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RESEARCH PAPER

The Utility of an Isolated Mitochondrial Fraction in the Preparation of Liposomes for the Specific Delivery of Bioactives to Mitochondria in Live Mammalian Cells

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ABSTRACT

Purpose To develop and evaluate liposomal formulations prepared with an isolated mitochondrial fraction as a mitochondria-specific delivery vehicle

Methods Liposomes were prepared with either a crude mitochondrial fraction extracted from cells or lipids extracted from the crude mitochondrial fraction and were then characterized by determining their size and zeta potential. The cell uptake of the liposomes and loaded bioactive was studied using flow cytometry and confocal microscopy. The cytotoxicity of the formulations was tested by MTS cytotoxicity assay.

Results Liposomes prepared with the mitochondrial extracts were non-toxic and colocalized with mitochondria in F98 cells. Addition of DOTAP to the liposomes facilitated DNA complexation and the DNA delivered intracellularly co-localized with mitochondria.

Conclusion The results from this study establish the potential of using a mitochondrial fraction isolated from cells to prepare liposomes capable of delivering biologically active molecules to mitochondria of live mammalian cells.

KEY WORDS DNA delivery · liposomes · mitochondria · sub-cellular · targeting

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INTRODUCTION

The effectiveness of a molecule as a drug depends on its ability to specifically act on its target molecule as well as its ability to specifically accumulate at the site of the target molecule (1,2). Improving the accumulation of a drug at the site of its target is therefore a major focus of efforts to improve drug therapy that collectively fall into the area of drug delivery (2). Within this field there is growing focus on controlling the sub-cellular accumulation of drug molecules, as many of these molecules are known to act on intracellular targets (2,3). These intracellular targets are either on or inside various organelle compartments within the cell. The mitochondrial network in particular is associated with several potential targets of biologically active molecules, and several strategies are being explored to improve the mitochondria-specific accumulation of drugs (4–7).

Liposomes have been studied by various investigators for their utility in mitochondria-specific delivery (8,9). Liposomes surface modified with residues known to accumulate in the mitochondria have been used to deliver various anticancer drugs to mitochondria (8,10). Liposomes determined to have improved fusion with mitochondrial membranes have also been explored for similar purposes (9). However, the most intriguing strategy suggested has been to use liposomes prepared in the presence of an isolated mitochondrial fraction (11). These liposomes can arguably be expected to contain the mitochondrial membrane lipids as well as membrane-associated mitochondrial proteins. This crude preparation was previously reported to co-localize with mitochondria upon microinjection into oocytes (11). The purpose of this study was to determine the utility of such a preparation in delivering drug molecules to the mitochondria of mammalian cells in culture and to shed some light on the component of the

mitochondrial fraction that is responsible for the co-localization of the liposomes with mitochondria.

MATERIALS AND METHODS

Rat glioma F98 cells (CRL-2397™) were purchased from ATCC® (Manassas, VA, USA). Chicken egg L-α-phosphatidylcholine (PC), 1,2-dioleoyl-3-trimethyl ammonium-propane (DOTAP), transphosphatidylated chicken egg L-α-phosphotidylethanolamine (PE), porcine brain L-α-phosphatidylserine sodium salt (PS), Bovine liver L-α-phosphatidylinositol sodium salt (PI), bovine heart cardiolipin disodium salt (Cdl), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (Rh-PE), N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (NBD-PE) were purchased from Avanti® Polar Lipids Inc. (Alabaster, AL, USA). The Molecular Probes® fluorescent dyes MitoFluor™ Green, Lysotracker Red and Mitotracker® Red CMXRoS were purchased from Invitrogen (Carlsbad, CA, USA).

