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21-Methylpyrenyl-cholesterol stably and specifically associates with lipoprotein peripheral hemi-membrane: A new labelling tool

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ABSTRACT

Lipoproteins are important biological components. However, they have few convenient fluorescent labelling probes currently reported, and their physiological reliability can be questioned. We compared the association of two fluorescent cholesterol derivatives, 22-nitrobenzoxadiazole-cholesterol (NBD-Chol) and 21-methylpyrenyl-cholesterol (Pyr-met-Chol), to serum lipoproteins and to purified HDL and LDL. Both lipoproteins could be stably labelled by Pyr-met-Chol, but virtually not by NBD-Chol. At variance with NBD-Chol, LCAT did not esterify Pyr-met-Chol. The labelling characteristics of lipoproteins by Pyr-met-Chol were well distinguishable between HDL and LDL, regarding dializability, associated probe amount and labelling kinetics. We took benefit of the pyrene labelling to approach the structural organization of LDL peripheral hemi-membrane, since Pyr-met-Chol-labelled LDL, but not HDL, presented a fluorescence emission of pyrene excimers, indicating that the probe was present in an ordered lipid micro-environment. Since the peripheral membrane of LDL contains more sphingomyelin (SM) than HDL, this excimer formation was consistent with the existence of cholesterol- and SM-enriched lipid microdomains in LDL, as already suggested in model membranes of similar composition and reminiscent to the well-described "lipid rafts" in bilayer membranes. Finally, we showed that Pyr-met-Chol could stain cultured PC-3 cells via lipoprotein-mediated delivery, with a staining pattern well different to that observed with NBD-Chol non-specifically delivered to the cells.

1. Introduction

Physiologically, lipoproteins fulfill the role of systemic shuttles in biological fluids for lipids delivery to target cells via specific receptors. Lipoproteins are multi-molecular assemblies that associate various lipids with apolipoproteins maintaining their structural stability, and they are usually classified according to their density, corresponding to various lipid/protein ratios [1]: the main ones are LDL (low-density lipoproteins, size 18–25 nm) and HDL (high-density lipoproteins, 5–12 nm). It is widely admitted that the lipoprotein core is made of the most hydrophobic lipids, essentially triglycerides and esterified cholesterol, while the particle periphery is a monolayer of less hydrophobic lipids, mainly phospholipids and unesterified cholesterol [2]. However, the structural features of lipoproteins periphery are rather poorly described, in particular regarding the lipid molecules arrangement. Among the phospholipids, sphingomyelin (SM) content is higher in LDL than in HDL (24% and 10% mol/mol, respectively [3]), which can have significant consequences on the structural organization of their peripheral hemi-membrane, in particular in relation with the presence of cholesterol. Indeed, in bilayer biological membranes, cholesterol and SM are known to be the two main components of the membrane microdomains, so-called "lipid rafts" [4].

Abbreviations: HDL, high-density lipoproteins; (V)LDL, (very) low-density lipoproteins; apo, apolipoprotein; NBD, nitrobenzoxadiazole; Pyr-met, methylpyrenyl; Chol, cholesterol; SM, sphingomyelin; LCAT, lecithin:cholesterol acyltransferase.

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It is thus essential to be able to quantify and qualitatively characterize lipoproteins, *in vitro* and *in vivo*. However, few biochemical tools are available, including apolipoprotein radiolabelling, fluorescent lipophilic dyes [5,6] and lipid derivatives [7,8], and immunoassays [9]. Fluorescent markers present many technical and practical advantages over radiolabelled compounds [10], and they also allow imaging approaches [6]. This is illustrated by the wide use of DiI for *in vitro* experiments [10,11], but since this lipophilic dye from carbocyanine family has only a very partial analogy with a physiologic lipid, its use as a lipoprotein marker can be questioned. Indeed, beyond lipoprotein fluorescent labelling, the question of the relevancy of the cell staining obtained is pivotal.

