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Qualitative and quantitative analysis of the uptake of lipoplexes by villous placenta explants

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Abstract

Controlled distribution of a drug by its association to a nanocarrier is a promising approach for the treatment of pregnancy disorders such as preeclampsia. For this application, tracking both the nanocarrier and the drug is necessary to ensure the safety of both the mother and the foetus. This study reports a method to visualize and quantify the uptake of liposomal formulations in placental tissue using fluorescent labelling and appropriate analytical tools. Lipoplexes were labelled with a fluorescent lipid, DOPE-NBD while the encapsulated siRNA was fluorescently labelled with rhodamine. Lipoplexes were incubated with villous placenta explants, explants were imaged with confocal microscopy, then DOPE-NBD was extracted from the explant and quantified by HPLC. Qualitative evaluation by confocal microscopy showed the presence of lipoplexes and siRNA into the outer layer of the placental explants, the syncytiotrophoblast. For quantitative evaluation, an HPLC method for the quantification of fluorescent lipid DOPE-NBD in placental tissue was developed and validated. The developed method was applied to quantify the DOPE-NBD uptake in the placental tissue. Increased amounts of DOPE-NBD were detected in placental explants when increasing the incubation concentration of lipoplexes. This study provides a method to evaluate the interactions between liposomal formulation and the placental barrier.

Keywords: Placenta, HPLC, Fluorescence, Liposomes, *Ex-vivo* model, Placenta explants

1. Introduction

Preeclampsia is a pregnancy complication causing a significant number of maternal and infant death every year around the world and there is still no effective treatment (Sibai et al., 2005). Delivering the baby and the placenta is currently the sole medical procedure to save the mother's life in case of severe preeclampsia. Such clinical choice may generate severe prematurity or infant death (Müller-Deile and Schiffer, 2014). New insights in biomedical research support the relationship between clinical symptoms of preeclampsia and the high abnormal serum levels of placental anti-angiogenic factors, including soluble fms-like tyrosine kinase-1 (sFlt1) (Verlohren et al., 2014). Recent papers have reported successful strategies to regulate sFlt-1 serum level by siRNA based gene therapy (Turanov et al., 2018; Yu et al., 2017). In a pregnant mouse model, up to 7% of siRNA dose was found in placental tissue using the chemical linkage of the oligonucleotide sequence to cholesterol (Turanov et al., 2018). Increasing siRNA concentration in target cells could further improve the clinical outcomes of this innovative strategy.

Controlled delivery of siRNA to the placental target cells, without trans-placental passage, will undoubtedly increase safety and efficacy of gene therapy. This goal could be achieved by a rationally designed nanomedicine that limits the off-target distribution of siRNA, protects it from degradation and enhances its concentration in target cells (Whitehead et al., 2014). Moreover, the potential of lipidic nanocarriers for siRNA-based gene therapy has recently been highlighted by the approvals of Patisiran, ONPATTRO™, for clinical use in the USA and EU. Patisiran is the first-ever RNAi therapeutic in the market (Hoy, 2018).

Liposomal formulation, including lipoplexes, are bio-inspired nanocarriers, whose biocompatibility and safety were largely reported (Fattal et al., 2004). Optimal formulation of lipoplexes in terms of size and surface properties could provide a carrier limiting the transplacental passage of siRNA while specifically delivering it to placental cells (Keelan et al., 2015; Muoth et al., 2016; Valero et al., 2018a). For such application, the safety of the treatment for both the mother and the foetus, combined to the efficacy of placental cell targeting should be evaluated. Reliable evaluation could be reached using experimental protocols and analytical tools that allow tracking both lipoplexes and siRNA.

Understanding the interactions between liposomes and the placental barrier plays a vital role in the development of an innovative liposomal formulation, leading to an improved biodistribution and an optimal safety profile. Due to species specificity of the placenta, human placenta models are usually used to evaluate the potential of liposomal

nanocarriers, as a therapeutic approach for pregnant women (Bajoria et al., 2013; Shah et al., 2018; Valero et al., 2018a). The villous placenta explant model preserves the *in vivo* architecture of human placenta while retaining several hours of viability (Martinez-Fierro et al., 2018; Mayhew and Barker, 2001). This *ex vivo* model provides pertinent information about liposomes penetration into the human placenta and appears to be suitable for assessing liposomes designed for the management of pregnancy complications.

In a previous study, dually labelled PEGylated liposomes were evaluated on both the villous placenta explants and the dually perfused placenta models (Valero et al., 2017). Experiments provided semi-quantitative data about a liposome penetration into the external layer of placental villi, the syncytiotrophoblast, with no detectable passage in the foetal circulation. Moreover, the localization of the fluorescent probe in villous explants was in agreement with transplacental passage results. This previous work demonstrated that the villous placenta explants model is a relevant model to evaluate the liposomal formulation ability to penetrate and cross the placental tissue.