Isolation and Characterization of the Mitochondrial Fraction

Rat glioma F98 cells were grown in standard T75 culture flasks in Dulbecco's modified eagle medium (DMEM) until they were about 70% confluent. The cells were then trypsinized, and the mitochondrial fraction was isolated using a Pierce protein research products Mitochondria isolation kit for cultured cells from Thermo Fisher Scientific (Rockford, IL, USA). The protein content of the isolated fraction was determined using a Pierce protein research products bichinconic acid (BCA) protein assay kit from Thermo Fisher Scientific (Rockford, IL, USA). The isolated fraction was then assayed for citrate synthase activity to assess the level of mitochondrial enrichment according to well-established protocols (12). Briefly, 100 μL of 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid);3-carboxy-4-nitrophenyl disulfide (DTNB) (final concentration 0.1 mM), 25 μL 10% Triton X-100 (final concentration 0.25%), 50 μL oxaloacetate (final concentration 0.5 mM), 25 μL Acetyl coenzymeA (final concentration 0.31 mM) were added to a 1 mL quartz cuvette and mixed. Then the mitochondrial fraction (equivalent to 300 μg of mitochondrial protein) was added into the cuvette, and the absorbance was recorded every 30 s for 300 s in a Spectronic Genesys5 spectrophotometer at 412 nm. The graph of absorbance *vs.* time was plotted. Citrate synthase purified from porcine heart was purchased from Sigma Aldrich (St Louis, MO, USA) and was used as a positive control at a final protein concentration of 0.0172 mg/mL in the assay cuvette.

Extraction of Lipids from the Isolated Mitochondrial Fraction

Lipids from the crude mitochondrial fraction were extracted by the method of Bligh and Dyer (13). Briefly, a 1:2 (v/v) chloroform: methanol solution was added to the mitochondrial fraction and was further mixed with one-third volume of distilled water and vortexed well. This was further centrifuged at 1,000 rpm for 5 min at room temperature to obtain a biphasic system. The bottom phase was recovered with a pasteur pipette by gentle positive pressure. The extracted lipid fraction was further concentrated by evaporating the chloroform using a rotary evaporator. The phospholipid enrichment was observed by subjecting both the crude fraction and the lipid-enriched fraction to one-dimensional thin layer chromatography (TLC) on Silica gel plates using chloroform: methanol:acetic acid:water (25:15:4:2) as the developing solvent. The phospholipids were visualized on the TLC plates by staining with iodine vapors and 40% sulphuric acid spray (14).

Preparation and Characterization of Liposomes

All liposomes used in this study were prepared by the thin-film hydration method. The appropriate amount of lipids from chloroform stock solutions were combined in a round-bottom tube to give the desired mole fraction of each lipid with a total lipid content of 5 mg/mL in the final hydrated preparations. Chloroform was then removed at 40°C under vacuum in a rotary evaporator to give a dry lipid film. The dried lipid film was then hydrated either with F98-derived mitochondrial fraction in phosphate-buffered saline (PBS) equivalent to 300 micrograms of mitochondrial protein or plain PBS to make up the desired volume. The resultant crude liposomal suspension was subjected to sonication for two 5-min periods at an interval of 1 min, in an ice-water bath using a probe sonicator. Where necessary, liposomes were fluorescently labeled by adding either 0.5% Rh-PE or 0.5% NBD-PE. Liposomes were also prepared with the following lipid composition that mimics the lipid composition of the mitochondrial membrane(15): PC (50 mol%), PE (31 mol%), PS (1.8 mol%), PI (7.7 mol%) Cdl (9.5 mol%).

The size distribution of the liposomes was determined by quasi-elastic laser light scattering using a Beckmann Coulter N4Plus particle size analyzer. One hundred μL of the liposomal preparation was added to 1 ml PBS. The zeta potential of liposomal formulations was determined using a Brookhaven Instruments Corporation Zetaplus zetasizer. For each measurement, 10 μL of the liposomal formulation was added into 1.5 mL of water.

Flow Cytometry

F98 cells were grown in T25 flasks in DMEM until 60–80% confluent. The cells were then incubated in DMEM containing Rh-PE-labeled liposomes for 6 h at an approximate final concentration of 250 μg liposomal lipid per ml. After incubation the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. Flow cytometry was performed on a Becton Dickinson FACSCalibur Flow Cytometer. All experiments were performed in triplicate.