Fluorescent derivatives of cholesterol are useful molecular tools for investigating the structure of biological lipid membranes. Among them, 21-methylpyrenyl-cholesterol (Pyr-met-Chol) and 22-nitrobenzoxadiazole-cholesterol (NBD-Chol) have been previously characterized on model bilayer membranes, and they were shown to present contrasting behaviors. Indeed, Pyr-met-Chol can insert into model bilayers just like cholesterol does with respect to its intramembrane distribution [12], whereas NBD-Chol inserts into them with a behavior largely differing from that of cholesterol [13,14], in particular not preserving the raft-type membrane microdomains. We thus investigated the respective interactions of these two molecular probes with lipoproteins, particularly HDL and LDL.

Here we report that (i) Pyr-met-Chol, at variance with NBD-Chol, can stably label serum lipoproteins without being esterified by LCAT, (ii) Pyr-met-Chol labelling is quantitatively and qualitatively distinguishable between HDL and LDL, (iii) the pyrene group senses an ordered membrane environment in Pyr-met-Chol-labelled LDL but not HDL, and (iv) Pyr-met-Chol-labelled lipoproteins can be used for staining cultured cells.

2. Materials and methods

2.1. Chemicals

Pyr-met-Chol was a kind gift from Dr Lopez (CNRS, France; patent WO/2006/100388), NBD-Chol (22-nitrobenzoxadiazole-cholesterol) was from Sigma-Aldrich, and stock solution were solubilized in chloroform/methanol (9:1 and 2:1, respectively).

2.2. Fluorescent cholesterol association to lipoproteins

F12 or DMEM cell culture medium containing 10-20% fetal calf serum was incubated with Pyr-met-Chol or NBD-Chol, each at 5 μM, for 48 hours at room temperature under gentle stirring and protection from light. Separation of the serum components was performed on a sepharose molecular sieve column (SuperoseTM6 10/300GL) with simultaneous recordings of emitted fluorescence (Pyr-met-Chol: excitation at 330 nm and emission at 385–400 nm; NBD-Chol: 490 nm and 535–560 nm respectively) and protein UV absorption (at 280 nm). LCAT activity was determined on human or fetal calf serum incubated for 48–72 h with either Pyr-met-Chol or NBD-Chol (20 μM).

Purified LDL and HDL were prepared from plasma of healthy donors by ultracentrifugation (120,000*g*, 4 °C, 66 h) onto a KBr layer of density 1.06 and 1.19, respectively. For fluorescent labelling, 1-7 mg/ml of lipoprotein was incubated with Pyr-met-Chol (using ethanol as a vehicle), either at 10 μM or at 5% mol/mol of the non-esterified cholesterol present in the lipoprotein fraction, corresponding to 20–50 μM for HDL and 130–300 μM for LDL, under gentle stirring and protection from light. Labelled lipoproteins were then submitted to ultracentrifugation in KBr (120,000*g*, 4 °C, 66 h), to eliminate unbound fluorescence, and then dialyzed using a semi-permeable membrane with 10 kDa cutoff, to eliminate KBr and weakly bound fluorescence. Bound Pyr-met-Chol fluorescence was measured by a fluorometric assay, using a Varioskan Flash fluorescence plate reader (Thermo-Electron), at 330 and 400 nm for excitation and emission, respectively. Protein concentration was determined by the Bradford protein assay, and cholesterol assay was performed using the Biolabo colorimetric kit. Quantitative experiments have been performed two to four times, each point being in triplicate.

To analyze the stability of fluorescent labelling, apoB-containing lipoproteins were precipitated with the phosphotungstene-containing Randox Laboratories kit, according to the manufacturer recommendations, and the fluorescence remaining in the supernatant was measured.

2.3. Fluorescent labelling of oxidized lipoproteins

Purified LDL (2–3 mg/ml) were oxidized by incubation with 5 μM $CuSO_4$ during 4 or 8 h at room temperature, and then fluorescently labelled by incubation with 10 μM Pyr-met-Chol according to the standard procedure. It is worth noting that Pyr-met-Chol labelling of oxidized lipoproteins was performed after the oxidating incubation, in contrast to other procedures reported in the literature that monitored the oxidation process by taking benefit of the simultaneous oxidation of the fluorescent probe [15,16].