Lipoplexes formulation, composed of the cationic lipid DMAPAP in combination with sodium alginate and encapsulating siRNA, was previously evaluated in our lab for several applications and showed high potential as gene therapy nanocarriers (Marimani et al., 2015). We have recently reported the ability of DMAPAP Lipoplexes to deliver a non-coding siRNA to a placental cells *in vitro* model of human primary villous cytotrophoblasts. The siRNA internalization rate achieved with DMAPAP lipoplexes was similar to that obtained by lipofectamine, a well-known transfection reagent (Valero et al., 2018b).

A quantitative assessment of the uptake of liposomes by placental tissue could be obtained using a fluorescent lipid that is then quantified by HPLC coupled to a fluorescence detection. The main objective of this study is the development of a suitable HPLC method allowing i) the separation of the fluorescent lipid from biological media and ii) the quantification of its concentration in the placenta villous tissue by fluorescence detection. This method is expected to provide quantitative data that complete qualitative microscopic observation of placenta interactions with liposomal formulations.

In this study, the uptake of lipoplexes by the human villous placenta explants model was evaluated qualitatively and quantitatively. Both siRNA moiety and liposomal nanocarriers were labelled by a fluorescent probe. A fluorophore-conjugated lipid, DOPE-NBD was added to the DMAPAP lipoplexes composition as a marker. Fluorescently labelled DMAPAP lipoplexes were incubated with human villous placenta model, confocal

microscopy imaging was used to assess qualitatively placental penetration. A liquid-liquid extraction protocol and an HPLC method based on fluorescent detection were developed and validated for fluorescent lipid DOPE-NBD analysis in biological media. Quantification of the internalized lipoplexes was then performed by quantifying fluorescent lipid in the placenta villous tissue using the developed method.

2. Materials and Methods

2.1. Materials

DMAPAP (Dimyristoylaminopropylaminopropyl), a cationic lipid was synthesized in our lab as mentioned in previous publications (Mignet et al., 2008). Dodagly-PEG ((2-dioctadecylcarbamoyl- methoxyacetyl amino) acetic acid-(α -methoxy)-polyethylene glycol 2000 ester), a PEGylated lipid was synthesized as reported previously (Mignet et al., 2012). Cholesterol, DOPE (1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine) and DOPE conjugated to fluorescent NBD, DOPE-NBD, (1, 2-dioleoyl sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1, 3-benzodioxadiazol-4-yl) were purchased from Avanti Polar Lipids (Alabaster, USA). Rhodamine (tetramethylrhodamine)-labeled and unlabeled non-coding siRNA (UUCUCCGAACGUGUCACGU) were acquired from (Eurogentec, Angers, France). Sodium alginate, Triton X100, Hank's balanced salt solution (HBSS), Dulbecco's modified eagle's medium, optiMEM medium and glutamine were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Bovine serum albumin (BSA) was purchased from Interchim (Montluçon, France). Primary and secondary Alexa Fluor antibodies and TOPRO-3-iodide were purchased from Life Technologies (Saint Aubin, France). Paraformaldehyde (PFA) was obtained from Electron Microscopy Sciences (Hatfield, UK). All solvents were of HPLC grade and were obtained from Carlo Erba (Val de Reuil, France). Milli-Q system was used to purify water and purchased from Millipore® synergy system (Millipore, Fontenay-sous-Bois, France).

2.2. Lipoplexes preparation and characterization

Liposomes and lipoplexes were prepared as previously described (Schlegel et al., 2011). Briefly, lipids were dissolved in chloroform with a molar ratio of DMAPAP/DOPE/DOPE-NBD/Dodagly-PEG; 50:40:5:5, then chloroform was evaporated using a rotatory evaporator to form a thin film. The thin film was then rehydrated with a 150mM NaCl solution. The liposome suspension was extruded, volume was adjusted to have a final lipid concentration of 2.5 mM. Lipoplexes were prepared by mixing equal

volumes of the cationic liposomal suspension and solution the siRNA and alginate in 150 mM NaCl, and then rapidly vortexing the mixture. The N/P charge ratio was 4, calculated using the molar ratio of charges of DMAPAP, siRNA and sodium alginate (Arruda et al., 2016). Lipoplexes were prepared with 50% of Rhodamine (3' TAMRA)-labelled and 50% of non-labelled siRNA.

Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) was used to determine the mean particle hydrodynamic diameter (Z-average), polydispersity index and Zeta potential. Dilution of (1/100) of liposomes or lipoplexes suspension was carried out in 150 mM NaCl prior to size measurement, or in 1mM KCl prior to zeta potential measurement, respectively. A RiboGreen® assay was used to determine the encapsulation efficiency of siRNA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

2.3. Lipoplexes incubation with villous explants model

The *ex vivo* suspended villous explants model was used according to experimental conditions previously reported (Valero et al., 2017). Briefly, placentae were obtained from a caesarean section of full-term pregnancy after obtaining a written consent from the patient and an approval from our local ethics committee (CCP- PRB, Paris Cochin, N 18–05, Paris, France). Small villi were dissected from the placenta, and each villous explant was hung by a needle in wells filled with Dulbecco's modified Eagle's medium containing 10% bovine serum albumin and 1% glutamine. Lipoplexes were added to the medium to obtain different concentrations 18 or 36 $\mu\text{mol/L}$ of total lipids corresponding to 815 and 1630 $\mu\text{g/L}$ of DOPE-NBD respectively. Wells were then incubated for 24 h at 37 °C and 5% CO₂ under gentle agitation. After incubation, placental villous explants were washed with 1% PBS. Samples for confocal microscopy analysis were directly analysed after incubation, whereas samples for HPLC experiments were stored at -20 °C until analysis.

2.4. Qualitative analysis by confocal microscopy

After 24 h of incubation with lipoplexes, villous placenta explants were washed 3 times using 1% PBS then fixed in 4% PFA and kept at 4 °C. Villi were embedded in agarose, then cut into slices 120 μm thick using a Vibratome (Technical Products International, Maryland Heights, USA). Slices were then treated with 500 μL of 0.5% Triton X-100 for 30 min. Unspecific sites were saturated using 4 % BSA at room temperature. To

visualize syncytiotrophoblast cells, primary antibodies against human cytokeratin 7 were incubated for 1 h at room temperature, then amplified with Alexa Fluor 405®-conjugated mouse secondary antibody for 1 h at room temperature. Nuclei were labelled by TOPRO-3-iodide stain. Mount was prepared using a fluorescent Dako mounting medium (Dako, Glostrup, Denmark), observed and imaged on a TCS SP2 confocal microscope (Leica, Wetzlar, Germany) of the Cellular and Molecular Imaging facility, INSERM UMS 025—CNRS UMS 3612, Faculty of Pharmacy, University of Paris Descartes, Paris, France.

2.5. Quantitative analysis by HPLC

2.5.1. HPLC instrumentation

HPLC analysis were carried out by using Shimadzu LC-20AD high-performance liquid chromatography (Duisburg, Germany) equipped with a quaternary pump, mobile phase degasser and autosampler connected to a C18 column (Kromasil, C18, 5 μ m, 4.6 mm \times 250 mm, 100 Å, Interchim, Montluçon, France). The column was protected by a pre-column (20 mm \times 3.9 mm) filled with the same stationary phase and operated at 40°C. Detection was performed using a fluorescence detector (RF-10AXL, Shimadzu) at an excitation wavelength of 465 nm and an emission wavelength of 535 nm. LabSolutions software was used for HPLC monitoring and data acquisition.

Analysis were performed using a gradient mobile phase composed of water, methanol, and isopropanol. For the first 5 min initial conditions with a mobile phase of water, methanol and isopropanol (10%, 80%, and 10%, respectively) were maintained, then changing to methanol and isopropanol (40% and 60%, respectively) in 5 min and was then maintained for 5 min and finally the conditions returned to the initial ones in 5 min and were maintained for the last 5 min of the run. The total time of the elution gradient was 25 min with a flow rate of 1 mL/min and an injection volume of 50 microliters.

2.5.2. Sample preparation for fluorescent lipid analysis in villous placenta explants

To extract lipids, a liquid-liquid extraction method was optimized. Each incubated explant was collected and washed then transferred into a 5 mL Eppendorf tube where it was sliced using a scalpel to facilitate homogenization. To the sliced explants, 0.4 ml of water and 0.1 ml of 10% Triton X-100 were added, and tubes were vortexed for 30 seconds. The mixture was sonicated for 10 min for two cycles while vortexing was performed again between the two cycles. The homogenate was transferred to a haemolysis tube

then 3 mL of chloroform were added. The mixture was vortexed for 20 min and centrifuged at 2000 RPM for 10 min. The organic phase was collected in a glass balloon and the process was repeated with another 3 mL of chloroform. The collected phase was evaporated via rotary evaporation under 1 mbar at room temperature. The remaining residue was reconstituted in 500 μ L of ethanol then filtered using a 0.2 μ m cellulose acetate filter Nalgene® (Thermofisher Scientific) and 50 μ L was then injected in HPLC.