Fluorescence Resonance Energy Transfer (FRET) Analysis

In order to assess the interaction between liposomal membranes and mitochondrial membranes, an assay was preformed with modifications of a previously reported procedure (16) that is based on the fluorescence resonance energy transfer (FRET) between NBD and rhodamine. Briefly, liposome formulations to be tested were labeled with 0.5 mol% each of Rhodamine-PE and NBD-PE. Fluorescence measurements were made on a Hitachi 2000 Fluorescence Spectrophotometer. The existence of FRET was verified by the detection of high levels of fluorescence at the Rh-PE emission maxima (Em_{max}) of 590 nm resulting from excitation at the 460 nm excitation maxima (Ex_{max}) of NBD-PE with low levels of fluorescence at the NBD-PE Em_{max} of 520 nm. The fluorescence emission of an aliquot of double-labeled liposomes containing 250 μg total lipid in 2 ml of water measured at both 590 nm and 520 nm (NBD Ex_{max}) with the fluorescence spectrophotometer set to an excitation wavelength of 460 nm 500 μl of a rat liver mitochondrial fraction corresponding to 300 μg total protein was then added, and the fluorescence was recorded again. All experiments were performed in triplicate, and data are expressed as the ratio of 590 nm to 520 nm fluorescence

Confocal Fluorescence Microscopy

F98 cells were grown in DMEM on 22 mm circular cover slips in 6-well culture plates until approximately 60–80% confluent and were then treated with fluorescently labeled liposomes in triplicate at an approximate final concentration of 250 μg liposomal lipid per ml. After incubation for 6 h, the cells were stained with sub-cellular stains as needed (mitochondria were stained with MitoFluor™ Green, lysosomes were stained with Lysotracker Red) followed by a PBS wash and then final mounting on slides in fluoromount medium. The processed samples were then examined on a Zeiss axioplan microscope with a LSM 510 META confocal unit. Images were processed using Image J Software and plugins downloaded for free from the National Institutes of Health (NIH) website.

Preparation and Characterization of Liposome DNA Complexes (Lipoplexes)

The 2.1 Kb linear DNA used in the DNA delivery experiments was prepared by polymerase chain reaction as previously reported (17). When necessary, the DNA was subsequently labeled using a *Label* IT® Fluorescein Labeling Kit from Mirus Bio LLC (Madison, WI, USA). The labeled DNA was purified by ethanol precipitation and then electrophoresed on a 1% agarose gel to confirm the presence of labeled DNA. The labeling was also verified using a fluorescence spectrophotometer.

A previously established SYBR dye exclusion assay (15) was used to determine the amount of liposomal lipids required to bind a unit weight of DNA. Briefly, a known amount of unlabeled DNA (~0.5 μg) was added to mixture of 250 μL SYBR® Green I and 750 μL of PBS in a 3 ml disposable cuvette. The fluorescence emission of the mixture was measured at 520 nm with a Hitachi 2000 Fluorescence Spectrophotometer set at an excitation wavelength of 497 nm. Liposomes were then added in 1–5 ml increments until the fluorescence of free DNA was completely lost. All measurements were done in triplicate. The DNA binding capacity was then calculated in terms of μg of liposomal lipids/μg DNA.

Lipoplexes were prepared by incubating the fluorescein-labeled DNA with liposomal preparations for 30 min in the lipid/DNA ratio determined from DNA binding experiments. The size distribution and Zeta potential of the resulting lipoplexes was determined in a similar manner to that described for liposomal characterization.

