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TITLE:

Uptake of New Lipid-coated Nanoparticles Containing Falcarindiol by Human Mesenchymal Stem Cells

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SUMMARY:

This article describes the encapsulation of falcarindiol in lipid-coated 74 nm nanoparticles. These particles get taken up by human stem cells into lipid droplets monitored by fluorescent and confocal imaging. Nanoparticles are fabricated by the rapid injection method of solvent shifting, and their size is measured with the dynamic light scattering technique.

ABSTRACT:

Nanoparticles are the focus of an increased interest in drug delivery systems for cancer therapy. Lipid-coated nanoparticles are inspired in structure and size by low-density lipoproteins (LDLs) because cancer cells have an increased need for cholesterol to proliferate, and this has been exploited as a mechanism for delivering anticancer drugs to cancer cells. Moreover, depending on drug chemistry, encapsulating the drug can be advantageous to avoid degradation of the drug during circulation in vivo. Therefore, in the study presented here, this design is used to

fabricate lipid-coated nanoparticles of the anticancer drug falcarindiol, providing a potential new delivery system of falcarindiol in order to stabilize its chemical structure against degradation and improve its uptake by tumors. Falcarindiol nanoparticles, with a phospholipid and cholesterol monolayer encapsulating the purified drug core of the particle, were designed. The lipid monolayer coating consists of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol (Chol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE PEG 2000) along with the fluorescent label DiI (molar ratios of 43:50:5:2). The nanoparticles are fabricated using the rapid injection method, which is a fast and simple technique to precipitate nanoparticles by good-solvent for anti-solvent exchange. It consists of a rapid injection of an ethanol solution containing the nanoparticle components into an aqueous phase. The size of the fluorescent nanoparticles is measured using dynamic light scattering (DLS) at 74.1 ± 6.7 nm. Afterward, the uptake of the nanoparticles is tested in human mesenchymal stem cells (hMSCs) and imaged using fluorescence and confocal microscopy. The uptake of the nanoparticles is observed in hMSCs, suggesting the potential for such a stable drug delivery system for falcarindiol.

INTRODUCTION:

Lipid-coated nanoparticles are seeing an increased interest regarding their function as drug delivery systems for cancer therapy[1]. Cancers have an altered lipid-metabolic reprogramming[2] and an increased need for cholesterol to proliferate[3]. They overexpress LDLs[1] and take in more LDLs than normal cells, to the extent that a cancer patient's LDL count can even go down[4]. LDL uptake promotes aggressive phenotypes[5] resulting in proliferation and invasion in breast cancer[6]. An abundance of LDL receptors (LDLRs) is a prognostic indicator of metastatic potential[7]. Inspired by the LDL and its uptake by cancer cells, a new strategy has been called: *Make the drug look like the cancer's food*[8]. Thus, these new nanoparticle drug delivery designs[8–10] have been inspired by the core- and lipid-stabilized design of the natural LDLs[11] as a mechanism for delivering anticancer drugs to cancer cells. This passive targeting delivery system supports the encapsulating of, especially, hydrophobic drugs, which are usually given in oral dosage form but provide only a small amount of the drugs to the bloodstream, so limiting their expected efficacy[12]. As with the stealth liposomes[13], a polyethylene glycol (PEG) coating helps to reduce any immunologic response and extends the circulation in the bloodstream for optimum tumor uptake by the purported enhanced permeation and retention (EPR) effect[14,15]. However, in addition to, in some instances, instability in the circulation and undesirable distribution in the system[16], some obstacles remain unsolved, such as how and to what extent such nanoparticles are taken in by cells and what is their intracellular fate. It is here that this paper addresses the nanoparticle uptake of a particular hydrophobic anticancer drug falcarindiol, using confocal and epifluorescence imaging techniques.

The aim of the project is to fabricate lipid-coated nanoparticles of falcarindiol and to study their uptake in hMSCs, thus assessing a new delivery system for this anticancer drug, in order to stabilize its administration and overcome the delivery difficulties, as well as to improve its bioavailability. Previously, falcarindiol has been administrated orally via a high concentration purified falcarindiol added to food[17]. However, there is need for a more structured approach to deliver this promising drug. Therefore, falcarindiol nanoparticles, with a phospholipid and

cholesterol encapsulating monolayer with the purified drug constituting the core of the particle, were designed. The rapid injection method of solvent shifting, as recently developed by Needham et al.[8], is used in this study to encapsulate the polyacetylene falcarindiol.