2.4. Spectroscopic analysis of fluorescently labelled lipoproteins

For the spectroscopic analysis of Pyr-met-Chol associated to lipoproteins, we used labelled purified lipoproteins diluted to 0.9 mg/ml for HDL and 0.4 mg/ml for LDL, and analyzed by recording fluorescence emission spectra (excitation at 335 nm) on an Aminco500 or a QuantaMaster-4 spectrofluorometer.

2.5. Cell culture, fluorescent staining and microscopy imaging

PC-3 cells [17], provided from ATCC, were cultured in Ham's F12 medium with 10% fetal calf serum and penicillin (100 U/ml) plus streptomycin (10 μg/ml) (Invitrogen) at 37 °C in 5% CO_2. PC-3 cells were seeded onto glass slides put in wells, at 250,000 per well in 12-well plastic plates, and cultured until confluency, washed and then incubated with 5 μM of both Pyr-met-Chol and NBD-Chol, using ethanol as a vehicle, for 48 h. Pyr-met-Chol and NBD-Chol were observed using a two-photon excitation (TPE) LSM 7MP Zeiss (Carl Zeiss, Germany) microscope equipped with a Chameleon Ultra II Ti:sappire laser (Coherent, CA, USA). Pyr-met-Chol was excited at 710 nm (TPE), and emission was recorded using a 370–485 nm band-pass filter; for NBD-Chol, excitation was at 940 nm (TPE), and emission recorded with a 500–550 nm filter. Acquisitions were performed with a 63× oil objective (1.4 NA).

3. Results

3.1. Preferential association of Pyr-met-Chol over NBD-Chol to serum lipoproteins

We first evaluated the capacity of Pyr-met-Chol to associate to serum components by following their separation on sepharose with the simultaneous detection of fluorescence emission and protein content. When incubated for 48 h in the presence of 20% fetal calf serum, Pyr-met-Chol (5 μM) was found in all three major lipoprotein fractions: VLDL, LDL and HDL (Fig. 1A). Part of the fluorescence was also found in the peak corresponding to albumin, the major protein component of serum. In contrast to Pyr-met-Chol, NBD-Chol associated only marginally with the HDL peak among

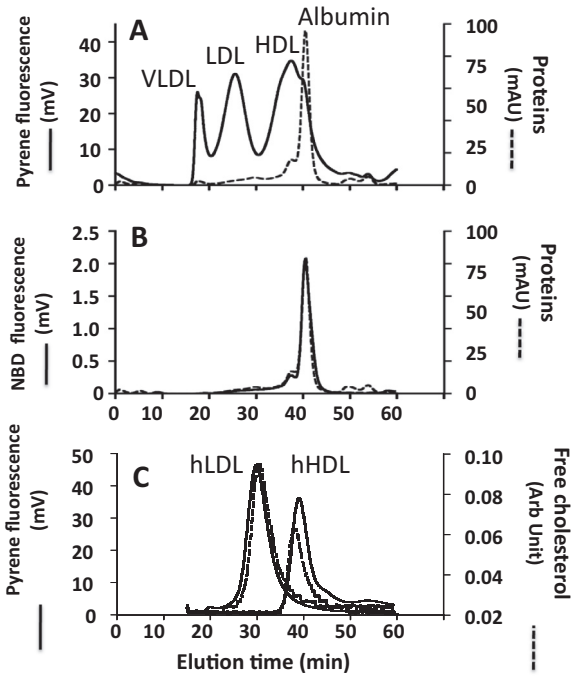

Fig. 1. Labelling of serum components with Pyr-met-Chol or NBD-Chol. Fetal calf serum (20% in DMEM) was incubated for 48 h with 5 μM Pyr-met-Chol or NBD-Chol, and separated on a sepharose column with simultaneous absorption and fluorescence detections : fluorescence emission of pyrene (385 nm, solid line in Panel A) and of NBD (560 nm, solid line in Panel B), and protein absorption (280 nm, dotted lines). Panel C: Pyr-met-Chol labelling of purified lipoproteins. Purified human LDL or HDL were incubated with Pyr-met-Chol (5% mol/mol of non-esterified cholesterol) for 48 h, and, after dialysis, pyrene fluorescence was detected after elution on sepharose (solid lines). On the same column, non-labelled purified human LDL or HDL were eluted and spectrophotometrically detected for their free cholesterol content (dotted lines).

all the lipoproteins, and was mainly found at the level of the albumin peak (Fig. 1B). Human serum lipoproteins gave a similar association profile for Pyr-met-Chol (not shown).

