Chapter 6

Cell Membrane Nanoparticles to Probe the Interactions between Stromal and Cancer Cells in Acute Myeloid Leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is one of the most common hematological cancers which remains difficult to treat because of chemoresistance and disease relapse. Increasing evidence has shown that by hiding in the bone marrow, a sub-population of cancer cells acquires chemoresistance and maintains stem cell-like properties for self-renewal. For this, tight interactions with the different cells composing the bone marrow niche and in particular the mesenchymal stromal cells are essential. Thus, novel strategies for therapy aim at targeting such interactions, although these are difficult to characterize. Within this context, here we have exploited cell membrane nanotechnology to prepare cell membrane nanoparticles from leukemia K562, THP-1 and MS-5 bone marrow stromal cells as a tool to probe the interactions between cancer and stroma. Nanoparticle preparation was optimized to increase the purity of extracted membranes and the obtained nanoparticles characterized to confirm inclusion of cell membrane components. Uptake studies were performed in order to compare the affinities between the different nanoparticles and target cells. Cell membrane-doped liposomes showed higher uptake efficiency in all tested cells than synthetic liposomes, and in particular the stromal MS-5 cell membrane-doped liposomes showed a preferential internalization in all the cell lines, including homotargeting effects on MS-5 cells themselves. This opens up the possibility of using stromal cell membrane nanoparticles not only as a tool to characterize the interactions of stromal cells with leukemia cells, but also to develop novel targeting strategies.

1. Introduction

Acute myeloid leukemia (AML) is a common and highly heterogeneous cancer of the myeloid line of blood cells characterized by proliferation and/or accumulation of abnormal clones, that interfere with normal blood cells. To date, traditional chemotherapy is used as the most efficient treatment of AML [1–5], but it can only cure 40-45% young adults and 10-20% older adults [6,7]. One of the key reasons for treatment failure is the acquisition of chemoresistance [8]. Additionally, relapses that often occur after the initial remission further decrease the poor survival rate [9]. Also,
the genetic and epigenetic heterogeneity of AML among or within individual patients further limits the possibilities of developing a general strategy to target AML [5,10,11]. Since Schofield first introduced the concept of ‘niche’ in 1978 [12], there has been increasing evidence indicating that the bone marrow microenvironment (the niche) plays an important role in the development and evolution of this disease [5,7,13]. It has been reported that in the niche, leukemia progenitor cells and bone marrow mesenchymal stromal cells are tightly associated through secreted factors, released exosomes, and via direct cell-cell interactions [13,14]. The complex interactions of leukemia cells with stromal cells can affect the development, progression, chemoresistance and relapse of AML [15–17]. Furthermore, compared with the high heterogeneity of AML cell populations, the heterogeneity of bone marrow mesenchymal stromal cells has been found to be minimal across subtypes and in general the AML-stromal interactions are altered as a consequence of upregulation of adhesion factors such as integrins and adherins [9,18,19]. Therefore, in order to efficiently treat AML, new strategies aiming at targeting the AML-stroma interactions have been proposed and several new drugs are already in different clinical trial stages [5]. However, the molecular details of the interactions between leukemia cancer cells and mesenchymal stromal cells remain poorly characterized. Identifying key players in such interactions can help the development of efficient targeting strategies. Nanotechnology may provide useful tools towards these goals.

Nano-sized materials have been widely developed as effective carriers for drug delivery [20,21]. Among them, cell membrane-camouflaged nanoparticles have emerged as a promising biomimetic platform, which exploits biologically derived components from cell membranes for the functionalization of synthetic nanoparticles [22–25]. Cell membranes which have been used to modify nanoparticles include plasma membranes from erythrocytes, platelets, leukocytes, mesenchymal stem cells and tumor cells [23–27]. These biomimetic nanoparticles, fabricated using different methods, allow to achieve specific interactions with complex biological environments. For example, it has been shown that leukocyte membrane-doped liposomes can mimic some of the features of leukocytes, thus allow to target inflamed vasculature [25]. Erythrocyte membrane-coated nanoparticles can camouflage nanoparticles, conferring them prolonged circulation time [28]. Additionally, it has
been proposed that cancer cell membrane coated nanoparticles allow cancer cell self-recognition and homotypic targeting [27].

In this study, cell membrane nanotechnology has been used as a novel tool to probe the interactions between leukemia cells and stromal cells. By preparing cell membrane nanoparticles from different cells, biomimetic nanoparticles with improved uptake efficiency in specific leukemia or stromal cells can be selected. These can be used on the one hand to identify specific proteins involved in the interactions of leukemia cells and its niche, and on the other hand to develop novel strategies to target them. Thus, as a first step, cell membranes extracted from human K562 and THP-1 leukemia cells and murine MS-5 mesenchymal stromal cells were used to prepare cell membrane nanoparticles and test preferential interactions with the different cell types. Procedures for cell membrane extraction were optimized to reduce the presence of impurities and contaminants. Then, the extracted cell membranes were incorporated on the surface of plain silica nanoparticles or in synthetic liposomes. To confirm the purity of cell membranes and their incorporation in synthetic nanoparticles, the derived cell membranes and the resulting cell membrane nanoparticles were characterized using cryo-TEM, UV-vis absorption, thin layer chromatography, and western blotting, as well as size distribution by dynamic light scattering and zeta potential measurements. Thus, uptake kinetics of the different cell membrane-coated nanoparticles on leukemia K562, THP-1 and TF-1 cells and stromal MS-5 cells were determined and uptake efficiency compared in order to select nanoparticles with improved affinity for specific cell types.