The method has been already used before for the fabrication of lipid nanoparticles to encapsulate diagnostic imaging agents[18,19], as well as test molecules (triolein)[27] and drugs (orlistat, niclosamide stearate)[8,27,28]. It is a relatively simple technique when carried out with the right molecules. It forms nanosized particles, at the limit of their critical nucleation (~20 nm diameter), of highly insoluble hydrophobic solutes dissolved in a polar solvent. The solvent exchange is, then, accomplished by a rapid injection of the organic solution with an excess of antisolvent (usually, an aqueous phase in a 1:9 organic:aqueous volume ratio)[20,21].

The compositional design of the nanoparticles is also designed to bring advantages. The DSPC:Chol components provide a very tight, almost impermeable, biocompatible, and biodegradable monolayer, and the PEG provides a sterically stabilizing interface which acts as a shield from opsonization by the immune system, slowing any uptake by the reticuloendothelial system (liver and spleen) and protecting against the mononuclear phagocyte system, preventing their retention and degradation by the immune system, and hence, increasing their circulation half-time in blood[22]. This allows the particles to circulate until they reach and extravasate at diseased sites, such as tumors, where the vascular system is leaky, allowing EPR. The lipid coat also has a function. It allows scientists to have more control over the nanoparticles' size by kinetically trapping the core at its critical nucleus dimension[27–28]. Lipids also provide for various surface properties (including peptide targeting, which was not yet available for this project), a pure drug core, and a low polydispersity[22,27–28]. The method used for particle size analysis is DLS, a technique that allows researchers to measure the size of a large number of particles at the same time. However, this method can bias the measurements to bigger sizes, if the nanoparticles are not monodispersed[23]. This issue is assessed with the lipid coat as well. More details of these fundamental designs and the quantification of all characteristics are given in other publications[27–28].

The drug encapsulated in the nanoparticles is falcarindiol, a dietary polyacetylene found in plants from the Apiaceae family. It is a secondary metabolite from the aliphatic C_{17}-polyacetylenes type that has been found to display health-promoting effects, such as anti-inflammatory activity, antibacterial effects, and cytotoxicity against a wide range of cancer cell lines. Its high reactivity is related to its ability to interact with different biomolecules, acting as a very reactive alkylating agent against mercapto and amino groups[24]. Falcarindiol has previously been shown to reduce the number of neoplastic lesions in the colon[17,25], although the biological mechanisms are still unknown. However, it is thought that it interacts with biomolecules such as NF-κB, COX-1, COX-2, and cytokines, inhibiting their tumor progression and cell proliferation processes, leading to arresting the cell cycle, endoplasmic reticulum (ER) stress, and apoptosis[17,26] in cancer cells. Falcarindiol is used in this study as an example anticancer drug since its anticancer potential and mechanism are being studied, and because it shows promising anticancer effects. The uptake of the nanoparticles is tested in hMSCs and imaged using

epifluorescence and confocal microscopy. This cell type was chosen due to its large size, which makes it ideal for microscopy.

PROTOCOL:

1. Nanoparticle synthesis by rapid solvent shifting technique

1.1. Set up the following for the nanoparticles' preparation: a block heater/sample concentrator, a desiccator, a digital dispensing system with a 1 mL glass syringe, a 12 mL glass vial, a magnetic stirrer, a magnetic flea (15 mm x 4.5 mm, in a cylindrical shape with polytetrafluoroethylene [PTFE] coating) inside the glass vial, and a rotatory evaporator.

1.2. Dispense 2.4 mL of 250 µM falcarindiol stock dissolved in 70% EtOH water mixture in a scintillation vial.

1.3. Evaporate the liquid fraction, using the sample concentrator for approximately 4 h, to obtain dry falcarindiol.

1.3.1. Insert the scintillation vial in the block heater; the sample concentrator delivers gas over the sample using stainless steel needles, concentrating the sample. Evaporate at room temperature; do not use heat.

1.4. Once dried, add the following components of the lipid coating into the above-mentioned scintillation vial: 16.3 µL of 31.64 mM DSPC chloroform stock solution, 3.4 µL of 17.82 mM DSPE PEG 2000 chloroform stock solution, 24 µL of 25 mM cholesterol chloroform stock solution, and 6 µL of 4 mM DiI chloroform stock solution.

CAUTION: Immediately close the vials containing the lipids so that the solvent does not evaporate and, thereby, modify the concentration. Work in a fume hood.

NOTE: The concentrations of chloroform stock solutions can vary, depending upon the chemical supplier or dilutions made in the lab.

1.5. Leave the sample overnight in the desiccator to evaporate the chloroform.

1.6. Dissolve the desiccated sample in absolute ethanol to make a final volume of 1.2 mL, which gives final concentrations of DSPC, DSPE PEG 2000, cholesterol, and DiI of 0.43 mM, 0.05 mM, 0.5 mM, and 0.02 mM, respectively. This solution represents the organic phase.