Incubation up to 72 h of Pyr-met-Chol with either human or fetal calf serum did not lead to any esterification of Pyr-met-Chol by LCAT, in contrast to NBD-Chol. Indeed, thin layer chromatography separation of lipid extracts allowed to detect fluorescent spots corresponding to NBD-Chol (r_F = 0.05), to Pyr-met-Chol (r_F = 0.17), and to esterified NBD-Chol (r_F = 0.19), whereas no upper fluorescent spot migrating above Pyr-met-Chol could be detected (not shown). Pyr-met-Chol molecules associated with lipoproteins were thus expected to remain anchored within their peripheral hemi-membrane thanks to the free hydroxyl group.

3.2. Differential association of Pyr-met-Chol with HDL and LDL

Since NBD-Chol virtually did not associate with lipoproteins, we further addressed the association of Pyr-met-Chol with purified human HDL and LDL. When Pyr-met-Chol was incubated for 48 h with LDL or HDL, at 5% mol/mol of the non-esterified cholesterol present in the lipoproteins, and then separated on sepharose, the pyrene fluorescence peaks were superimposed to the non-esterified cholesterol peaks that revealed the non-labelled lipoproteins (Fig. 1C), without change in the elution time. This indicated that Pyr-met-Chol-labelled lipoproteins did not exhibit significant structural perturbations.

We then incubated Pyr-met-Chol (at 5% mol/mol of the non-esterified cholesterol content) with purified HDL or LDL during various times, and assayed by fluorometry their Pyr-met-Chol content before and after dialysis. After either a 8 or 48 h incubation, dialysis eliminated less than 5% of Pyr-met-Chol associated with HDL, whereas it eliminated ca 50% of Pyr-met-Chol associated with LDL (not shown). When we determined (after dialysis) the kinetics of Pyr-met-Chol association to purified human LDL or HDL up to 48 hours, an increasing association with both lipoprotein classes was measured, with a higher amount (about twice when normalized to protein content) of Pyr-met-Chol associated with HDL than with LDL (Fig. 2). Similar results (slow kinetics, comparable amounts) were obtained when performing incubations with 10 μM Pyr-met-Chol (not shown). Roughly 30% of Pyr-met-Chol was not associated to the purified LDL or HDL. However, after a 48 h incubation, when the amount of Pyr-met-Chol associated with lipoproteins was determined as the fraction of non-esterified cholesterol content, it was measured to be largely higher for HDL than for LDL (4200 FU/μmol and 640 FU/μmol, respectively). This could be assigned to a clearly lower apparent affinity for LDL than for HDL, because LDL were not saturated at 200 μM (0.3% of non-esterified cholesterol in the lipoproteins *vs.* 0.08% at 10 μM), while HDL were virtually saturated at 30 μM (2.0% of non-esterified cholesterol *vs.* 1.7% at 10 μM). Nevertheless, Pyr-met-Chol appeared tightly associated to LDL since specific precipitation of the apoB-containing lipoproteins led to the simultaneous precipitation of all the pyrene fluorescence (not shown). These data indicated that the association of Pyr-met-Chol with the lipoproteins, although rather slow, was stable and independent of the presence of other serum components.