2.5.3. Method validation

A stock solution of DOPE-NBD was prepared at concentration of 1 g/L by dissolving 1 mg DOPE-NBD powder in 1 ml of ethanol and stored at -20 °C. The linearity of the method was checked by using 2 series of freshly prepared calibration standard samples at six different concentrations of DOPE-NBD 2.5, 5, 10, 20, 30 and 60 μ g/L. A calibration series was prepared in ethanol and in the presence of control villi extracts. Another calibration series taking into account the extraction procedure was also prepared by spiking control villi before extraction using known concentrations of DOPE-NBD, the extraction procedure was then performed, and the reconstituted residue was analysed by HPLC.

The specificity of the method was tested by comparing the chromatogram of the control villi with the one of the villi incubated with lipoplexes at a 36 μ mol/L total lipids concentration. The accuracy and precision of the method were evaluated by analysing the inter-day variability of the three sets of villi samples prepared on three different days and spiked using 3 different concentrations of DOPE-NBD 7.5, 15 and 45 μ g/L. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined based on signal-to-noise ratios of 3:1 and 10:1, respectively.

3. Results and Discussion

In this study, qualitative and quantitative analyses of interactions between lipoplexes and human placenta have been performed. Qualitative assessment of lipoplexes penetration into placental explants was performed by confocal microscopy imaging. For the quantitative assessment, a HPLC method for quantification of fluorescent lipid DOPE-NBD in human placental tissues was developed and validated. The developed method was then applied in order to determine the amount of lipoplexes in human placental explants.

3.1. Liposomes and lipoplexes characterization

Fluorescently-labelled liposomes formulation exhibited a mean hydrodynamic diameter of 163 ± 10 nm, a Pdl of 0.19 ± 0.04 and a positive Zeta potential of $+ 22 \pm 3$ mV. Lipoplexes were obtained after adding siRNA and sodium alginate solution. siRNA solution was composed of 50% of rhodamine-labelled siRNA and 50% of unlabelled non-coding siRNA sequence. The hydrodynamic diameter of lipoplexes was of 225 ± 45 nm, Pdl was 0.20 ± 0.05 and neutrality was reached with a Zeta potential of $- 3 \pm 2$ mV. A full complexation of siRNA into lipoplexes was confirmed by RiboGreen® assay. Fluorescent liposomes and lipoplexes showed a slight increase in size in comparison with non-fluorescent ones having similar composition as it has been reported in a recent study (Valero et al., 2018b).

3.2. Qualitative assessment by confocal microscopy

Confocal microscope images were obtained from explants incubated with lipoplexes and compared with control explants incubated in culture media without lipoplexes. Figure 1A shows representative images of control placental explant observed with confocal microscopy. Immunostaining of the cytokeratin-7 marker with cyan color allowed to visualize the syncytiotrophoblasts, i.e. the outer cell layer of placental villous. Cell nuclei of syncytiotrophoblasts and mesenchyma were labelled and visualized in blue color. As expected, the control explant images showed the absence of the green and red fluorescence of DOPE-NBD and encapsulated siRNA, respectively. Figure 1B showed an explant incubated with the labeled lipoplexes at a concentration of $36 \mu\text{mol/L}$ of total lipids. Co-localization of green and red fluorescences signaled the presence of the fluorescent lipid and the siRNA respectively, into the outer layer of the villi, the syncytiotrophoblast. No fluorescence associated with lipid or siRNA was observed in the mesenchyma, where fetal capillaries are situated. This observation is confirmed by using channels of red and green fluorescence on the same image (figure 1C). Fluorescent lipids of lipoplexes and complexed siRNA seemed to adhere to and penetrate into the syncytiotrophoblast, i.e. placental specific cell layer in direct contact with maternal blood. Besides, results showed that lipoplexes (lipids and siRNA) actually do not gain access to the mesenchymal axis. These observations could be strengthened by using an advanced imaging technique such as the Light Sheet Fluorescent Microscopy (LSFM). The latter technique can provide deeper imaging and a better resolution compared to confocal microscopy for such application (Lazzari et al., 2019).

Our previous study showed that siRNA encapsulated into lipoplexes made with DMAPAP were internalized by primary villous cytotrophoblasts, representing a syncytiotrophoblast model *in vitro* (Valero et al., 2018b). In this study, we complete and confirm the previously collected data by reporting the interaction between DMAPAP lipoplexes and the placental barrier on a 3D structure represented by the placental villous explants. Our previous results showed that the fluorescent probe localization into placental villous mesenchyma is consistent with its transplacental passage which was measured using a dual circulation perfused placental model (Valero et al., 2017). We could infer that lipoplexes and associated siRNA penetrated the outer layer of placental barrier and did not reach the fetal circulation as no fluorescent signal was detected in the mesenchymal axis. It should be noted as previously observed (Bajoria and Contractor, 1997; Menezes et al., 2011) that the uptake into syncytiotrophoblasts and transplacental transport to the fetus are two distinct processes. However, additional experiments using the complementary *ex vivo* model of perfused placenta should be performed to confirm the absence of lipoplexes and siRNA transplacental passage.