1.7. Take the 12 mL glass vial, fill it with 9 mL of purified water, add the magnetic flea into the vial containing 9 mL of water, and keep the vial on the magnetic stirrer, stirring at 500 rpm (**Figure 1**).

1.8. Attach the 1 mL glass syringe to the dispensing system and clean it with chloroform to avoid any contamination. For this, slowly pull the chloroform in and push it out of the glass syringe, manually, at least 10x, dispensing the chloroform into its waste collector.

CAUTION: This must be done under a fume hood.

1.9. Prime the syringe with ethanol. Priming replaces the old solvent, as well as removes any air bubbles.

CAUTION: This must be done under a fume hood.

1.10. Using the syringe, aspirate 1 mL of the organic phase.

1.11. Insert the syringe into the glass vial, up to the middle of the 9 mL watermark, and maintain it steady in the middle of the vial (as shown in **Figure 1**).

1.12. Inject the solution at the selected speed of injection (833 µL/s) by pressing the dispense button on the dispensing system (**Figure 2**). This generates 10 mL of 50 µM lipid-coated nanoparticles of falcarindiol in 10% ethanol-containing water.

NOTE: This injection speed has been found to achieve the finest particles, obtaining a narrow particle size distribution. It is critical to make sure that the syringe is in the center, steady, and straight when dispensing the solution.

1.13. Immediately after the injection, remove the vial from the stirrer and transfer the sample to a 50 mL round-bottom flask (RBF).

1.14. Attach the RBF to the rotary evaporator and evaporate 1 mL of the organic solvent, using the rotary evaporator at room temperature. Avoid excess bubble formation.

NOTE: This step will take ~5 min.

1.15. Transfer the nanoparticle suspension from the RBF to another 12 mL glass vial. Ensure that the volume is 9 mL, and then, split the sample in two 12 mL glass vials (put 4.5 mL in each).

1.16. Add 0.5 mL of ultrapure water in one of the vials and 0.5 mL of 10x phosphate-buffered saline (PBS) in the other vial. Take out 1 mL of each sample for the particle size measurement.

2. Particle size analysis using the DLS technique

NOTE: Size measurements were carried out by using a DLS analyzer which determines particle size distributions. It is equipped with a 100 mW laser that operates at a wavelength of 662.2 nm and with an avalanche photodiode detector placed at a 90° angle to the incident angle. The beam is scattered by the nanoparticles and detected by the photodetector.

2.1. Turn on the DLS instrument and set the desired temperature at 20 °C, until it stabilizes.

2.2. Set the instrument parameters as follows: the data acquisition time = 4 s, the number of acquisitions = 30, the auto-attenuation function = On, and the auto-attenuation time limits = 0.

2.3. Fill the plastic cuvette with 1 mL of nanoparticle suspension and start the measurement.

2.4. Report the measured size depending on the used solvent (water or PBS).

NOTE: The measurement in PBS is made to have an approximate idea of the size of the cells in the medium when treating the cells. The cell treatment will be done with the nanoparticles dissolved in water.

2.5. Repeat the measurements 24 h after the synthesis, to check for particle aggregation.

3. Cell treatment

3.1. Grow hMSCs in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humified chamber at 37 °C with 5% CO_2.

3.2. Seed approximately 50,000 cells to obtain a cell density of approximately 30% on previously absolute-EtOH–sterilized #1.5 coverslips placed in 6-well plates. Add MEM in order to have a final volume of 3 mL in each well. Incubate for 24 h under the same conditions as in step 3.1. Seed the cells 24 h before the treatment.

NOTE: It is critical the cells are seeded at least 24 h before the nanoparticle treatment, to make sure that the cells are in an adequate confluence.

3.3. Without removing the medium, add 3 µL of the nanoparticle solution, for a final falcarindiol concentration of 5 µM. Incubate for 24 h in the same conditions as in step 3.1.

NOTE: The nanoparticles' preparation was carried out on the day of the cell treatment, to avoid particle aggregation.

3.4. Subsequently, after 24 h of treatment, wash the cells 2x with PBS, fix them in 4% formaldehyde for 10 min at room temperature, and store them in PBS at 4 °C for up to several months until imaged.

3.4.1. Alternatively, after fixation, a 4',6-diamidino-2-phenylindole (DAPI) nuclear staining can be performed. For this, after fixing the cells, permeabilize them with 0.1% Triton X-100 for 30 min, wash them 2x with PBS, and stain them with 250 µL of 300 nM DAPI.

4. Microscopy

4.1. Fluorescence microscopy

4.1.1. Use a widefield fluorescence microscope equipped with an electron-multiplied CCD camera to acquire images. Use the 150x NA 1.45 oil objective and the EGFP LP channel.