3.3. Spectroscopic analysis of Pyr-met-Chol excimer formation in labelled LDL

We then used the specifique features of pyrene fluorescence emission to further study the interaction of Pyr-met-Chol with LDL peripheral hemi-membrane. When purified LDL were incubated for 48 h with Pyr-met-Chol (200 μM, i.e. 5% of LDL non-esterified cholesterol content) and then dialyzed, fluorescence emission sprectrum showed the presence of a broad peak in the range 440–500 nm (Fig. 3A), typical of pyrene excimers (the excimer to monomer ratio, I_E/I_m, was 0.55, as evaluated by the fluorescence emission intensities at 450 and 375 nm, respectively). By comparison, the spectrum of Pyr-met-Chol-labelled HDL (after an incubation at 30 μM, i.e. 5% of HDL non-esterified cholesterol content) showed a much lower, if any, presence of excimers (I_E/I_m = 0.05), in spite of a higher amount of Pyr-met-Chol associated (see above). Noteworthilly, after a 8 h incubation, the Pyr-met-Chol-labelled LDL displayed a spectrum with an even higher excimer peak than after 48 h (not shown), thus well reflecting some characteristics

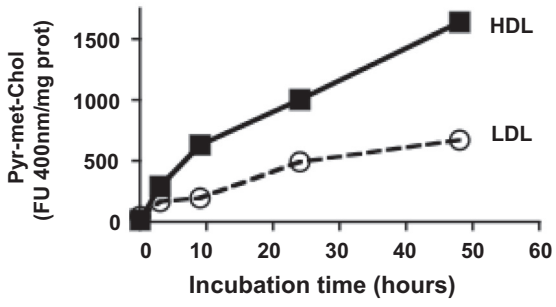

Fig. 2. Kinetics of association of Pyr-met-Chol with HDL or LDL. Purified human LDL (3.0 mg/ml, open circles) and HDL (2.7 mg/ml, closed squares) were incubated (37 °C) with Pyr-met-Chol (5% mol/mol of non-esterified cholesterol), then dialyzed, and associated Pyr-met-Chol amount was quantified by fluorescence emission measurement.