RESULTS

Liposomes Prepared in Presence of Isolated Mitochondrial Fraction Associate with Cells, Co-localize with Mitochondria Within the Cells

The mitochondrial isolation protocol used in this study routinely yielded a preparation with a total protein content of 600 μg from a starting cell pellet of ~250 million cells. The fraction was always subjected to an assay for citrate synthase activity and showed significant enrichment of citrate synthase activity over that of the starting cell suspension confirming the enrichment of the mitochondrial fraction. Preparation of liposomes in the presence of this crude mitochondrial fraction (cMf) yielded liposomes (cMf-liposomes) with reproducible size and zeta potential (Table I). Flow cytometry analysis revealed that these liposomes associate strongly with cells in a manner similar to positively charged liposomes prepared with DOTAP that are known to show strong cell association (Fig. 1a). Addition of mitochondria to cMf-liposomes labeled

Table I Composition and Characteristics of Liposome Formulations Used in the Study

Type of liposome	Composition	Polydispersity index	Average Size (nm)	Zeta Potential (m V)
nt-liposome	PC (100 mol%)	0.14 ± 0.02	127 ± 33	−7 ± 9
DOTAP liposome	PC (90 mol%),DOTAP(10 mol%)	0.17 ± 0.01	123 ± 40	38 ± 10
cMf-liposome	PC (100 mol%), crude mitochondrial fraction	0.20 ± 0.02	154 ± 34	−23 ± 2
lMf-liposome	PC (100 mol%)+enriched mitochondrial lipid extract	0.21 ± 0.03	151 ± 23	−38 ± 4
sMf-liposome	PC (50 mol%), PE (31 mol%), PS (1.8 mol%), PI (7.7 mol%) and CDL (9.5 mol%).	0.20 ± 0.02	135 ± 24	−53 ± 5

Data represent mean±sd, $n = 3$

with rhodamine and NBD resulted in the loss of FRET-associated fluorescence at EX/EM of 460/590 nm, while no loss of the FRET-associated fluorescence was seen in the case of liposomes without an incorporated mitochondrial fraction (nt-liposomes), suggesting that cMf-liposomes undergo rapid lipid mixing with mitochondria to a sufficient extent to dilute the FRET pair. Pearsons coefficient values for co-localization calculated from confocal micrographs (Fig. 2a and b) indicated that cMf-liposomes have a greater tendency to co-localize with mitochondria than with lysosomes within the cell. The levels of mitochondrial co-localization were found to be significantly higher than those seen with liposomes prepared without the cMf (Fig. 2a and b).