4.2. Confocal microscopy

4.2.1. Acquire confocal microscopy images, using the 63x NA 1.4 oil objective, an Argon laser (514 nm) for DiI, and a two-photon laser (780 nm) for DAPI, to verify the uptake of the nanoparticles into the cells.

REPRESENTATIVE RESULTS:

Two different types of nanoparticles were fabricated, namely pure falcarindiol nanoparticles and lipid-coated falcarindiol nanoparticles. Various concentrations of lipids and cholesterol were tested. As shown in **Table 1**, uncoated nanoparticles formed in water and measured in PBS had a diameter of 71 ± 20.3 nm with a polydispersity index (PDI) of 0.571. Those parameters were measured on a DLS analyzer. The lipid-coated nanoparticles of falcarindiol used in the experiments, and so including the fluorescent dye, DiI, were of a similar size, namely 74.1 ± 6.7 nm; however, they were found to be relatively monodispersed and had a lower PDI of 0.182, which indicates a smaller distribution of particle sizes, since PDI describes the size distribution of the nanoparticle population. Generally, when fabricating nanoparticles for pharmaceutical purposes, a PDI below 0.3 is desired.

Following the fabrication, the particle size was measured after 3 h and after 24 h, times based on the delay required for the addition of nanoparticles to the cell culture. The data of the 24 h measurements is not shown in this manuscript as it will be reported in another study, but no aggregation was observed, but it is recommended to test for particle stability after 24 h. After confirming the size stability of the lipid-coated nanoparticles, DiI-labeled, lipid-coated nanoparticles were fabricated by following the protocol and, eventually, used for the uptake study. For every study, a fresh nanoparticle sample was prepared. A schematic of the final falcarindiol nanoparticles' structure is shown in **Figure 3**, and the particle's size data after fabrication is shown in **Table 1**, as well as the measurement taken 3 h after fabrication.

As a first observation of the nanoparticles inside the cells, epifluorescence microscopy images were acquired after 24 h of treatment. The nanoparticles were visualized as white bright dots, and it could be hypothesized that nanoparticles were located inside the cells, surrounding the nucleus (**Figure 4A**).

To verify that falcarindiol nanoparticles had entered the cells, confocal microscopy was performed on hMSCs treated for 24 h. It is shown that nanoparticles had entered the cells, and a large number of nanoparticles were scattered in the cytoplasm in every cell (**Figure 4B-D**). These results show that nanoparticles act as a stable drug delivery system for falcarindiol.

FIGURE AND TABLE LEGENDS:

Figure 1: Nanoparticles preparation setup showing assembly for injection under stirring[27]. The setup consists of the autopipette with a 1 mL glass syringe filled with 1 mL of the ethanolic solution containing the nanoparticles' components. The glass vial contains 9 mL of water and the magnetic flea is placed on the magnetic stirrer.

Figure 2: Schematic of the mixing of solvents in the rapid injection method of solvent shifting[27]. Panels show the injection of 1 mL of ethanolic phase containing nanoparticles' components at a speed of 833 µL/s into 9 mL of water while stirring at 500 rpm. The rapid injection with chaotic mixing of the ethanolic solution containing the nanoparticles' components (falcarindiol, DSPC, cholesterol, DSPE PEG 2000, DiI) into the antisolvent (water), leadd to the formation of the nanoparticles. The color is given by DiI. It can be observed how the ethanolic solution is mixed, rapidly increasing its concentration. The nanoparticles are formed.

Figure 3: Schematic of the final falcarindiol nanoparticles' structure. This figure shows the nanoparticle structure, including DSPC, DSPE PEG 2000, cholesterol, DiI, and falcarindiol. The different components are scaled according to their concentrations.

Figure 4: Images of lipid-coated falcarindiol nanoparticles in human mesenchymal stem cells. (**A**) Epifluorescence microscopy image of hMSCs treated with falcarindiol nanoparticles for 24 h. The following panels show confocal microscopy images of the hMSCs treated with falcarindiol nanoparticles for 24 h: (**B**) DAPI stain of nuclei, (**C**) DiI nanoparticles, and (**D**) an overlay of both images, in which the nuclei are shown in blue and the nanoparticles in red. The scale bars are 10 µm. Nanoparticles are visualized as white bright dots in the cell cytoplasm. The images show that a great number of nanoparticles had already entered the cells after 24 h of incubation.

Table 1: Different designs of the fabricated nanoparticles. The size and polydispersity index of the synthesized falcarindiol nanoparticles, depending on the solvent and nanoparticle type. Falcarindiol nanoparticles with and without a lipid coat were fabricated. Various concentrations of the coat components were tested. The particle aggregation was tested after 3 h.