2. Materials and methods

2.1 Cell culture

The myelogenous leukemia cell line K562 cells (ACC-10, DSMZ), the acute monocytic leukemia cell line THP-1 cells (ATCC TIB-202) and the human erythroleukemic cell line TF-1 cells (ACC-334, DSMZ) were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco Thermo Fisher Scientific) supplemented with 10% v/v Fetal Bovine Serum (FBS) (Gibco Thermo Fisher Scientific). For TF-1 cells 5 ng/ml granulocyte-macrophage colony stimulating factor
(GM-CSF) (Genetics Institute, Cambridge, MA, USA) was also added. The murine stromal cell line MS-5 cells (ACC-441, DSMZ) were grown in alpha MEM (Lonza) supplemented with 10% v/v FBS. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37 ºC and used for maximum 20 passages. Mycoplasma testing was performed every month to exclude mycoplasma contamination.

2.2 Cell membrane extraction

In order to extract the cell membrane, at least 20 million cells were collected for each cell line. K562 and THP-1 cells growing in suspensions were harvested from completed RPMI 1640 medium by centrifugation at 500 g for 5 min followed by resuspension in PBS and centrifugation at 500 g for 5 min for other 3 times. Adhering MS-5 cells were rinsed with PBS once, detached by incubation with 2 mM Ethylenediaminetetraacetic acid (EDTA) (Merck) in PBS for 30 minutes, followed by resuspension in PBS and centrifugation at 500 g for 5 min for other 3 times. The recovered cells were then suspended in a cold hypotonic lysing buffer (pH = 7.5) containing 20 mM Tris-HCl (Promega Corporation), 2 mM MgCl₂ (Merck) and 1×EDTA-free protease inhibitor (Roche) followed by cell disruption using a Dounce homogenizer for 30 times. Then, the homogenized solution was centrifuged at 3200 g for 5 min, the supernatant was collected for further use and the pellet was resuspended in lysis buffer followed by other 2 homogenization for 30 times and centrifugations in the same way (thus a total of 3 homogenizations). All the collected supernatants were pooled together and centrifuged at 20’000 g for 20 min. The obtained supernatant was further centrifuged at 100’000 g for 1 h and the pellet containing the isolated cell membrane fraction was collected and stored in -20 ºC for further characterization.

In order to optimize the cell membrane isolation protocol and increase the purity of the cell membrane sample, a sucrose cushion was applied as an extra step for sample cleaning. For this, 0.2 M sucrose (Mp Biomedical, LLC) was added to the cold hypotonic lysing buffer (pH = 7.5) and all following steps were performed as described above. Thus, the supernatants recovered from the homogenization steps followed by centrifugation at 3200 g for 5 min and 20’000 g for 20 min were layered on the top of 2 M sucrose contained hypotonic lysing buffer and centrifuged for 2 h at 100’000 g. The top layer was diluted 6 times with 10 mM Tris-HCl (pH = 7.5) and
centrifuged for 1 h at 100’000 g. Finally, the obtained pellet containing the cell membrane fraction was collected and stored in -20 °C for further use. All the procedures mentioned above were performed at 4 °C.

2.3 Preparation of cell membrane nanoparticles

An extrusion method developed by Zhang et al. [29][24] was used to prepare cell membrane coated silica nanoparticles. Briefly, cell membranes containing a total of 1 mg proteins were incubated with 1 mg 100 nm plain silica nanoparticles (Sicastar, from Micromod Partikeltechnologie GmbH) followed by 5 min sonication in a bath sonicator and co-extrusion through a 400 nm polycarbonate membrane for 21 times and 200 nm polycarbonate membrane for at least 11 times using an Avanti Mini-Extruder (Avanti Polar Lipids). The extruded solutions were centrifuged at 10’000 g for 12 min. The supernatant containing free membrane lipids was discarded and the nanoparticle pellet was collected and resuspended in PBS. Silica nanoparticles coated with synthetic lipid bilayers were also prepared with the same procedure, as a control, by co-extruding 1 mg 100 nm plain silica nanoparticles and 1 mg lipids of 100 nm diameter liposomes containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol (Avanti Polar Lipids) in a 2:1 molar ratio. All the procedures were performed at 4 °C.

In order to prepare cell membrane doped liposomes, DOPC, cholesterol and 1,1´-Dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich) were first dissolved in chloroform and mixed in a molar ratio of 2:1:0.015. The mixed lipids were dried under a nitrogen stream and incubated under vacuum overnight. The cell membrane solution diluted in water was then used to rehydrated the dried lipids with a mass ratio of membrane proteins to synthetic phospholipids of 1:50 or 1:10, followed by 5 min sonication in a bath sonicator, snap freezing in liquid nitrogen and lyophilization using a Freeze Dryer ALPHA 1-4 (Martin Christ Gefriertrocknungsanlagen GmbH). The freeze-dried samples were dissolved in water, sonicated for 5 min in a bath sonicator and extruded through 400 nm and 200 nm polycarbonate membranes for 21 times each using an Avanti Mini-Extruder. Lipid films were also rehydrated with only water to prepare fully synthetic liposomes using the same procedures as a control. All the procedures were performed at 4 °C.
As an additional control on the cell membrane extract, the cell membrane obtained from roughly 50 million K562 cells were first suspended in 50 µl 10 mM Tris-HCl, then were diluted 10 times in PBS, and extruded through 200 nm polycarbonate membrane for at least 11 times using the Avanti Mini-Extruder (Avanti Polar Lipids).

