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Long-term biophysical stability of nanodiamonds combined with lipid nanocarriers for non-viral gene delivery to the retina

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ABSTRACT

Nanodiamonds were combined with niosome, and resulting formulations were named as nanodiasomes, which were evaluated in terms of physicochemical features, cellular internalization, cell viability and transfection efficiency both in *in vitro* and in *in vivo* conditions. Such parameters were analyzed at 4 and 25 °C, and at 15 and 30 days after their elaboration. Nanodiasomes showed a particle size of 128 nm that was maintained over time inside the ± 10% of deviation, unless after 30 days of storage at 25 °C. Something similar occurred with the initial zeta potential value, 35.2 mV, being both formulations more stable at 4 °C. The incorporation of nanodiamonds into niosomes resulted in a 4-fold increase of transfection efficiency that was maintained over time at 4 and 25 °C. *In vivo* studies reported high transgene expression of nanodiasomes after subretinal and intravitreal administration in mice, when injected freshly prepared and after 30 days of storage at 4 °C.

1. Introduction

The pharmaceutical science has directed considerable efforts towards discovering and developing safe and efficient vectors for gene therapy purposes. While most studies focus on overcoming specific issues related to conventional gene delivery platforms, such as unpredictability, incompatibility with biological systems or low efficiency, few studies conduct an exhaustive assessment of the storage stability of gene carriers, a critical quality to achieve both large-scale production and clinical application (Suzuki et al., 2015).

Nowadays, few gene therapy drugs have been marketed globally, and most of these products are based on viral vectors (Al Qtaish et al., 2020; Shahryari et al., 2019). However, because of specific issues associated to viral gene carriers, including low DNA packing capacity, high costs and complex production, non-viral vectors are gaining increasing interest (Do et al., 2019; Ibraheem et al., 2014; Ginn et al., 2018). In addition to overcoming these specific challenges, non-viral vectors offer high versatility due to the wide variety of available nanomaterials that can be used to produce gene delivery systems (Grijalvo et al., 2019; Riley and Vermerris, 2017). Among these, niosomes have been reported in repeated occasions as efficient vehicles for gene delivery to brain (Mashal et al., 2018) and retina (Puras et al., 2015), among others.

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Niosomes are cationic lipid nanoparticles with a bilayer distribution similar to liposomes, but, additionally, niosomes can also contain a "helper" component and a non-ionic surfactant to obtain more stable colloidal dispersions. All these mentioned components, provide niosomes superior chemical and storage stability than liposomes (Bartelds et al., 2018; Ojeda et al., 2016). All the components of niosome formulations influence on their biocompatibility and transfection efficiency. In particular, the characteristics of the "helper" component influence directly on relevant biological processes, such as the cellular uptake and the subsequent intracellular disposition, which are critical factors that determine successful gene delivery efficiency (Ojeda et al., 2016). Among the most studied "helper" components, lipid-based ones such as lycopene, cholesterol, squalane, squalene and sphingolipids (Al Qtaish et al., 2021; Mashal et al., 2017; Ojeda et al., 2016) have been the most employed to date, but also non-lipid ones such as chloroquine are gaining interest with encouraging results (Mashal et al., 2019).

Recently, nanodiamonds (NDs) have emerged as an interesting material to elaborate non-viral vectors for gene delivery applications. The high biocompatibility, low toxicity, along with their versatile surface chemistry (Lim et al., 2016), which allows multiple combination forms as "helper" components with other nanomaterials such as polymers or lipids have captured the interest of scientifics. NDs are allotropes of carbon that contain a core diamond crystalline structure and present unique physicochemical properties, such as almost spherical shape, low size polydispersity and high specific area. Additionally, NDs can be easily functionalized with many chemical compounds (Chauhan et al., 2020). In previous research for gene therapy purposes, authors combined NDs with hydrophilic cationic polymers such as polyethylenimine 800 (PEI 800) (Alhaddad et al., 2011); Chen et al., 2021; Zhang et al., 2009) and polyallylamine hydrochloride (PAH) (Alhaddad et al., 2011), or with cationic monomer such as lysine (Alwani et al., 2016) by electrostatic interactions. On the other hand, covalent derivatization of NDs has been performed with silane-NH_2 groups (Edgington et al., 2018; Zhang et al., 2009) and polyamidoamine (PANAM) (Lim et al., 2017). In other study, Bi et al designed and synthetized a complex structure of ND-$CONH(CH_2)_2NH$-VDGR/survivin-siRNA with antitumoral effect (Bi et al., 2016). Finally, our research group combined NDs with niosomes, demonstrating their superiority in enhancing the transfection efficiency of these non-viral vectors (Al Qtaish et al., 2022). However, to the best of our knowledge, the combination of NDs with niosomes to evaluate their stability along with their retinal gene delivery efficiency has not been explored yet.