3.3. Quantitative assessment by HPLC equipped with fluorescence detector

Quantifying placental uptake of liposomal formulation is an information of high interest for the safety evaluation of liposome-based innovative therapy. Lipid quantification is challenging in terms of detection methods, specificity and sensitivity of analysis. Although several HPLC methods have been reported to separate and quantify lipids using evaporative light scattering detection (ELSDs) (Jeschek et al., 2016; Oswald et al., 2016; Wöll et al., 2018). However, this detection method is not specific, and cannot distinguish liposomal formulation lipids from biological medium lipids which appears as a major limitation in using HPLC-ELSDs for our application. Specificity could be improved by coupling lipid to fluorescent probes and measuring lipid concentration indirectly via fluorescence detection. In the following sections, we will first be developed and validated an HPLC method with fluorescent detection for DOPE-NBD quantification in placental tissue, then the results of the method application will be presented.

3.3.1. Method development

The mobile phase (water, methanol, and isopropanol) was chosen based on the lipophilic property of DOPE-NBD and reported methods for lipids separation and quantification using HPLC-ELSDs (Jeschek et al., 2016; Wöll et al., 2018). Many gradients were tested to elute effectively DOPE-NBD with a sharp peak improving the

limit of detection. The main concept was to start with a gradient with more polar mobile phase with a higher percentage of methanol and water to elute most of the polar constituents in the extract in the first minutes of the run, and therefore minimize interference with DOPE-NBD peak. The gradient then gradually shifted to a more non-polar mobile phase to start eluting lipids including DOPE-NBD. The optimal gradient was chosen based on its ability to elute DOPE-NBD with a retention factor between 1 and 5 and tailing factor close to 1. The chosen gradient starts with 10% of water, 10% of isopropanol and 80% of methanol, methanol percentage is then decreased to 40% and isopropanol is increased to 60% which allowed to elute the analyte, the gradient was then returned to the initial conditions to equilibrate the column for the next run. DOPE-NBD was eluted in symmetric and sharp peak with a retention time of 12.3 min, retention factor k' was equal to 4.62 and tailing factor was of 1.12 (figure 2A). A small peak was observed around 7 min, it could be attributed to side products of DOPE-NBD synthesis or post-synthesis degradation products.

In order to apply this method for quantifying DOPE-NBD in placental villous explants, an extraction protocol was developed to recover fluorescent lipids from lipoplexes and biological matrix. A liquid-liquid extraction method using chloroform was optimized. The choice of chloroform is based on its high ability to dissolve lipids, immiscibility with water and its easy evaporation under vacuum conditions. After the extraction process, extracts were reconstituted with ethanol and injected into the HPLC system. The ability of extraction method to recover the analyte and the specificity of the HPLC method were then checked. Villi were incubated with a known lipoplexes concentration of 36 $\mu\text{mol/L}$ of total lipids, Figure 2B displays the chromatogram of a-10-time diluted extract of villous explant incubated with lipoplexes as well as the chromatogram of extracted control explant incubated in the same medium without lipoplexes. The DOPE-NBD peak was eluted with the same retention time ($t_r=12.4$ min) and well separated from the interfering peaks of the matrix (peaks eluted with retention time around 5 min). This indicates that the matrix did not induce interferences in the quantification of DOPE-NBD using our optimal protocol and conditions.

3.3.2. Method validation

The method was validated in terms of linearity, recovery, detection and quantification limits. The precision and accuracy of the method were also calculated using quality control samples.

Linearity was first checked by preparing triplicates of DOPE-NBD standard solutions at six concentrations 2.5, 5, 10, 20, 30 and 60 $\mu\text{g/L}$ in ethanol as the solvent and in the presence of dry extracts of control villous in order to evaluate the matrix effect on linearity. Regression linearity between surface area and concentration was assessed showing R-squared of 0.989 ($y=55291x + 28676$, RSD of the slope = 7%, RSD of the intercept = 34%; where y is the peak area and x the concentration ($\mu\text{g/L}$) of DOPE-NBD).

Linearity was also assessed taking into account the extraction process (figure 3). Villous explants were spiked before extraction by adding known concentrations of DOPE-NBD standard solutions, the reconstituted extracts were injected into the HPLC system (fig. 3A) and peaks surface area were plotted as a function of DOPE-NBD concentrations (fig. 3B). Linear regression was obtained at concentrations between 2.5 and 60 $\mu\text{g/L}$ with R-squared of 0.995 ($y=33713x - 11660$, RSD of the slope = 5%, RSD of the intercept = 39%; where y is the peak area and x the concentration ($\mu\text{g/L}$) of DOPE-NBD). This equation is considered the recovery yield of the extraction process and will be used in this study to calculate the amount of fluorescent lipid in villous explant.