DISCUSSION:

A detailed protocol for fabricating lipid-coated nanoparticles for drug delivery with the simple, fast, reproducible, and scalable rapid injection method of solvent shifting was followed[27–28] and is presented in this paper, as applied to falcarindiol. By controlling the speed of the injection of the organic phase into aqueous phase and by using coating lipids at appropriate concentrations to coat the falcarindiol core, fine sub-100 nm particles could be obtained successfully. The possibility of induced polydispersity due to the involvement of turbulent mixing for the falcarindiol precipitation alone was controlled by the presence of coating lipids. The structure of these lipid-coated nanoparticles resembled the low-density lipoproteins (except for the obvious exclusion of the native 500,000 kDa ApoB100 itself and the additional presence of PEG-lipids for steric stability). This passively targeted drug delivery system allows the encapsulation

of a broad range of especially hydrophobic drugs and diagnostic materials[18,19], reducing the immunologic response and accumulating in cancerous tissues[16,18]. Furthermore, depending on the drug degradation reactions (e.g., hydrolysis, enzymolysis), it also avoids degradation of the drug during circulation in vivo.

Therefore, falcarindiol nanoparticles, with a lipid-encapsulating monolayer containing a pure core of the purified drug, were designed and fabricated. The lipid monolayer coating consisted of DSPC, cholesterol, and DSPE-PEG 2000, with the fluorescent label DiI. The rapid injection method of solvent shifting was used to fabricate them, which consists of a rapid injection of an ethanolic solution containing the nanoparticles components into an excess of aqueous phase (1:9). The size of the nanoparticles was measured using DLS, and the uptake of the nanoparticles was tested in hMSCs and imaged using fluorescence and confocal microscopy.

While uncoated nanoparticles can also be obtained, their sizes were 71 ± 20.3 nm. However, after following the protocol described above, nanoparticles of 74.1 nm ± 6.7 nm, with polydispersity values of 0.182, were obtained. Thus, after modifying the nanoparticles by adding the lipid coat, the size of the nanoparticles was reduced, as well as the PDI of the nanoparticles, making them more suitable for drug delivery.

It is important to be highly aware of the critical steps in the protocol, such as the importance of the syringe position when injecting the organic phase, the preparation of the nanoparticles on the same day of treatment to avoid aggregation, and the seeding of the cells the day before to ensure enough confluence. As a matter of fact, all the steps in the first part of the protocol can be considered critical since they either affect the size of the nanoparticles or the final concentration of the active compound or coating lipids. Considering 'concentration' as an important parameter, steps 1.2, 1.4, 1.6, 1.15, and 1.16 are critical. Considering 'nanoparticle size' as an important parameter, steps 1.7, 1.11, and 1.12 are critical.

Fluorescence and confocal microscopy showed that nanoparticles had entered the cells, and a large number of nanoparticles were scattered in the cytoplasm of every cell. These results suggest that nanoparticles are a new, stable drug delivery system for falcarindiol.

This technique provides a simple, fast, and reproducible approach to encapsulate different cancer drugs, and the limitations of the method are assessed with the lipid coat.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 4