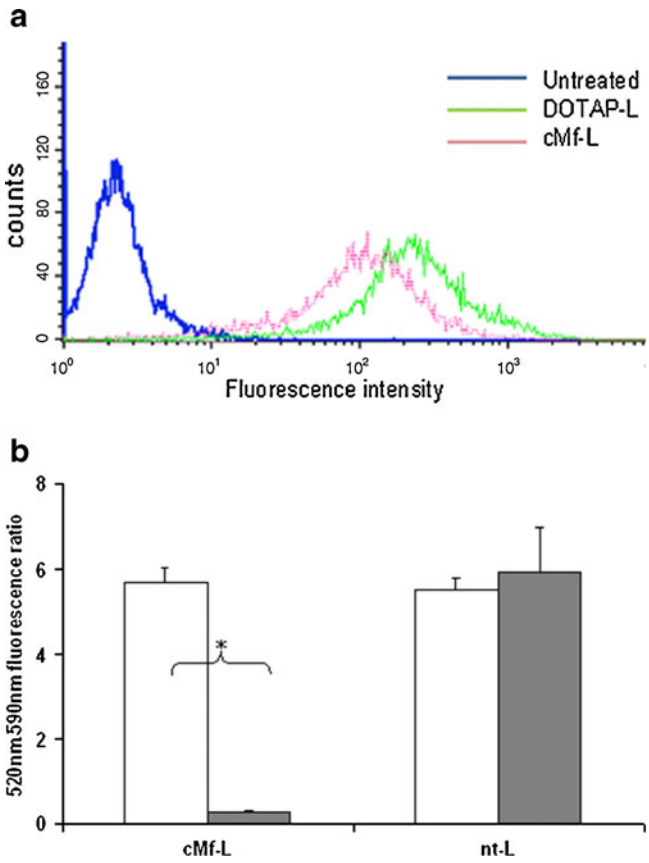

Fig. 1 Assessment of liposomal interaction with intact cells and isolated mitochondria. (**a**) Representative flow cytometry histograms of untreated F98 cells (untreated) and F98 cells treated with fluorescently labeled DOTAP liposomes (DOTAP-L), liposomes incorporating a crude mitochondrial fraction (cMf-L). (**b**) Effect of the presence (shaded bars) or absence (open bars) of mitochondria on the fluorescence resonance energy transfer between NBD and Rhodamine in double-labeled liposomes incorporating a crude mitochondrial fraction (cMf-L) or liposomes without an incorporated mitochondrial fraction (nt-L). The data are expressed as the mean±standard deviation ($n = 3$) of the ratio of fluorescence at 590 nm to fluorescence at 520 nm with an excitation of 460 nm. *Significant difference ($p < 0.05$) between indicated groups.

To further study the role of the lipid component of the mitochondrial fraction in mediating the sub-cellular disposition of the liposomes the crude mitochondrial fractions were subjected to a Bligh and Dyer lipid extraction to yield an enriched lipid mitochondrial fraction (lMf). Liposomes (lMf-liposomes) prepared in the presence of this fraction also showed reproducible size and zeta potential (Table I). Interestingly, these liposomes showed significantly higher levels of co-localization with mitochondria than either the cMf-liposomes or nt-liposomes (Fig. 2a and b). Further, we investigated whether the high levels of mitochondrial co-localization could be achieved by using a liposome formulation prepared with a lipid composition known to be similar to that of the mitochondrial membrane. This liposome formulation prepared with a "synthetic" mitochondrial lipid fraction (sMf) was also established to be reproducible in size and zeta potential before further investigation. The sMf-liposomes did co-localize with mitochondria but to a significantly lower extent than was recorded with lMF-liposomes (Fig. 2a and b).

Mitochondriotropic Liposomes Prepared Using Isolated Mitochondrial Fractions Well Tolerated by Cells in Culture

A methyl tetrazolium salt (MTS) cytotoxicity assay was performed to measure and compare the cytotoxicity of the liposomes developed in this study on mammalian cells in culture. Incorporation of either the cMf or lMf into liposomes did not significantly change the cytotoxixity profile over