2.4 Cryo-TEM

Cryo-TEM was used to check the purity of the recovered cell membranes, extracted from K562 cells with and without the use of sucrose cushions. For this, cell membrane pellets obtained from both procedures were suspended in 200 µl 10 mM Tris-HCl (pH = 7.5).

A few microliter of the suspension was deposited on a holey carbon coated copper grid (Quantifoil 3.5/1, Quantifoil Micro Tools). The grids were vitrified in liquid ethane after blotting the excess liquid (Vitrobot, FEI) and transferred to a FEI Tecnai T20 cryo-electron microscope equipped with a Gatan model 626 cryo-stage operating at 200 keV, with images recorded under low-dose conditions using a slow-scan CCD camera. Cryo-TEM was also used to check the coverage of K562 cell membrane coated silica nanoparticles and DOPC coated silica nanoparticles using the same procedure.

In order to detect eventual impurities in the extracted membranes, UV-vis absorption was used. For this, 2 µl of the K562 cells membrane suspension were loaded on a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) and the absorbance from 220 nm to 350 nm wavelength was recorded to detect eventual impurities from nucleic acids with absorption at around 260 nm. Thin layer chromatography (TLC) was used to confirm the presence of lipids in the recovered samples [30][31]. Briefly, 6 µl of each sample, including a control 10 mg/ml DOPC liposomes, were deposited on non-activated TLC plates (silica gel 60 F254, aluminum sheets, Merck) and developed in a mixed solution of chloroform/methanol/water in a ratio of 45:45:10 (v/v). The plate were then dried in the air and stained by spraying with 2 M H2SO4 and heating at 100 ºC for 15 min.

2.5 Membrane protein concentration

In order to determine cell membrane protein concentration, cell membranes extracted with sucrose cushions from K562, THP-1 and MS-5 cells were resuspended
in 200 µl 10 mM Tris-HCl (pH = 7.4). Their protein concentration was determined using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc.) following manufacturer instructions. Briefly, a series of solutions containing 0.1 mg/ml to 3.2 mg/ml bovine serum albumin (BSA) were prepared in PBS to build a calibration curve. Samples of 5 µl were then mixed with the working reagents, incubated for 15 min at room temperature and their absorbance at 650 nm measured using a ThermoMAX microplate reader (Molecular Devices, LLC). The BSA standard curve was used to calculate the protein concentration of the different samples.

2.6 Western blot and SDS-PAGE of extracted cell membranes

In order to determine the potential enrichment of membrane proteins, western blot analysis was performed on cell membrane extracted from K562 cells, the whole cell lysate and the supernatant obtained after the 1 h 100'000 g centrifugation step. To prepare whole cell lysate, K562 cells were pelleted by centrifugation at 500 g for 5 min and lysed in 1 mL RIPA buffer (Merck) containing 100 µL 1×EDTA-free protease inhibitor solution. After 30 min incubation at 4 ºC, the suspension was centrifuged at 16'000 g for 2.5 min and the supernatant was collected. Samples corresponding to 28 µg proteins were run on a 10% SDS-PAGE gel at 120 V and the separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane at 300 mA for 90 min on ice. The membranes were blocked at room temperature for 2 h with 5% non-fat dry milk (Bio-Rad Laboratories, Inc.) or 5% bovine serum albumin (Sigma-Aldrich) dissolved in TBST (1 x Tris buffered saline (TBS) mixed with 0.1% v/v Tween-20) and incubated overnight at 4 ºC with a primary antibodies including mouse monoclonal anti-transferrin receptor (1:1000, Thermo Fisher Scientific), mouse monoclonal anti-GAPDH (1:10000, Cell Signaling Technology) and rabbit polyclonal anti-ERK 1/2 (1:1250, Cell Signaling Technology). The membranes were washed with TBST for 30 min, incubated with HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit, 1:2000) at room temperature for 2 h. The blotted proteins were stained with enhanced chemiluminescence (ECL) solution (GE Healthcare) and images were recorded using a ChemiGenius II bio-imaging system.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the membrane proteins of the cell membranes
extracted from K562, THP-1 and MS-5 cells. Samples corresponding to 20 µg membrane proteins were mixed with 4 × loading buffer (200 mM Tris-HCl, 400 mM DTT, 8% SDS, 0.4% bromophenol blue and 40% glycerol) to a final volume of 30 µl. The mixture was then boiled at 95 °C for 5 min and 30 µl loaded on a 10% polyacrylamide gel followed by a 1 h run at 120 V at room temperature. The gel was stained with a water/methanol/glacial acetic acid (5:4:1, v/v) solution containing 0.1% Coomassie blue R-250 under gentle shaking for 30 min and de-stained in hot ultrapure water until the gel background disappeared. Images were recorded using a ChemiDoc XRS (Bio-Rad).

2.7 Size distribution and zeta potential measurements

The size distribution by dynamic light scattering and zeta potential of the cell membrane nanoparticles, together with controls were measured using a Malvern Zetasizer Nano ZS. Additionally, the size distribution of the cell membrane isolated from 50 million K562 cells was also measured after suspension in 50 µl 10 mM Tris-HCl, 10 time dilution in PBS, and extrusion through a 200 nm polycarbonate membrane for at least 11 times using an Avanti Mini-Extruder (Avanti Polar Lipids).

Samples corresponding to 0.1 mg/ml silica for cell membrane coated silica nanoparticles or corresponding to 0.1 mg/ml lipids for cell membrane doped liposomes and synthetic liposomes were measured. For all samples, 3 measurements were performed at 20 °C with an automatic setting for the measurement duration.