Click here to access/download;Figure;Final Figure 4.tif

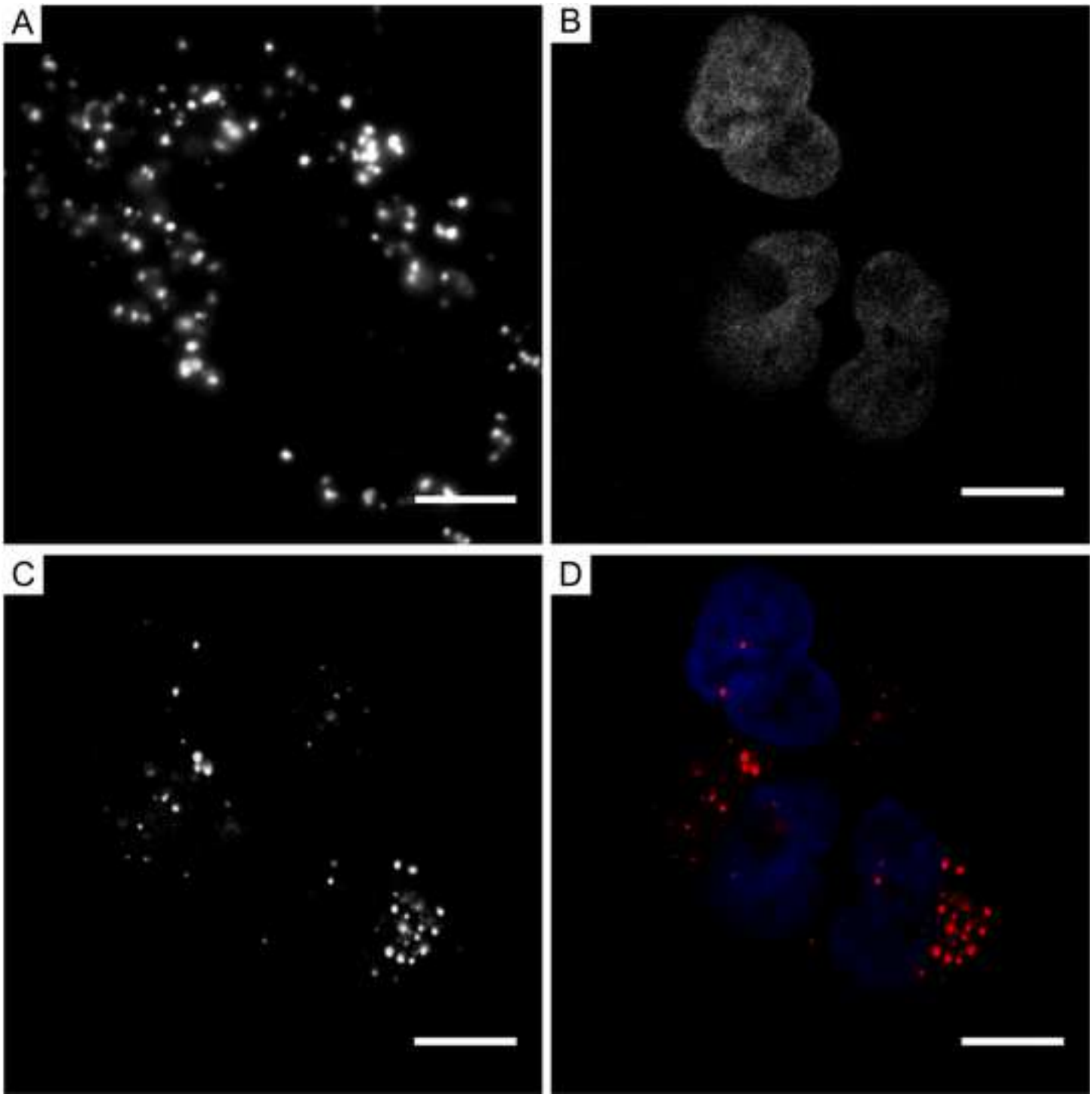

fig 1

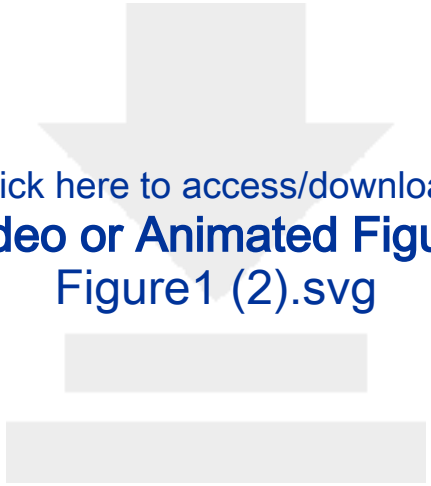

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Figure1 (2).svg

fig 2

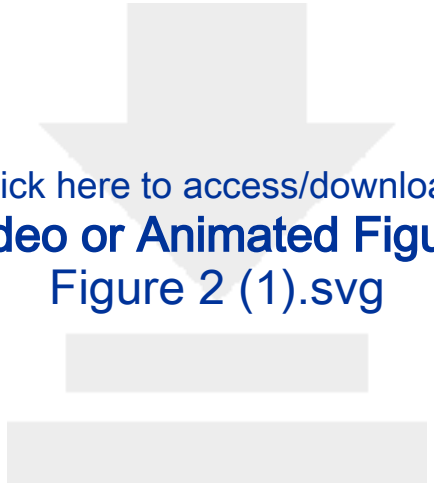

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Figure 2 (1).svg

fig 3

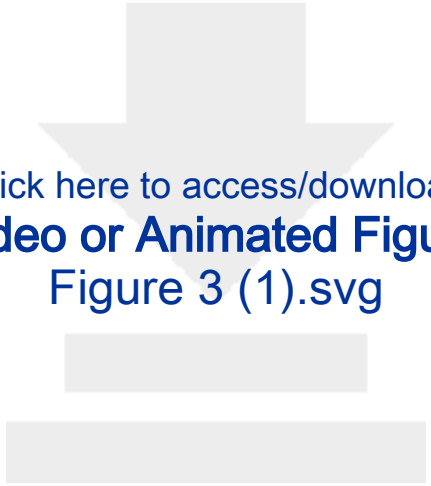

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Figure 3 (1).svg

Table

Type of nanoparticles	Solvent	Nanoparticle size (nm)	Polydisperisty (nm)
Uncoated Falcarindiol	Water	83.9	± 23,9
Test	PBS	71	± 20,3
Lipid coated Falcarindiol	PBS	91.6	± 6,4
	PBS after 3h	93.5	± 5,7
Lipid coated Falcarindiol + DiI	PBS	74.1	± 6,7