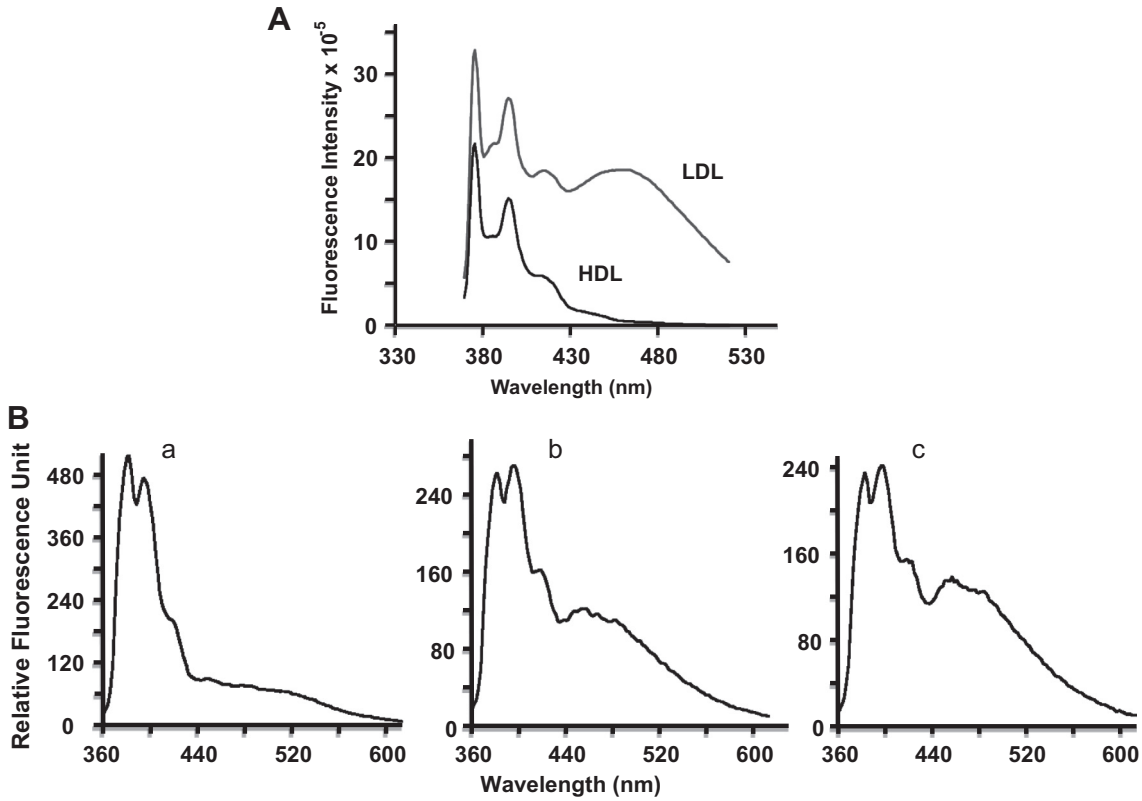

Fig. 3. Spectroscopic analysis of the fluorescence emission of Pyr-met-Chol associated with purified lipoproteins. Panel A: Difference between labelled HDL and LDL. Pyr-met-Chol was incubated for 48 hours with purified HDL and LDL as in Fig. 2, and fluorescence emission spectrum was recorded. Panel B: Effect of *in vitro* oxidation of labelled LDL. Purified LDL were incubated with 5 μM $CuSO_4$ during 4 (b) or 8 (c) hours at room temperature, or not (a), and then they were labelled by 10 μM Pyr-met-Chol, and fluorescence emission spectrum was recorded.

of the structural organization of the peripheral hemi-membrane, independently of the amount of the incorporated fluorescent dye. Otherwise, Pyr-met-Chol-labelled VLDL presented a very similar I_E/I_m ratio than LDL (not shown).

In order to test the effect of a perturbation of the LDL peripheral lipids on this pyrene fluorescence characteristics, we performed an *in vitro* oxidation of LDL by treating them with $CuSO_4$ for 4 or 8 h, before labelling them with Pyr-met-Chol (at 10 μM). At that concentration, the excimer peak of untreated LDL (Fig. 3Ba) is much lower than previously observed after a labelling with 200 μM (see Fig. 3A). After the LDL treatment by $CuSO_4$, the total LDL-specific precipitation of pyrene fluorescence indicated that the oxidized LDL were still able to tightly associate Pyr-met-Chol (not shown). Interestingly, the excimer peak of Pyr-met-Chol-labelled LDL dramatically increased as a function of oxidation time (Fig. 3Bb and c). This clearly showed that the chemical modifications of lipids in the LDL peripheral hemi-membrane modified its structural organization, as probed by pyrene.

3.4. Feasibility of a specific cell staining using Pyr-met-Chol-labelled lipoproteins

We finally addressed the potential use of Pyr-met-Chol-labelled lipoproteins as a new tool for cell staining. As a first test, we thus incubated cultured PC-3 cells for 48 h with 5 μM Pyr-met-Chol directly added to the culture medium, that is under similar conditions than those shown in Fig. 1A to induce a marked binding on the lipoproteins. After washing, observation of the treated cells using a TPE fluorescence microscope showed a cellular pattern presenting well-stained punctate intracellular structures (Fig. 4A). For the sake of comparison, we simultaneously added NBD-Chol (5 μM) to the PC-3 cells culture medium, and observed the same field of stained cells. In the channel reporting NBD-Chol fluorescence, the cellular staining pattern was clearly different, with irregularly distributed intense spots (Fig. 4B). Since data in Fig. 1A showed that NBD-Chol did not significantly associate with lipoproteins, and thus should follow a non-specific cellular uptake pathway, NBD-Chol cell staining provided a lipoprotein-independent, control image for the cellular incorporation of Pyr-met-Chol mediated by the lipoproteins.

4. Discussion

4.1. Stable and specific association of a fluorescent derivative of cholesterol to lipoproteins

The NBD fluorophore group provides a partially hydrophilic character to NBD-Chol, making it a true amphiphilic molecule, at variance with Pyr-met-Chol that is more hydrophobic. This difference of lipophilicity is likely a driving force for their respective preferential binding to albumin and to lipoproteins. Indeed, albumin is well-known to efficiently chelate amphiphilic, often polycyclic, drug molecules, while lipoproteins are frequently reported to play the role of vector particles for hydrophobic compounds [2].