Extraction recovery was calculated by comparing the peak areas obtained by spiking villi with DOPE-NBD before liquid-liquid extraction to corresponding ones obtained by preparing the standard solution in presence of the biological extract. The mean extraction yield using this protocol was of $58 \pm 3\%$. A similar value was obtained by comparing the slope of standard curve of spiked samples to the one of standard curve of samples prepared in presence of the biological extract and it was equal to 61%. Extraction recovery was relatively low, this might be due to degradation and/or the loss of fluorescent lipid during the extraction process. However, this did not influence the quantification of fluorescent lipid because of the low variability in extraction yield and the linearity of the standard curve considering the extraction process.

The limit of quantification (LOQ) was determined experimentally from the lowest concentration which was included in the range of linearity and had a signal to noise ratio superior to 10. The LOQ was found to be 2.5 $\mu\text{g/L}$. The limit of detection (LOD) for a signal to noise ratio equal to 3 was found to be 0.5 $\mu\text{g/L}$, i.e. 25 picogram per injection. These relative low values are of high interest for the use this quantification method in the safety evaluation and quality control of liposomal formulation (Wöll et al., 2018).

Inter-day variability was assessed through analysis of quality control samples (QCs) on

three different days. QCs were prepared by spiking of villous explants with lipoplexes at 3 known concentration of DOPE-NBD 7.5, 15 and 45 $\mu\text{g/L}$. Accuracy values for the three concentration were 105.2, 102.2 and 96.6%, respectively. The precision, i.e the relative standard deviation of the accuracy values, was less than 7.5% for all concentrations

3.3.3. Method application for the quantification of lipid uptake in the placenta

Quantifying placental uptake of lipoplexes was performed on several villous explants dissected from two full-term placentae. Villous explants were incubated with two concentrations of lipoplexes, 18 and 36 $\mu\text{mol/L}$ of total lipids, in order to evaluate the relation between the lipoplexes concentration and the placental uptake. Villous explants were classified by placenta (with the letter A or B) and by villi number (1 to 4). Table 1 summarizes the results including the normalization of the DOPE-NBD amount found in villous explants to its mass. First, no difference was observed between villous explants that were dissected from the same placenta and incubated with the same concentration of lipoplexes. These results confirm the robustness of our experimental protocol and the validity of the developed HPLC method. Second, a higher uptake was observed when villous explants were incubated with a higher concentration of lipoplexes. The magnitude of uptake increase seems to be different between the two placentae, this can be explained by the interindividual variability of placentae. At term, thickness of the syncytiotrophoblast and permeability to nutrients and xenobiotics vary as a function of different parameters such as fetus characteristics (e.g. sex, weight) and mother parameters (e.g. weight, diabetes) (Thornburg et al., 2016). More samples, in terms of villi and placentae holding different characteristics, would be needed to confirm this observation.

4. Conclusion

In this study, we developed an experimental protocol for qualitative and quantitative analysis of the interactions between liposomes and *ex vivo* human placenta explants. Confocal microscopy imaging provided information about the localization of the carrier and the therapeutic agent into the placental tissues. The developed and validated HPLC method allowed the quantitative analysis of the placental uptake of lipoplexes with high specificity, sensitivity and reproducibility. The application of the developed method to the lipid uptake within the placenta confirmed the placental uptake of lipoplexes observed by confocal microscopy. Moreover, quantitative analysis showed that the amount of internalized fluorescent lipid increased with increased concentration of incubated

lipoplexes. These data combining qualitative microscopy observations and quantitative analysis i) confirmed previous results on human primary cytotrophoblasts highlighting the use of lipoplexes as gene delivery vector for human placental cells and ii) contributed to the design of efficient lipoplexes' formulation as promising and safe gene delivery strategy for preeclampsia therapy. Furthermore, the developed HPLC method could potentially be applied to other liposomal formulations to acquire more quantitative data about safety and biodistribution of liposomal nanocarriers in placental tissue.

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Abbreviations

DOPE-NBD: 1, 2-dioleoyl sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,3-benzodioxadiazol-4-yl)
siRNA: Small interfering RNA
sFlt1: Soluble fms-like tyrosine kinase-1
PEG: Polyethylene glycol
DMAPAP: Dimyristoylaminopropylaminopropyl
Dodagly-PEG : (2-dioctadecylcarbonyl-methoxyacetyl amino) acetic acid-(α -methoxy)-polyethylene glycol 2000 ester
TAMRA : Carboxytetramethylrhodamine
LOD : Limit of detection
LOQ : Limit of Quantification
ELSDs : Evaporative light scattering detection
QCs : Quality control sample