Polydispersity Index (PDI)
0.571
0.571
0.141
0.122
0.182

Name of Material/ Equipment	Company	Catalog Number
12 mL Screw Neck Vial (Clear glass, 15-425 thread, 66 X 18.5 mm)	Microlab Aarhus A/S	ML 33154LP
6 well plates	Greiner Bio One International GmbH	657160
Absolute Ethanol	EMD Millipore (VWR)	EM8.18760.1000
Chloroform	Rathburn Chemicals Ltd.	RH1009
Cholesterol	Avanti Polar Lipids, Inc.	700000P
Confocal Microscope	Zeiss LSM510	
Cover Slips thickness #1.5	Paul Marienfeld GmbH & Co	117650
Desiccator	Self-build	
DiI	Invitrogen	D282
DLS	Beckman Coulter	DelsaMAXpro 3167-DMP
DSPC (Chloroform stock)	Avanti Polar Lipids, Inc.	850365C
DSPE PEG 2000 (Chloroform stock)	Avanti Polar Lipids, Inc.	880120C
eVol XR	SGE analytical science, Trajan Scientific Australia Pty Ltd.	2910200
Fetal Bovine serum	Gibco	10270-106
Fluorescence Miccroscope	Olymous IX81	
Incubator	Panasonic	MCO-18AC
Magnetic flea	VWR Chemicals	15 x 4.5 mm
Magnetic stirrer	IKA	RT-10
Minimum Essential Media	Gibco	32561-029
PBS tablets for cell culture	VWR Chemicals	97062-732
Pen/strep	VWR Chemicals	97063-708
Phosphate Buffer Saline (PBS, pH 7.4)	Thermo Fisher	10010031
Rotary Evaporator	Rotavapor, Büchi Labortechnik AG	R-210
Sample concentrator	Stuart, Cole-Parmer Instrument Company, LLC	SBHCONC/1

Comments/Description

With Manual TIRF and Andor iXon EMCCD

Cylindrical shape with PTFE coating

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Author(s): E PIBIRI-OLLE, P WALKE, M NOTABI, RB EL HOURI, M OSTERMANN D NEEDHAM, EL ARNSPANG.

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Dear Editor. Thank you for all the comments. We have changed the manuscript according to all the comments, below are answers for the questions in italic.

Answers are in italic.

1. Protocol language: change to the imperative tense.

Protocol language has been changed to the imperative tense.

2. What are the concentrator parameters/settings? Mention centrifugation speed (in g), filter size etc.

The concentration is not done by centrifugation. A more accurate description of the process has been added.

3. How much of each is added?

The exacts amount have been added, although the final concentrations needed were mentioned in 1.5.

4. Is this under a fume hood? Please add a note of caution from chloroform usage.

Caution notes for chloroform and other organic solvents usage have been added were needed.

5. Unclear how the cleaning is performed. Mention care to be taken. Is this done under a hood?

A more detailed description of the cleaning procedure has been added.

6. Please add citations to the previous studies. Incubate under same conditions as in 3.1?

The citation has been added. It is a not published yet manuscript. And same conditions has been specified.

7. What is the temperature for storage? For how long can they be stored?

The information has been added.

8. Protocol highlight:

Our protocol is no longer than 3 pages, therefore no text has been highlighted, everything should be included.

9. Discussion:

The different parts have been clarified, and some information has been added.

   a. *Modifications and troubleshooting: adding lipid coat.*
   b. *Limitations of the technique:*
   c. *Significance with respect to existing methods*
   d. *Future applications: approach to encapsulate cancer drugs*
   e. *Critical steps within the protocol: organic phase injection, time of preparation of nanoparticles and time of seeding cells. All the steps are critical as they either affect the size of the nanoparticles or final concentration of the active ingredient or coating lipids. Considering 'concentration' as important parameter, step 1.1, 1.3, 1.5, 1.13 and 1.14 are critical. Considering 'nanoparticle size,' step 1.6, 1.9 and 1.10 are critical steps.*

10. Commercial language

It has been deleted. Abbreviations have been defined, all figures are original.

Answers to all peer review comments follow:

11. It is not clear whether this protocol is extended from a previous publication validating the efficacy of falcarindiol-coated nanoparticles or this manuscript is their first report describing this particular nanoparticle.