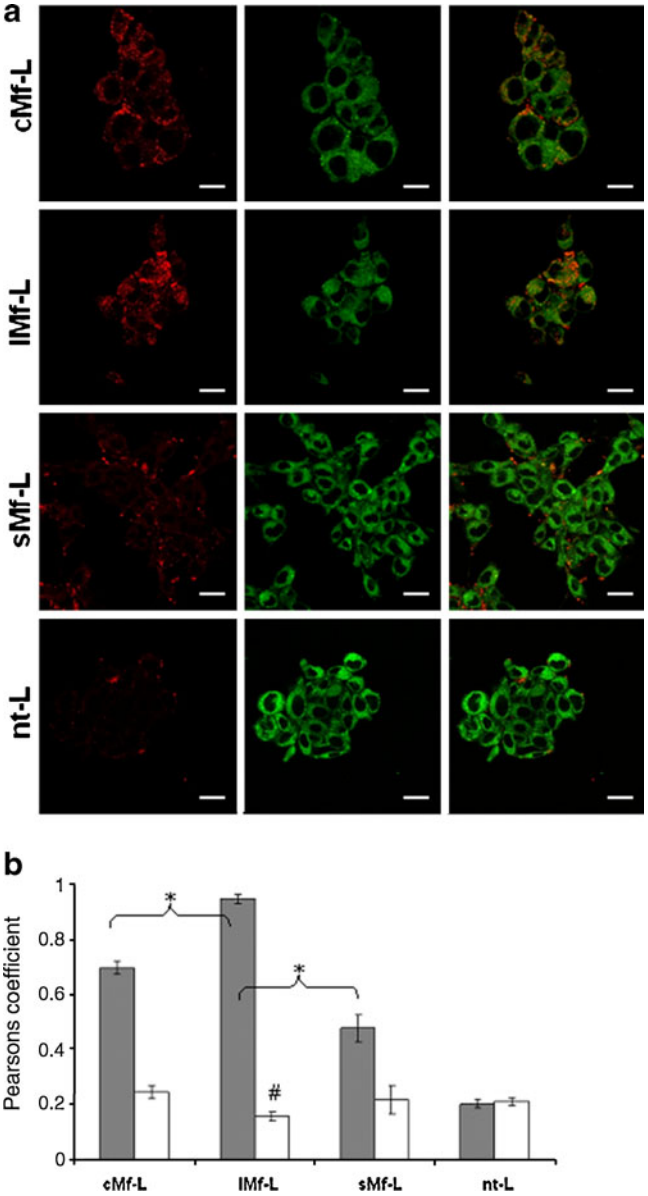

Fig. 2 (**a**) Determination of sub-cellular distribution of liposomes. Representative confocal micrographs at 60x magnification of cells treated with liposomes incorporating a crude mitochondrial fraction (cMf-L), liposomes incorporating an enriched mitochondrial lipid fraction (lMf-L), liposomes incorporating a synthetic mitochondrial lipid fraction (sMf-L) and liposomes without an incorporated mitochondrial fraction (nt-L). Left column shows the red channel indicating the distribution of the labeled liposomes, middle column shows the green channel indicating the cellular mitochondria stained with Mitofluor® green, right column shows the merged red and green images with co-localization appearing yellow. Scale bar in all images corresponds to 30 μm. (**b**) Quantitative analysis of fluorescence co-localization expressed as Pearson's coefficient ±standard deviation ($n=9$) calculated using ImageJ software for co-localization of rhodamine-labeled liposomes with Mitofluor-green-stained mitochondria (shaded bars) and co-localization of NBD-PE-labeled liposomes with Lysotracker® red (open bars). *Significant difference ($p<0.05$) between indicated groups. #Significant difference ($p<0.05$) compared to all other groups.

liposomes prepared without a mitochondrial fraction (Fig. 3). However, it was seen that the sMf liposomes were significantly more cytotoxic ($p<0.05$) at all concentrations compared to the other formulations tested (Fig. 3).

Mitochondriotropic Liposomes Prepared Using Isolated Mitochondrial Fractions to Deliver Bioactives to Mitochondria in Mammalian Cells

Liposomes in general are well suited to delivering poorly soluble molecules into cells. The high levels of co-localization seen with the liposomes prepared in this study and bearing a fluorescently labeled lipid strongly support their utility in delivering poorly soluble molecules to mitochondria, similar to formulations described in the literature. In order to study the ability of the liposomes to deliver DNA to mitochondria, they first had to be supplemented with the DNA binding lipid DOTAP. Incorporation of 10 mol% DOTAP into cMf-liposomes and lMf-liposomes conferred the necessary DNA binding ability (Table II). The amount of liposomes required to bind a given amount of DNA was not significantly different for any of the liposome preparations tested, and the size and zeta-potential of the liposome-DNA complexes formed with each of the liposome preparations tested, was not significantly different (Table III). Any differences in the intracellular distribution are therefore not due to differences in the physical dimensions of the complex but are most likely due to the specific effects of the lipid composition. Confocal microscopy analysis confirmed that the incorporation of DOTAP did not significantly reduce the mitochondriotropic nature of the liposomal formulation (data not shown). Using

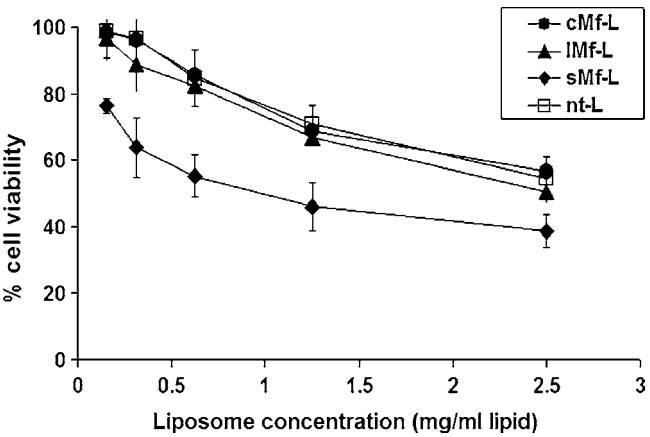

Fig. 3 Evaluation of liposome cytotoxicity. Percent cell viability upon exposure of F98 cells for 6 h to various concentrations of liposomes incorporating a crude mitochondrial fraction (cMf-L), liposomes incorporating an enriched mitochondrial lipid fraction (lMf-L), liposomes incorporating a synthetic mitochondrial lipid fraction (sMf-L) and liposomes without an incorporated mitochondrial fraction (nt-L). Data represent mean±standard deviation.