2.8 Size exclusion chromatography

Size exclusion chromatography (SEC) was used to detect the DiI label and proteins in the cell membrane doped liposomes. Briefly, K562 cell membrane doped liposomes prepared as described above were loaded on a 12 × 1.5 cm column packed with Sepharose CL-4B (Sigma-Aldrich) in PBS. Fractions of 0.5 mL eluent were collected up to a total volume of 13 ml. For each fraction the absorbance at 280 nm and 550 nm was measured using a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) in order to detect, respectively, proteins and the DiI label.

2.9 Lipid concentration
A lipid assay was used to determine the concentration of DOPC and other phospholipids in the cell membrane doped liposomes based on the Stewart assay [32]. Briefly, a series of lipid standards containing from 0 mg/ml to 1 mg/ml DOPC in chloroform was prepared. A ferrothiocyanate reagent was prepared by dissolving 30.4 mg ammonium thiocyanate (Sigma Aldrich) and 27.0 mg ferric chloride hexahydrate (Sigma Aldrich) in 1 mL Milli-Q water. Samples of 20 µL liposomes or standards were then mixed with 1 mL chloroform and 1 mL ferrothiocyanate reagent, vortexed for 1 min and centrifuged at 300 g for 10 min. The organic phase at the bottom layer was transferred to a quartz cuvette and the absorbance at 470 nm was measured using a Unicam UV500 Spectrophotometer (Unicam Instruments). The DOPC standard curve was used to calculate the lipid concentration.

2.10 Uptake studies by flow cytometry

Nanoparticle uptake by cells was determined by flow cytometry. K562 cells, THP-1 cells and TF-1 cells were seeded at a density of 1 × 10^5 cells/well in a 24 well plate in complete RPMI medium. For MS-5 cells, 2.5 × 10^4 cells/well were seeded in complete alpha MEM. Twenty four hours after seeding, 10 µg/ml silica of membrane coated silica nanoparticles or plain silica were exposed to K562 cells, THP-1 cells and MS-5 cells after dispersion in the corresponding medium. In the case of cell membrane doped liposomes, cells were exposed to samples corresponding to 50 µg/ml DOPC lipid and – as a control – 50 µg/ml pure DOPC liposomes. After different exposure times, cells were washed once with complete medium and twice with PBS to remove excess nanoparticles outside cells. Cells in suspension were then collected and adhering MS-5 cells harvest by incubation for 5 min with 0.05% trypsin-EDTA at 37 °C. The collected cells where then pelleted by centrifugation for 5 min at 300g, resuspended in 100 µl PBS, and measured immediately using a Cytoflex S Flow Cytometer (Beckman Coulter) using a 561 nm laser for quantification of nanoparticle fluorescence. Gates were set in the two dimensional plots of forward scattering against size scattering in order to choose live cells and singlets. For each condition 2 samples were prepared and for each sample at least 20k cells were recorded. FlowJo software (FlowJo, LLC) was used to extract the median cell fluorescence of the obtained distributions and the average and standard deviation over the two replicate samples calculated.
3. Results and discussion

3.1 Optimization of cell membrane extraction

Because of their fast proliferation, K562 cells were used to optimize the procedure for cell membrane extraction. Generally, most protocols start with cell lysis, followed by removal of nuclei and cell debris by low speed centrifugation, and isolation of a crude membrane fraction containing all types of membranes by ultracentrifugation [29,33–38] (Some studies using the pellet after the first low speed centrifugation to prepare nanoparticles are also reported [27,39,40]). Here, as a first step, similar procedures were followed, as previously published and illustrated in Fig. 1A(i) [29].

However, cryo-TEM of the recovered samples showed a strong background with objects of around 30 nm diameter (Fig. 1B-C and Supplementary Fig. S1A) and UV-vis absorption spectra had a clear peak at around 260 nm, usually attributed to nucleic acids (Fig. 1D). These results suggested that the impurities observed by cryoTEM were possibly ribosomes, which have a diameter between 25 and 30 nm [41]. Similar contaminations were also observed by Tasciotti et al. [42][25]. In their studies, mass spectrometry analysis revealed that around 50% of the proteins identified on leukocyte membrane coated nanoporous silicon particles or leukocyte membrane protein integrated liposomes belonged to the cell membrane, while the rest of the proteins were primarily from ribosomes and mitochondria [25,42]. The presence of these impurities could complicate the following preparation of cell membrane nanoparticles.

Thus, further purification steps have been introduced. Usually, the purity of cell membrane preparations can be increased by separating either the post-nuclear supernatant or the crude fractions by ultracentrifugation in a discontinuous sucrose gradient or on a sucrose cushion, taking advantage of the different ratios between lipids and proteins in different cellular membranes [33,43–45]. Thus, here, the post-nuclear supernatant containing the crude cell membrane extract was layered on top of a sucrose cushion for further cell membrane isolation as described in Methods and shown in Fig. 1A(ii). The cell membrane pellet collected after sucrose purification and ultracentrifugation were characterized again with cryo-TEM and UV-Vis.
absorption (Figs. 1C-D and Supplementary Fig. S1B). Cryo-TEM clearly confirmed isolation of membranes and in this case no background was observed (Fig. 1C and Supplementary Fig. S1B), and similarly, no absorption at 260 nm (Fig. 1D). Thin layer chromatography (TLC) was also used to further compare the two isolation procedures. As shown in Supplementary Fig. S1C, in both cases the presence of lipids was clearly confirmed, however in the sample isolated without using sucrose cushions, a dark deposition that did not transfer with the organic phase was also visible where the sample was loaded, suggesting a contamination of proteins and other components [46]. Based on these results, the procedure including the additional purification step (Fig. 1A(ii)) was used to extract the cell membranes for nanoparticle preparation.