Yes, there are two publications that involve the nanoparticle fabrication method mentioned in the current paper. (References 5 and 6).

12. Critical steps or potential technical issues of the described technique have not been addressed through the protocol section.

Organic phase injection, time of preparation of nanoparticles and time of seeding cells. A part from these, there are no other critical steps. All the steps are critical as they either affect the size of the nanoparticles or final concentration of the active ingredient or coating lipids. Considering 'concentration' as important parameter, step 1.1, 1.3, 1.5, 1.13 and 1.14 are critical. Considering 'nanoparticle size,' step 1.6, 1.9 and 1.10 are critical steps.

13. Additional intro or discussion should be included to explain why human stem cells (MSC) instead of cancer cell is chosen for in vitro study. The author has mentioned that falcarindiol is an anticancer drug, and lipid coated nanoparticles can target cancer cells specifically due to the need of cancer cells for cholesterol. Therefore, it is not quite logical to use human stem cells as in vitro system to test how falcarindiol-coated nanoparticle can be taken by cells.

They were chosen because they are large and suitable for microscopy.

14. Falcarindiol is not the common anticancer drug, why would you choose it?

It is the drug that the group was working with, it is used as an example anticancer drug to fabricate the nanoparticles.

15. Why used hMSC to verify the delivery of anticancer drugs?

They were chosen because they are large and suitable for microscopy.

16. Please explain the reason that 833μL/s was selected as the injection flow rate.

Optimization of the protocol in Prasad Walke's PhD thesis. Its the highest speed that can be achieved with eVol XR® system. We tested the effect of all the speeds that can be achieved using this system and the finest particles with narrow particle size distribution were obtained at the highest speed, which is 833 μL/s.

17. According to the protocol of cell treatment, I suggest the time point of stability experiment in vitro should be extended to the 24h at least.

This time point was also checked and no aggregation was observed. Data is shown in another manuscript under preparation.

18. In Figure 3, Please pay attention to the difference between drawing DSPE-PEG and PEG.

It has been changed.

19. In Figure 4, I can't clearly distinguish between cells and nanoparticles. It is recommended that the nucleus be stained with DAPI, and the Dil should emit orange-red fluorescence after being stimulated.

Figure 4 now has the nucleus DAPI stain and the DiI nanoparticles separate images and the overlay of the two.

Dear Editor. Thank you for your work. We have answered all your comments.

Answers are in italic.

1. The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review (preferably use a proficient English speaker) the manuscript and edit any errors.

Grammar has been checked.

2. Why new?

Because the rapid injection method is new.

3. Please fill and sign the attach License agreement. Please also confirm the correct access type: Open vs Standard.

The file was sent to the editor on email. It is uploaded again.

4. I have reduced this to 50 words

Thank you for that.

5. All the cells you image have been fixed, please change this.

It has been changed.

6. I think this can be deleted.

It has been deleted.

7. I added this, is it correct?

It is correct.

8. What is the source? Please add it to the table of materials.

Cells were provided by Dr. Moustapha Kassem, it is mentioned in acknowledgments.

9. At what cell density? What are the incubation conditions for the 24 h hours after seeding? Is MEM used? If so, how much?

It has been clarified.

10. Is the culture media removed prior to this addition?

It has been clarified.

11. Are the fixed cells mounted on microscopy slides?

Yes, on the #1.5 cover slips where the cells were seeded in step 3.2.

12. Please remove/replace the commercial name

It has been removed.

13. DAPI has an absorption peak at ~400 nm. It is unclear how the 780 nm laser can be used to excite it.

Thank you for this comment. We have clarified in the text, it is a two-photon laser.

14. It is unclear that both methods were demonstrated in the protocol (only lipid coated). Please reference the steps where pure falcarindiol particles were fabricated, and the steps where lipid-coated NPs were fabricated.

The protocol only shows the lipid-coated fabrication, but the same technique was used, only without adding the lipids for the pure falcarindiol nanoparticles.
Data for pure falcarindiol nanoparticles size is in table 1.

15. How was this measured?

The software gives this value.

16. What about the 3 h measurement?

The 3 h measurement is shown in table 1.

17. At 3 h?

Table 1 shows the sizes of the nanoparticles after fabrication and also a measurement after 3 h. We have tried to clarify it in the text.

18. At least a sample image must be provided. Unclear why it cannot be shown. You can include this as a figure or a supplementary file

It has been added.

19. Please provide this as a separate excel file.

It is uploaded now.

20. The file provided is clipped on the left and right sides. Please fix this.

We fixed it.

21. Define the pseudocolors red and blue.

They have been defined now.