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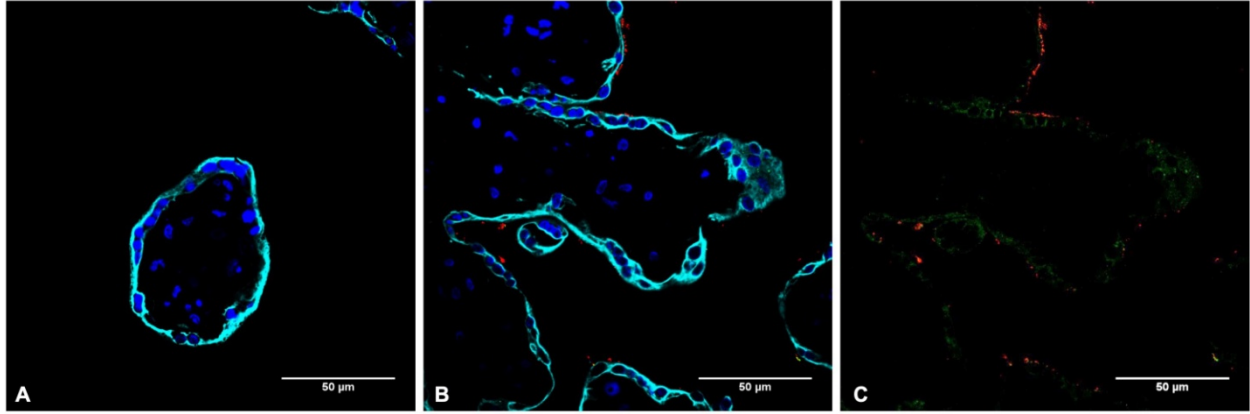
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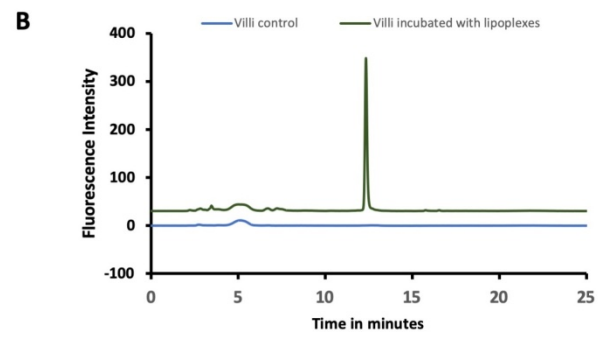
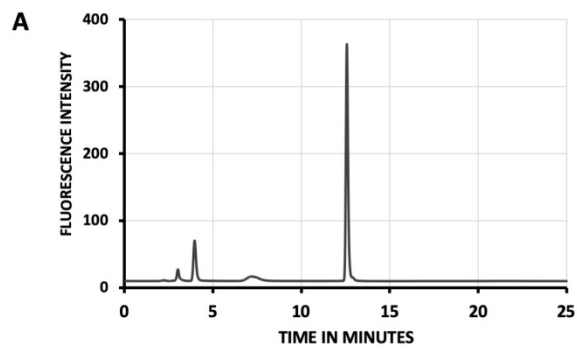
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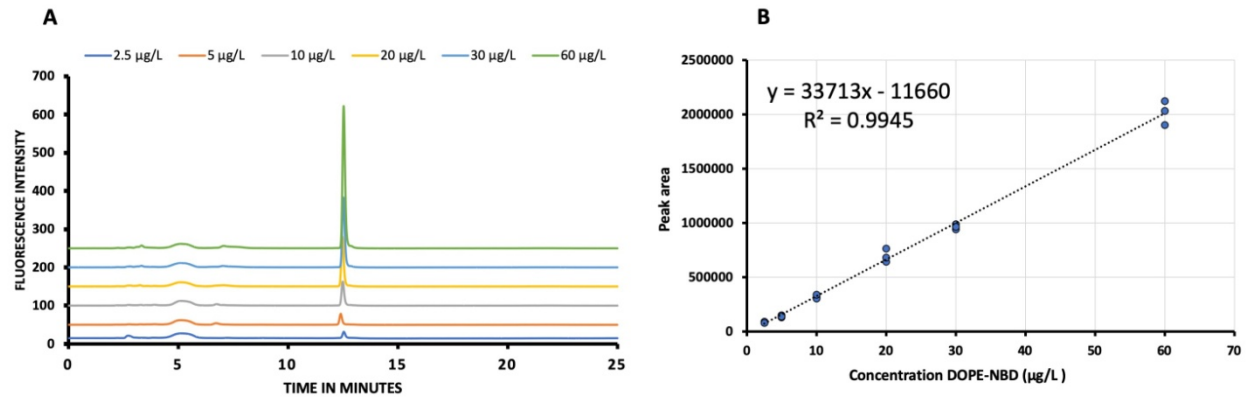
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Figure 1 : Confocal microscope images of explants; A) control explant, overlay of all four channel: only blue (Nuclei) and cyan (trophoblasts) were detected, B) and C) explant incubated with lipoplexes at a concentration of 36 $\mu\text{mol/L}$ of total lipids, B) overlay and C) channels of red and green fluorescence to better observe liposomes labelled with NBD (green) and siRNA with Rhodamine (red). Scale bar =50 μm .