**Figure 1.** Optimization of cell membrane extraction. (A) Overview of the cell membrane extraction procedure without (i) and with (ii) an additional purification step using sucrose cushions. (B-C) Cryo-TEM images of the cell membrane samples isolated without and with the use of sucrose cushions, respectively (as illustrated in panels (i) and (ii)). (D) UV/Vis absorption spectra of the cell membrane samples isolated following the procedure illustrated in panel A(i) or (ii). The results showed that without the additional purification step using sucrose cushions a background of many objects of around 30 nm was observed (B), as well as absorbance at 260 nm (D), suggesting the
presence of contaminants including nucleic acids, likely ribosomes. These were not present after the additional purification step (ii).

Thus, the purified membranes were further characterized after extrusion through 200 nm filters (note that cell membranes are very hard to extrude, because of the presence of membrane proteins). Dynamic light scattering (DLS) showed that objects with homogeneous size distribution around 160 nm (Fig. 2A) were obtained. Western blotting analysis of transferrin receptor (TfR) and the cytosolic proteins extracellular signal-regulated kinase (ERK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) confirmed enrichment of cell membrane proteins, in comparison to the whole cell lysate or the supernatant obtained after ultracentrifugation (Fig. 2B). However, the cytosolic proteins ERK and GAPDH were detected in all samples, including the cell membrane sample. This kind of impurities was already observed in similar studies, and is probably due to the self-assembly of cell membrane fragments and resulting encapsulation of cytosolic proteins during the isolation procedure [25]. SDS-PAGE of the extracted cell membranes showed that very different proteins were isolated from K562, THP-1 and MS-5 cell membranes (Fig. 2C).

Figure 2. Characterization of extracted cell membranes. (A) Size distribution by intensity of extracted K562 cell membranes after extrusion through 200 nm filters (see Methods for details). (B) Western blotting analysis of membrane and cytosolic markers. Western blots against the transferrin receptor (TfR), and the cytosolic ERK and GAPDH proteins on cell membranes extracted from K562 cells (line 1), the supernatant obtained after ultracentrifugation with sucrose cushions (line 2) and
a whole K562 cell lysate (line 3). (C) SDS-PAGE image of cell membranes isolated from K562, THP-1 and MS-5 cells. Homogeneous dispersions were obtained after extrusion (A) and western blot analysis confirmed enrichment of membrane proteins in the cell membrane extract (B). Very different proteins were recovered in the cell membranes extracted from different cells (C).

3.2 Preparation and characterization of cell membrane coated silica nanoparticles

As a next step, the extracted cell membranes were deposited on plain silica nanoparticles. A previously reported extrusion approach was used [24,29], thus the isolated cell membranes were coextruded with 100 nm plain silica nanoparticles (si-plain), first through 400 nm and then through 200 nm polycarbonate membranes with 1:1 mass ratio between membrane proteins and silica. As previously noted, extrusion of pure cell membranes is very hard because of the presence of proteins. As a consequence of this, quantification of silica fluorescence after extrusion showed that a large amount of silica (around 50-80% was lost in the extruder during the procedure. Nevertheless, DLS showed a slight increase of size of around 10 to 20 nm after coextrusion (Fig. 3A) and a decrease in the zeta potential to values comparable to K562 cell membrane vesicles (from –38 mV for plain silica, to –21 mV). The decrease in zeta potential was even lower when silica was coextruded with THP-1 and MS-5 cell membranes (Fig. 3B). Overall, the change in size and zeta potential suggested successful deposition of the cell membrane around the silica nanoparticles. However, cryo-TEM imaging showed that in many nanoparticles the membrane coverage was only partial and some silica without lipid bilayer were present (Figs. 3C-E). Increasing the amount of membranes in the extrusion (up to a mass ratio of 2:1 between membrane proteins to silica nanoparticles) did not improve these outcomes (data not shown). In contrast, as shown in Fig. 3F, coextrusion of DOPC liposomes and silica nanoparticles was much easier to perform and led to a complete coverage of the nanoparticle surface with a lipid bilayer. The different coverage efficiency was probably a consequence of the different rigidity between pure phospholipids (DOPC liposomes) and the cell membranes, because of the many proteins in the bilayer [47].

Uptake studies on K562, THP-1 and MS-5 cells of the membrane coated silica (named si-k562, si-thp1 and si-ms5, respectively) and – as an additional control for comparison - the plain silica nanoparticles (si-plain) showed that in all cases the
membrane coated silica had higher uptake efficiency than plain silica in all cells (Supplementary Fig. S2). This effect was reproduced in multiple experiments using different cell membrane extracts to prepare replicate cell membrane coated silica. However, for each nanoparticle preparation the 3 types of cell membrane coated nanoparticles had very different uptake efficiencies in each of the cells (also in Supplementary Fig. S2), suggesting that the overall approach and results were not reproducible. Many factors could lead to this irreproducibility, including - for instance - the incomplete silica coverage observed (Figs. 3C-E), or different orientation of the cellular membrane on the particles surface in different preparations [48].

