BY and PGPE-BY were rapidly taken up into the plasma membrane and/or the cell interior. Incubations with the cells were preformed in PBS to avoid interference with serum lipid components including phospholipases. It has been shown in previous studies that both phospholipids are entirely hydrolyzed in FCS within a few hours. After 1 h, already 50% of the oxidized lipids were degraded in FCS [4]. In contrast, both POVPE-BY and PGPE-BY showed long term stability in PBS (Figure 3.3, C and Figure 3.6, B).

Phospholipid-labeled (mm)LDL showed a different behavior depending on the constituent oxPL. POVPE-BY was stable in LDL (Figure 3.3, B) whereas LDL-associated PGPE-BY was efficiently hydrolyzed. The respective lysophospholipid was the only fluorescent degradation product [4] (Figure 3.6, C and D).

It was the intention of our study to determine the role of the oxPL carrier in cellular uptake and to find out whether LDL internalization is required for lipid uptake. Therefore, we cultured the cells in the presence of 10 % FCS in order to suppress expression of the LDL receptor and to minimize the possibility that entire lipoprotein particles are internalized.

Fluorescence microscopy provided evidence that both oxPLs are taken up from lipid micelles by macrophages within 5 minutes localizing to the plasma membrane (POVPE-BY and PGPE-BY) and the cell interior (mainly PGPE-BY). After 30 minutes incubation, PGPE-BY is only seen inside the cells, preferentially in the lysosomes [4]. On the other hand, POVPE-BY stays captured in the plasma membrane, presumably as a consequence of the formation of covalent adducts with free amino and sulfhydryl groups of proteins and phospholipids [4]. There was no effect of the delivery system on oxPL uptake into the cells. POVPE-BY-labeled LDL and micelles led to the same fluorescence staining of macrophages (Figure 3.5, B-D). On the other hand, LDL-associated PGPE-BY showed lower lipid uptake rates than the micelle bound phospholipid (Figure 3.8, A-C).

To determine the amount of oxidized phospholipid, that is only loosely associated with the cells [4], labeled macrophages were treated with 0.1% Triton solutions. Specifically, cells were labeled with POVPE-BY- and PGPE-BY and treated with 0.1% Triton for 5 seconds which is enough for extraction of lipids from the plasma membrane. After the Triton wash, we still found considerable PGPE-BY fluorescence inside the cells. Whereas POVPE-BY was still mainly detectable in the plasma membrane, very likely due to the Schiff base formation with proteins and lipids [6,7].

Analysis of lipid extracts from cells after incubation with POVPE-BY-labeled LDL points to more than one “adduct” (Figure 3.4, B). Lipid extracts from the supernatants just contained one “adduct” (Figure 3.4, A). The chemical structure of these compounds is still subject to identification. They can be covalent conjugates of the fluorescent oxPL with aminophospholipids. In addition, we have to take into account that aldehydolipids might undergo aldol self-condensation under certain circumstances [5].

Lipid extracts from cells incubated with PGPE-BY and PGPE-BY-labeled LDL showed hydrolytic degradation products (Figure 3.7) [4]. These lipids may be due at least to two enzymatic processes. On the one hand, PGPE-BY is subject to hydrolysis by intracellular phospholipases, including PAF- acetylhydrolase [4]. On the other hand, the oxPL is already degraded by the phospholipases in LDL (e.g. extracellular PAF- acetylhydrolase) followed by transfer of the degradation product(s) to the cells [3]. Thus, transfer and metabolic stability of PGPE-BY inside and outside cells is a complex process involving various molecular species that ultimately may trigger the individual cell response.

Finally we addressed ourselves to the question, whether the POVPE-BY which shows covalent reactivity with amino groups of lysine and sulfhydril groups [4] would exchange not only

between the individual carriers and the cells but between the carriers themselves also (Figure 3.11). We found that POVPE-BY was transferred from LDL to albumin and vice versa. This phenomenon can be explained by the fact that Schiff bases between lysines and lipid aldehyds are unstable around their pKs (~7) [43] and thus may be released and bound again. If the lipid conjugates are stabilized by $NaCNBH_4$ reduction, their exchangeability is abolished.

We were able to demonstrate that LDL and mmLDL may act as delivery systems for the transport of oxidized phospholipids (POVPC and PGPC) into murine macrophages. Depending on their chemical reactivates towards lipids and proteins they showed different characteristics of internalization. Since POVPC is able to form Schiff bases with amino groups of lysine it was mainly found in the plasma membrane even after prolonged incubation time. In contrast, PGPC can only physically interact with lipids and proteins. Thus it easily passes through the plasma membrane ending up in intracellular membranes. Since it does not form covalent conjugates with other membrane components it is subject to efficient degradation by hydrolytic enzymes (phospholipase).

Summarizing our observations, we may conclude on the one hand POVPC mainly elicits its biological effects as a conjugate. In order to understand the cellular activities of PGPC, we have to take into account that multiple molecular species are involved, including the intact oxPL and the products of phospholipase-mediated hydrolysis (phospholipase).

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