Pyr-met-Chol molecule has the same hydrophilic OH group and the same lateral chain length as unesterified cholesterol, which is determining for its anchoring within the peripheral lipid monolayer of lipoproteins. In addition, Pyr-met-Chol, in contrast to NBD-Chol, is not esterified by LCAT, known to more or less esterify cholesterol and various sterols depending on their chemical structures [18]. Thus, no Pyr-met-Chol molecule is likely to be present in the hydrophobic core of lipoproteins, and this determines the stable association of Pyr-met-Chol to their hemi-membrane, making it a true label of lipoprotein periphery.

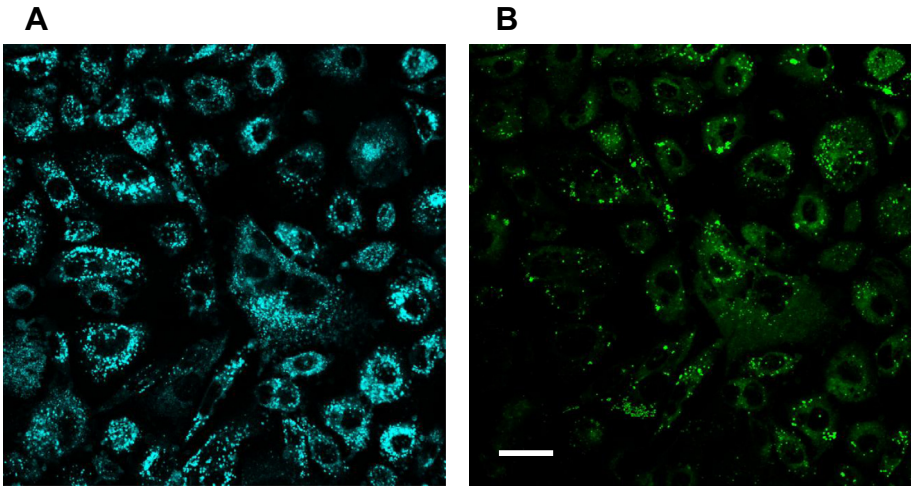

Fig. 4. Staining of cultured PC-3 cells by Pyr-met-Chol in lipoprotein-containing medium. PC-3 cells at confluency were further cultured for 48 h in the presence of 5 μM Pyr-met-Chol and 5 μM NBD-Chol added to the medium containing 10% fetal calf serum. After washing, the stained cells were observed with a TPE fluorescence microscope. Panel A: blue channel, Pyr-met-Chol; panel B: green channel, NBD-Chol. Scale bar is 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Insertion of a cholesterol fluorescent derivative in the peripheral hemi-membrane of HDL and LDL

Since the peripheral monolayer of LDL contains higher amounts of SM and cholesterol than HDL [1,3], we addressed the question of their respective structural organizations using two fluorescent derivatives of cholesterol. NBD-Chol association to lipoproteins being very poor, we focused on Pyr-met-Chol interaction with HDL and LDL. In both cases, association kinetics are slow, consistent with a high energetic barrier for the transfer of a hydrophobic molecule into a membrane via the aqueous bulk medium where it is introduced (Fig. 2). This should indicate a stable association since the release kinetics is expected to be also very slow. However, HDL and LDL are clearly distinguishable since (i) Pyr-met-Chol has a higher affinity and association capacity for HDL, and (ii) no Pyr-met-Chol associated with HDL can be dialyzed, in contrast to LDL, showing that a part of Pyr-met-Chol can be first adsorbed on LDL hemi-membrane rather than inserted in it, before being desorbed during the subsequent dialysis. These data are indicative that HDL and LDL do not present the same "structural state" as an acceptor lipid monolayer for Pyr-met-Chol, HDL being largely more permissive. As a matter of fact, NBD-Chol association to HDL, even if much lower than that of Pyr-met-Chol, is also higher than to LDL (which is even not detectable). HDL are characterized by a marked curvature, and higher amounts of the polyunsaturated arachidonic acid [16] (hence more easily oxidized) than LDL. Accordingly, in-silico molecular dynamics of HDL particles have shown that the high fraction of phospholipid molecules that in contact with apoA-I display a much disordered state [19]. The HDL peripheral lipid hemi-membrane can thus be envisioned as a highly disordered monolayer that is well tolerant for incorporating exogenous amphiphilic/hydrophobic molecules.