**Figure 3.** Characterization of cell membrane coated silica nanoparticles. Briefly, 1 mg silica nanoparticles and cell membranes containing 1 mg proteins were co-extruded to prepare cell membrane coated silica as described in the Methods. (A) Size distribution by intensity (diameter, nm) of 0.1 mg/ml silica nanoparticles before and after cell membranes coating. (B) Zeta potential of isolated K562 cell membranes after extrusion through 200 nm filters (K562 cm) and 0.1 mg/ml plain silica nanoparticles before (si-plain) and after cell membrane coating (si-k562, si-thp1 and si-ms5 silica nanoparticles coated with K562, THP-1 and MS5 cell membranes, respectively). The increase in size and decrease of zeta potential suggested deposition of cell membrane on the silica. (C-F) Cryo-TEM images of K562 cell membrane coated silica (C-E) and DOPC-coated silica (F). Scale bar: 50 nm. Cryo-TEM of K562 cell membrane coated silica showed that some bare silica nanoparticles were present (D) together with particle covered with a lipid bilayer (E). Complete coverage instead was observed on all DOPC-coated silica (F).
3.3 Preparation and characterization of cell membrane doped liposomes

Because of the low reproducibility of the uptake behavior on cells (Supplementary Fig. S2) and the large loss of material during sample preparation, a different approach to prepare cell membrane nanoparticles was pursued. Thus, cell membrane doped liposomes were prepared, by mixing synthetic phospholipids with cell membrane extracts without the use of nanoparticle cores.

![Figure 4](image)

**Figure 4.** Characterization of cell membrane-doped liposomes. (A) Size distribution by intensity (diameter, nm) and (B) zeta potential of DOPC liposomes (lipo), and DOPC liposomes doped with K562 cell membrane at a synthetic phospholipid to membrane protein weight ratio of 50:1 and 10:1 (lipo/cm 50:1 and 10:1, respectively). The zeta potential of K562 cell membranes (K562 cm) in PBS was also measured for comparison. Samples corresponding to 0.1 mg/ml synthetic lipids in PBS were measured. For zeta potential, each sample was measured twice and the results are the average and standard deviation over 2 replicate measurements. (C) Elution profile of lipo/cm 10:1 by size exclusion chromatography. Briefly, 1 ml 0.1 mg/ml lipo/cm 10:1 was loaded on a column, fractions of 0.5 ml eluent were collected and their absorbance at 280 nm and 550 nm were measured. (D) Uptake kinetics by flow cytometry of lipo, lipo/cm 50:1 and lipo/cm 10:1 in K562 cells. Samples corresponding to 50 µg/ml synthetic lipids in complete RPMI medium were added to cells and uptake at different times was measured by flow cytometry. The results are the average and standard deviation over 2 replicates of the median cell fluorescence intensity.

Also, in this case, cell membranes extracted from K562 cells were first used to optimize the procedures. As a first step, different amounts of cell membranes were added to DOPC liposomes (lipo) corresponding to mass ratios of 50:1 and 10:1 between the synthetic lipids and cell membrane proteins (lipo/cm 50:1 and lipo/cm...
DLS measurements showed liposome size slightly decreased from 184 nm to around 144 nm when the extracted cell membranes were added, possibly owing to a tighter packing of the bilayer when cell membrane proteins are present (Fig. 4A) [25]. The drop of zeta potential from +4 mV for the pure liposomes to values comparable to the pure extracted K562 cell membranes (around – 21 mV) suggested that very different bilayers were obtained by doping the liposomes with the cell membranes (Fig. 4B). Size exclusion chromatography was then used to separate the cell membrane doped liposomes from eventual residual lipids or proteins of smaller sizes. Surprisingly, elution profiles at 280 nm and 550 nm (for protein and DiI label detection, respectively) showed that labelled liposomes containing proteins eluted out of the column roughly at fractions from 6 to 11, but very low absorbance was detected at both wavelengths in the following fractions (Fig. 4C and Supplementary Fig. S3), suggesting high purity of the final preparation. Importantly, uptake studies on K562 cells exposed to the same amount of lipids (based on lipid assays) showed that the uptake efficiency of the liposomes increased when cell membrane components were included in the bilayer and the increase was higher for liposomes including larger amount of cell membranes (Fig. 4D). This suggested that the inclusion of membrane components promoted nanoparticle interactions with cells (possibly cell receptors), leading to higher nanoparticle uptake. Given their higher internalization efficiency, further studies were performed with cell membrane doped liposomes at synthetic lipid to membrane protein weight ratio of 10:1. This ratio was used to prepare cell membrane doped liposomes from K562, THP-1 and MS-5 cells (named as lipo-k562, lipo-thp1 and lipo-ms5, respectively). As already observed with lipo/cm preparations, also with the other cell membranes the size of the liposomes was reduced and the zeta potential was changed from slightly positive to negative values after incorporation of the cell membrane (Fig. 5A-B).

When incubated with K562, THP-1 and MS-5 cells, all the cell membrane-doped liposomes showed higher internalization efficiency than DOPC liposomes (lipo) in all cell lines (Fig. 5C-E). As opposed to what observed for the cell membrane coated silica (Supplementary Fig. S2), the uptake trend was qualitatively reproducible also for replicate preparations of cell membrane doped liposomes, when the same cell membrane extracts were used (Supplementary Fig.S4). Interestingly, in all cell lines
the doped liposomes containing stromal cell membranes had the highest uptake, including in the stromal cells themselves (Fig. 5C-E). Many studies have shown that the mesenchymal stromal cells are one of the central components of the bone marrow niche and that they strongly interact with leukemia cells [7,49–51]. This might explain the high uptake of MS-5 cell membrane-doped liposomes in all leukemia cells, while the highest uptake of lipo-ms5 in the stromal cells themselves suggests these cells may show homotypic targeting [27]. These data also suggest that the use of stromal cell membrane-doped liposomes might be an interesting avenue to pursue in the near future for targeted drug delivery.