Table II DNA Binding Capacity of Liposomes after the Incorporation of 10mole% DOTAP

Type of liposome	DNA binding capacity (μg lipid/μg DNA)
nt-liposome	55[a]
cMf-liposome	55 ± 4.5
lMf-liposome	65 ± 4.0

Data represent mean±sd, $n = 3$.

[a] indicates no SD recorded as all three measurements yielded exactly the same value.

DNA labeled with fluorescein and confocal micoroscopy, it was then observed that both cMf-liposomes and lMf liposomes are able to mediate the cell uptake of the fluorescent DNA. Further, the DNA that is delivered intracellularly co-localizes with mitochondria (Fig. 4). Liposomes not containing a mitochondrial fraction mediated significantly lower levels of co-localization of delivered DNA with mitochondria inside the treated cells (Fig. 4).

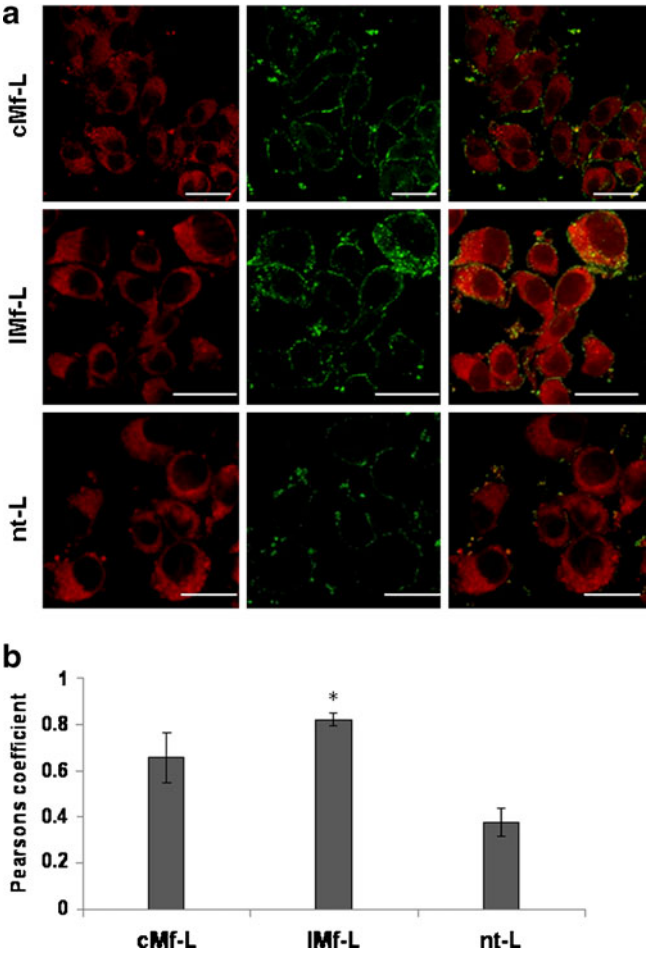

Fig. 4 Evaluation of liposome-mediated mitochondria specific DNA delivery. (**a**) Representative confocal micrographs at 60× magnification of cells treated with fluorescein-labeled DNA complexed with liposomes incorporating a crude mitochondrial fraction (cMf-L), liposomes incorporating an enriched mitochondrial lipid fraction (lMf-L) and liposomes without an incorporated mitochondrial fraction (nt-L). Left column shows the red channel indicating the cellular mitochondria stained with Mitotracker® red, middle column shows the green channel indicating the distribution of the fluorescein-labeled DNA delivered intracellularly by the indicated liposomes, right column shows the merged red and green images with co-localization appearing yellow. Scale bar in all images corresponds to 20 μm. (**b**) Quantitative analysis of fluorescence co-localization expressed as Pearson's coefficient ±standard deviation ($n = 9$) calculated using ImageJ software for co-localization of fluorescein-labeled DNA with Mitotracker®-red-stained mitochondria. * Significant difference ($p < 0.05$) compared to cMf-L.