4.3. Pyr-met-Chol inserted in the peripheral hemi-membrane of LDL senses an ordered micro-environment

Here we have shown that LDL and HDL are also remarkably differing by the fact that Pyr-met-Chol-labelled LDL, but not Pyr-met-Chol-labelled HDL, present a pyrene excimer fluorescence emission (Fig. 3A). Such excimers, formed from two instantaneously interacting pyrene molecules, probe the probability of intermolecular meetings, which depends on their close environment. Indeed, in a bilayer membrane, pyrene excimer formation is the consequence of favorable local conditions, such as overconcentration or preferential insertion into ordered domains [12,20]. Here, this gives insights on the local structural state of the peripheral lipid monolayer in the labelled lipoprotein. Since Pyr-met-Chol content is lower in LDL than in HDL, the excimers observed in LDL cannot be due to a simple global overconcentration effect. Rather, their presence reveals a greater local dynamics, with a locally increased number of monomers inducing a more effective excimer formation, consistently with the reported high miscibility of Pyr-met-Chol in ordered lipid domains in model membranes [12]. Finally, taking into account (i) the richness of SM and cholesterol in LDL monolayer, (ii) the reluctance of NBD-Chol, known to be incompatible with the preservation of lipid raft structure [13,14], to associate with LDL, and (iii) the pyrene excimer fluorescence emission of the Pyr-met-Chol-labelled LDL (but not HDL), our observations indicate that the LDL peripheral monolayer presents some ordered microdomains. The existence of such lipid domains is consistent with a previous NMR study, which had shown that LDL particles presented different populations of PC and SM at their surface [21], and this is also consistent with some observations on model monolayers of similar lipid compositions [22–24]. This is a clue for the structural organization of the peripheral hemi-membrane of LDL, presently poorly described at the molecular level, although pivotal for determining the specific interaction with their cognate receptors on target cells. However, this does not preclude the otherwise easy binding of Pyr-met-Chol to HDL peripheral monolayer since (at variance with a bilayer) it appears as very tolerant to exogenous hydrophobic molecules, as discussed above.

As an illustration of its possible use as a probe for the "structural state" of the lipoprotein periphery, excimer emission of Pyr-met-Chol-labelled LDL was observed to dramatically increase after a progressive $CuSO_4$-induced oxidative *in vitro* stress (Fig. 3B). This is consistent with the progressive modification of unsaturated acyl chains, providing a relatively more saturated and ordered environment that is more prone to pyrene excimer formation. Pyr-met-Chol labelling of LDL thus provides a convenient means for witnessing the monolayer membrane perturbation induced by the progressive formation of peroxidized lipids during such an oxidative stress.

4.4. Potential uses of Pyr-met-Chol-labelled lipoproteins

Pyr-met-Chol-labelled lipoproteins could thus be proposed as a new way for staining cultured cells by a fluorescent probe aimed at contributing to study lipoprotein receptor-mediated cell incorporation and trafficking of cholesterol. Indeed, the fact that Pyr-met-Chol is a close derivative of cholesterol, presenting a similar behavior in model membranes [12], makes it a promising probe. A clear advantage of the pyrene fluorophore in this context is its photochemical stability. However, the nature of the intracellular structures stained remains to be determined, and whether the incorporated probe can actually trace for the physiological fates of cellular cholesterol.

Otherwise, Pyr-met-Chol-labelled lipoproteins should also be proposed to be used for *in vivo* experiments designed to determine the systemic kinetics of a given class of lipoproteins after *ex vivo* labelling and subsequent infusion. Tissue imaging experiments could also be performed to assess their richness in lipoprotein receptors.

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