Figure 5. Characterization and cellular uptake of cell membranes doped liposomes in different leukemia and stromal cells. (A) Size distributions by intensity (diameter, nm) and (B) zeta potential of DOPC liposomes (lipo) and liposomes doped with cell membranes extracted from K562, THP-1 and MS-5 cells at a synthetic phospholipid-to membrane proteins weight ratio of 10:1 (lipo-k562, lipo-thp1 and lipo-ms5, respectively). Samples corresponding to 0.1 mg/ml DOPC lipid (based on lipid assay) in PBS were measured. For zeta potential, each sample was measured twice and the results are the average and standard deviation over 2 replicate measurements. (C-E) Uptake of lipo, lipo-k562, lipo-thp1 and lipo-ms5 in K562, THP-1 and MS-5 cells. Briefly, K562, THP-1 or MS-5 cells were exposed for 7 h to liposomes corresponding to 50 µg/ml lipids (based on lipid assay) prior to uptake measurements by flow cytometry. The results are the average and standard deviation of the median cell fluorescence intensity of 2 replicate samples. In all cell lines, cell membrane doped liposomes had higher uptake than synthetic DOPC liposomes (lipo), and lipo-ms5 showed the highest internalization efficiency.
In order to confirm these observations, further uptake studies with lipo-ms5 liposomes were performed in all different cell lines and additionally also in TF-1 human leukemia cells, providing a different type of leukemia cells with different genetic backgrounds (Fig. 6). As already observed (Figs. 5C-E), adding stromal cell membranes in the bilayer strongly increased liposome uptake in all cells. Thus, we have normalized the uptake of lipo-ms5 liposomes by that of the synthetic liposomes (lipo) in the same cells (Fig. 6E). In this way we could directly compare the effect of the addition of stromal cell membranes on liposome uptake efficiency, taking into account that different cells may have different uptake levels. The results showed that for the leukemia cells, doping the liposomes with the stromal cell membranes increased uptake roughly between 3 to 10 times. It is interesting to note that there are differences in liposome uptake efficiency between the different cell line models, with the stronger effects observed on K562 cells as opposed to the the THP-1 cells. While the underlying mechanisms are not yet clear, it is quite possible that differences in expression of adhesion factors such as integrins, cadherins or G protein-coupled receptors might be related to this. With our novel experimental setup, these differences can now be studied functionally by CRISPRing out individual candidates. Interestingly, in the stromal cells instead uptake increased up to 20 times, further suggesting that stromal cells may show homotypic targeting.

**Figure 6.** Uptake efficiency of lipo-ms5 in different leukemia and stromal cells. (A-D) Uptake kinetics of synthetic liposomes and liposomes doped with cell membranes from MS-5 stromal cells at a synthetic phospholipid to membrane proteins weight ratio of 10:1 (lipo and lipo-ms5, respectively) in leukemia (A) K562, (B) THP-1, (C) TF-1 cells and (D) stromal MS-5 cells. Briefly, cells were exposed for increasing time to liposomes corresponding to 50 µg/ml lipids (based on lipid assay) prior to uptake measurements by flow cytometry. The results are the average and
standard deviation of the median cell fluorescence intensity of 2 replicate samples. (E) Comparison of lipo-ms5 uptake efficiency across different cell lines. The results shown in panels A-D for lipo-ms5 are normalized by the results obtained for the synthetic liposomes (lipo) in the same cells at the same exposure time. Addition of stromal cell membranes increased liposome uptake of 3-10 times in the different leukemia cells and up to 20 times in the stromal cells.

4. Conclusions

Cancer AML cells have been shown to reside in the bone marrow in specific niches where they can acquire and maintain chemoresistance and stem-cell like properties thanks to the local microenvironment and tight interactions with the stromal cells. In this study we have exploited the use of cell membrane nanotechnology to prepare cell membrane nanoparticles as a tool to test the interactions between cancer and stromal cells, and ultimately to use such nanoparticles to enhance drug delivery.

Procedures to extract cell membranes were optimized in order to avoid contaminations from other cell compartments and different methods were followed to prepare cell membrane nanoparticles using a nanoparticle core or doping synthetic liposomes. Co-extrusion with silica nanoparticle cores led to loss of high amounts of materials and often incomplete nanoparticle coverage, with uptake behaviors on cells which were difficult to reproduce. On the contrary, doping synthetic liposomes with cell membranes allowed us to improve sample preparation and to obtain good formulations with reproducible uptake behaviors on the different cell lines tested. Cell membrane-doped liposomes showed higher uptake efficiency in all tested cells than the synthetic liposomes, suggesting that addition of cell membrane components favors interactions with cells (possibly cell receptors), thus leading to increased uptake levels. Liposomes doped with the stromal cell membranes showed the highest uptake, and of the leukemic cell lines uptake in K562 was more efficient compared to TF-1 and THP-1. Possibly, differences in adhesion proteins underly these differences and this opens up interesting possibilities in two directions. On one hand, stromal cell membrane nanoparticles can be used as a nanotechnology tool to characterize the interactions between stromal and leukemia cells and identify the proteins involved. For instance, liposomes doped with stromal cell membranes could be used to isolate leukemia clones showing highest interactions with the nanoparticles, thus likely to have higher propensity for interactions with the stromal
cells [11,13,49]. Their phenotypes could be characterized, as well as key proteins involved in the interactions with the stromal membranes. In this way, potentially, novel strategies to target such clones or the identified interactions with stromal cells could be designed [5,7,13]. Secondly, our nanotechnology approach might allow the improvement of drug delivery. Chemotherapy, as a one-size-fits-all strategy is currently still widely used to treat AML patients but does not distinguish between healthy and cancer cells, with severe side effects as consequence. Our future experiments will include analyses comparing uptake of stroma-coated particles in healthy CD34+ stem/progenitor cells compared to leukemic cell lines and primary AML cells from patients to determine whether preferential uptake in the leukemic cells is indeed observed.