In this work, we prepared and comparatively evaluated the transfection efficiency and long-term stability at different storage temperatures of two niosome-based formulations that only differed on the use or not of NDs as "helper" components. Formulations were based on cationic lipid N-[1-(2,3dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and non-ionic surfactant polysorbate 20. NDs were added as "helper" components to one of the two formulations. Resulting formulations were named as niosomes and nanodiasomes depending on their ND content and were incubated with pCMS-EGFP plasmid in order to obtain nanocomplexes, named as nioplexes and nanodiaplexes, respectively. Formulations were evaluated in terms of physicochemical properties, including size distribution, superficial charge and polydispersity index at different periods of time (0, 15 and 30 days) and storage temperatures (4 °C and 25 °C). In addition, *in vitro* biological studies were performed to evaluate the toxicity of the formulations along with their cellular uptake and gene delivery efficiency over time at different storage temperatures in HEK-293 cells. Further assays were carried out in primary retinal cells and in mice after both intravitreal and subretinal administration of the formulations in order to determine the effect of NDs as "helper" components on the gene delivery efficiency and long-term storage of the formulations.

2. Materials and methods

2.1. Preparation of nanodiasome and niosome formulations

For the preparation of the nanodiasome formulations, the water in oil emulsion technique was used as previously described. Briefly. 250 μL of NDs (10 mg/ml in H_2O, Sigma-Aldrich Madrid, Spain, product ID: 900180) were ultrasonicated for 30 min and mixed with an aqueous phase composed of 2 mL of 0.5% polysorbate 20 (Sigma-Aldrich Madrid, Spain) plus 1.75 mL of MilliQ water. On the other hand, 5 mg of DOTMA (Avanti Polar Lipids, Inc., Alabama, USA) were accurately weighted and diluted in 1 mL of the organic solvent dichloromethane (DCM) (Panreac, Barcelona). This oil phase was incorporated into the aqueous phase and sonicated for 30 s at 50 W (Branson Sonifier 250, Danbury). The emulsion was maintained under magnetic stirring for 2 h at room temperature (RT) until evaporation of DCM to obtain the nanodiasome formulation. The preparation of the niosome formulation followed the same procedure, but the aqueous phase did not contain NDs. Fig. 1 summarizes the main components and their disposition in both formulations.

2.2. Preparation of the nanocomplexes

Nanocomplexes were obtained by incubating both niosomes and nanodiasomes with the previously propagated pEGFP plasmid, as described elsewhere (Ojeda et al., 2016), to obtain complexes (nioplexes and nanodiaplexes, respectively) at 5/1 cationic lipid/DNA ratio (w/w).

2.3. Physicochemical studies

Niosomes, nanodiasomes, and their corresponding complexes were physicochemically characterized by means of mean particle size, dispersity index (Đ) and zeta potential, following previously reported methodology (Mashal et al., 2017).

Microscopy studies were carried out to determine the morphology and the disposition of NDs in the nanodiasomes, by cryo-electron tomogram, as previously described (Al Qtaish et al., 2022).

2.4. Biophysical stability studies of formulations

Stability studies were performed with all formulations by means of physicochemical characterization and biological performance. For that purpose, particle size, dispersity, zeta potential, cell viability and transfection were evaluated at 0, 15 and 30 days with stored formulations at 4 °C and 25 °C. Cellular uptake, transfection in primary retinal cell cultures, along with *in vivo* retinal assays were performed with freshly prepared formulations and with formulations stored at 4 °C for 30 days.