DISCUSSION

Drug delivery strategies that improve the cell uptake and target specific sub-cellular accumulation of drugs have the potential to significantly improve drug therapy. Additionally, there is a need for such specific delivery systems as research tools to elucidate mechanisms of various sub-cellular processes. It stands to reason that such delivery vehicles should be specific in mediating the accumulation of their cargo, be simple and cost effective to use and be well tolerated by cells. With this in mind, we sought to further explore the utility of liposomes bearing components of an isolated mitochondrial fraction as mitochondria-specific delivery vehicles. In addition to the report that liposomes bearing a mitochondrial fraction co-localize with mitochondria upon microinjection into cells (11), it has also been reported that isolated mitochondria can be internalized by cells and that they co-localize with cellular mitochondria (11,18,19). Further, exogenous mitochondria that have been introduced into cells have also been shown to fuse with intracellular mitochondria (20). Taken together, these observations suggest that there are distinct elements of the mitochondrial membrane that might serve as mitochondria-specific localization signals and that the presence of such components on the surface of any vesicular compartment within the cell can lead to the association and eventual fusion of the vesicular compartment with the mitochondrial compartment. It would also be reasonable to postulate that following the interaction of liposomes with the cell surface and the subsequent endocytic internalization of the liposome, some of the liposomal membrane components are transferred to the surface of the endosomal membrane. The fate

Table III Average Size and Zeta Potential of the Liposome-DNA Complexes Prepared with the Indicated Liposomes

Type of liposome	Average Size (nm)	Zeta Potential (mV)
nt-liposome	446 ± 44	21 ± 10
cMf-liposome	483 ± 83	12 ± 2
lMf-liposome	465 ± 48	11 ± 2

Data represent mean±sd, $n = 3$.

of the endosome could then be dictated by its membrane composition, which if sufficiently similar to that of a membrane-bound intracellular compartment like the mitochondria could result in the trafficking of the endosome and its contents towards that compartment before the maturation of the endosome to a late endosome and its subsequent fusion with lysosomes. The results from this study do indeed seem to support this notion, especially the observations that cMf-liposomes differ dramatically from nt-liposomes in terms of their interaction with isolated mitochondria and that it is the lipid component of the mitochondrial fraction that demonstrates the best co-localization with the mitochondria and finally that a synthetic lipid formulation does not exhibit similar behavior. Of course, a significant limitation of this study has been the practical difficulties of being able to extract a usable amount of the protein fraction of the mitochondrial membrane for comparison with the lipid component. However the observations that the lipid component has a significant effect seem to be in agreement with an earlier study that reported the use of liposomes prepared with a lipid composition that was previously determined to promote fusion with the mitochondrial membrane resulted in improved mitochondrial delivery of a water-soluble protein cargo (9).

Based on this study, the use of an isolated mitochondrial fraction also appears to be a more cell-compatible alternative to mitochondriotropic formulation than the use of synthetic lipids, and as such any perceived inconvenience of having to isolate a mitochondrial fraction to prepare the formulation is certainly justified, at least in a research setting. The liposome formulation and preparation procedure described in this study is based on the use of commercially available lipids and the use of a commercially available mitochondrial isolation kit, thereby allowing wide access to an easily adaptable liposome formulation as a research tool for the delivery of poorly soluble molecules or DNA to mitochondria.

CONCLUSIONS

Mitochondriotropic liposomes can be reproducibly prepared using mitochondria isolated from the cell type being studied, and these liposomes can be used to deliver either water-soluble or water-insoluble molecules to mitochondria in live mammalian cells.

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