Figure 2: A) Chromatogram of DOPE-NBD at concentration of 50 $\mu\text{g/L}$ in ethanol B) Chromatogram of extract of control villi (incubated in culture medium) compared to chromatogram of extract of villi incubated with lipoplexes labelled by DOPE-NBD.

Figure 3: A) Chromatograms of samples obtained by spiking biological extract with DOPE-NBD before liquid-liquid extraction to obtain a standard curve integrating the extraction process. B) Standard curve integrating the extraction process.

Table 1: DOPE-NBD quantification in placental tissue after incubation with various lipoplexes concentrations. The amount of DOPE-NBD is normalized to the mass of each villus.

Villi number	Weight of villi (g)	Incubated concentration of lipoplexes		Concentration of DOPE-NBD in extract ($\mu\text{g/L}$)	Total mass of DOPE-NBD extracted (μg)	Amount of DOPE-NBD in 1g of villi (μg)
		Total lipids ($\mu\text{mol/L}$)	DOPE-NBD ($\mu\text{g/L}$)			
A1	0.035	36	1630	585	0.29	8.4
A2	0.040			726	0.36	9.1
B1	0.018			1103	0.55	30.6
B2	0.030			1845	0.92	30.7
A3	0.025	18	815	204	0.10	4.1
A4	0.013			100	0.05	3.8
B3	0.026			88	0.04	1.7

Qualitative and quantitative analysis of villous placenta explants uptake of lipoplexes

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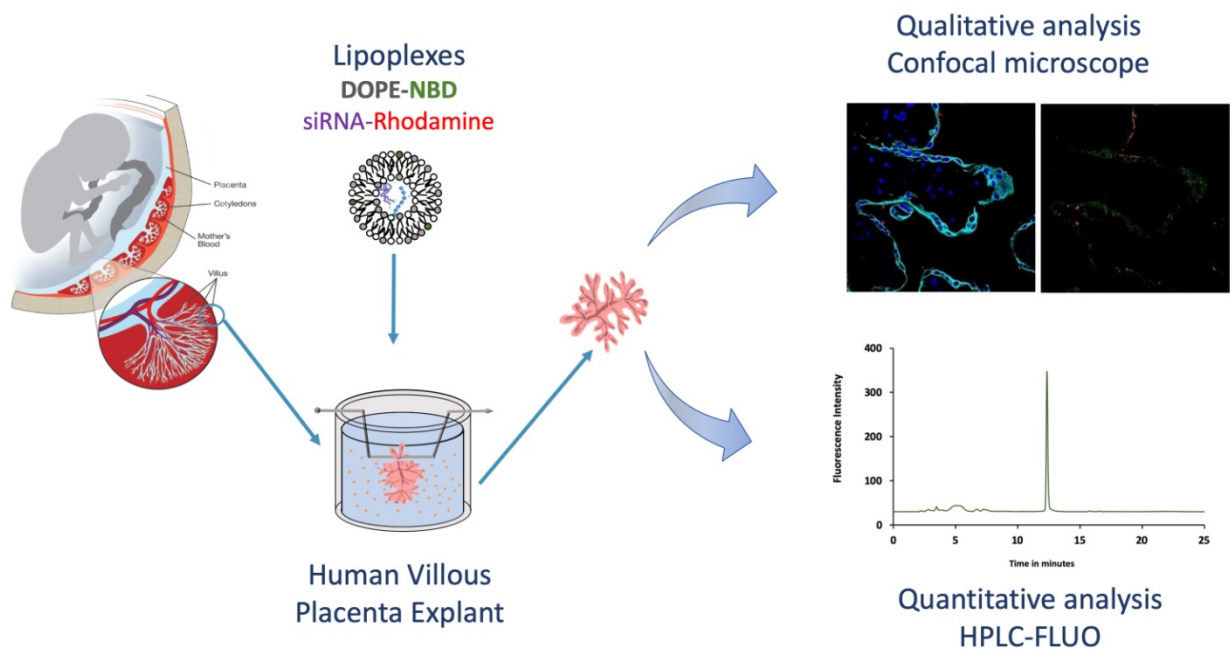
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Author contributions

KAL, KA, TF and NM designed the experiments and managed the project. LV, KEM, and KAL performed HPLC experiment. SG and TF provide the material and the expertise on placental tissue. LV and LF realized ex vivo studies. KAL and LV wrote and the manuscript. TF, CR, LF, and KA participated to manuscript edition.



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