An additional observation is that the highest uptake of stroma-coated particles was observed in MS-5 stromal cells themselves. On the one hand, this homotypic targeting might be not surprising since these stromal cells are known to be able to bind to the extracellular matrix, other cells types, and under laboratory conditions to e.g. plastic tissue culture dishes. The exact molecular basis for this remains to be elucidated but our nanoparticles are ideally suited to identify such molecules. Of course, this also means that in terms of drug delivery it will have to be determined whether potential off-target effects will also include targeting the niche, which is potentially unwanted since it might also impact on normal hematopoiesis. It would be important to confirm these observations by using stromal cells isolated from patients to prepare similar cell membrane-doped liposomes.

On a more general level, identifying the mechanisms leading to higher uptake of cell membrane doped liposomes (either with leukemia or stromal cells) both in leukemia and stromal cells could allow to discover key players in the interactions between cancer cells and their nice, as well as identify potential receptors for improved drug delivery.

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Supplementary Materials

Supplementary Figure S1. Characterization of extracted cell membranes. Cryo-TEM images of K562 cell membrane samples isolated without (A) or with (B) an additional purification step using sucrose cushions (as illustrated in Fig.1A (i) and (ii), respectively). In the sample isolated by ultracentrifugation a strong background of small objects of around 30 nm was visible, together with isolated cell membranes forming vesicles (A). The background was strongly reduce when an additional purification step using ultracentrifugation in sucrose cushions was added (B). (C) Thin layer chromatography of DOPC liposomes (lane 1) and K562 cell membrane samples isolated without (lane 2) and with (lane 3) sucrose cushion purification. In all lanes, a smear of lipids travelling with the organic phase could be detected. However, cell membranes extracted by ultracentrifugation also showed a large dark deposition that did not transfer with the organic phase where the sample was loaded (lower part of the image), suggesting a contamination of proteins and other components (lane 2). This was strongly reduced by addition of the purification steps with sucrose cushions (lane 3). Thus, this method was followed to extract cell membranes of higher purity for nanoparticle preparation.
Supplementary Figure S2. Uptake kinetics of cell membrane coated silica nanoparticles by K562 (a), THP-1 (b) and MS-5 (c) cells. Nanoparticles included bare silica nanoparticles (si-plain) as a control and K562, THP-1 and MS-5 cell membranes coated silica nanoparticles (si-k562, si-thp1 and si-ms5, respectively). Briefly, nanoparticles corresponding to 10 µg/ml silica nanoparticles were exposed to cells in complete medium and collected at different times for flow cytometry measurements (see Method for details). For each condition 2 samples were measured and the data are presented as the average and standard deviation of the median cell fluorescence obtained in the 2 replicate samples. In panels A-D, the results obtained with 4 replicate nanoparticle samples prepared using different cell membrane extracts are shown. For all preparations and replicate experiments (A-D), cell membrane coated nanoparticles always had higher uptake than the plain silica (si-plain) in all 3 cell lines. However, in each replicate experiment, the different cell membrane nanoparticles had different uptake efficiency, thus results obtained with the particles prepared in this way were not reproducible.
Supplementary Figure S3. Elution profile of lipo/cm 50:1 by size exclusion chromatography. Briefly, cell membranes extracted from K562 cells were added to DOPC liposomes (lipo) corresponding to mass ratios of 50:1 between the synthetic lipids and cell membrane proteins (lipo/cm 50:1). Then, 1 ml 0.1 mg/ml lipo/cm 50:1 was loaded on a column, fractions of 0.5 ml eluent were collected and their absorbance at 280 nm and 550 nm were measured. Elution profiles at 280 nm and 550 nm (for protein and DiI label detection, respectively) showed that labelled liposomes containing proteins eluted out of the column roughly at fractions from 6 to 11, but very low absorbance was detected at both wavelengths in the following fractions, suggesting no residual lipids and proteins were present.
Supplementary Figure S4. Uptake kinetics of replicate preparations of cell membrane doped liposomes. (A-B) Uptake kinetics of synthetic liposomes (lipo) and liposomes doped with cell membranes extracted from leukemia K562 and THP-1 cells (lipo-k562 and lipo-thp1, respectively) in K562, THP-1 and MS-5 cells. Briefly, K562, THP-1 or MS-5 cells were exposed for increasing times to liposomes corresponding to 50 µg/ml lipids (based on lipid assay) prior to uptake measurements by flow cytometry. The results are the average and standard deviation of the median cell fluorescence intensity of 2 replicate samples. In all cell lines, cell membrane doped liposomes had higher uptake than the synthetic DOPC liposomes (lipo). As opposed to what observed for the cell membrane coated silica (Supplementary Fig. S2), the trend was qualitatively reproducible also for replicate formulations of cell membrane doped liposomes, prepared using a same batch of cell membrane extract. Thus in all preparations, the uptake of lipo-k562 was higher than that of lipo-thp1 and both were higher than the uptake of lipo.