2.5. Transfection studies

To perform transfection experiments, human embryonic kidney 293 cell line (HEK-293; ATCC® CRL1573™) was cultured and maintained as previously described (Ojeda et al., 2016). For this, HEK-293 cells were seeded at 20×10^4 cells per well in 24 well plates and incubated to reach 70% of confluence the next day. After discarding the medium from the wells, cells were transfected using OptiMEM (Gibco, San Diego, CA, USA) transfection medium, for 4 h with nioplexes and nanodiaplexes, from freshly prepared and stored for 15 and 30 days at 4 °C and 25 °C formulations, at the cationic lipid/DNA mass ratio 5/1, as previously reported (Al Qtaish et al., 2022). Positive control of transfection consisted in cells transfected with Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA), while negative control were non-treated cells but in OptiMEM for 4 h. Each condition was carried out in triplicate.

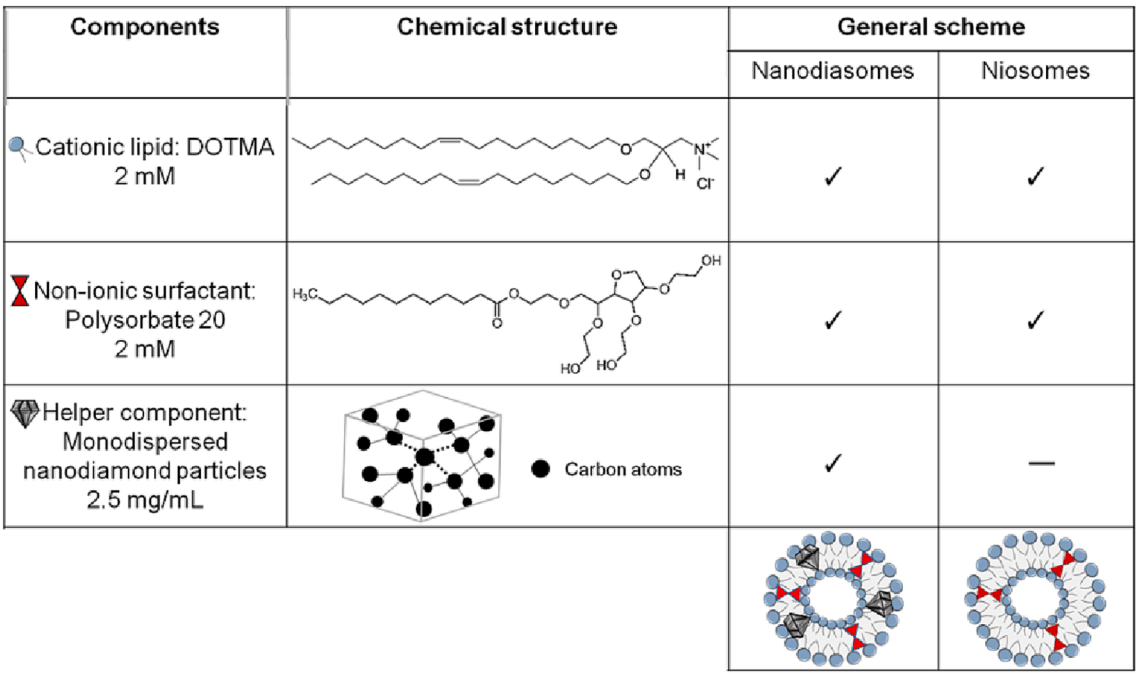


Fig. 1. Overview of formulations and their components.

2.6. EGFP expression and cell viability assays

The efficiency of the transfection process was assessed both qualitative and quantitatively 48 h after the transfection assay. Qualitative determination of EGFP signal was performed using an inverted fluorescence microscope (Eclipse TE2000-S, Nikon). Quantitative studies of plasmid expression, cell viability and mean fluorescence intensity (MFI) were carried out by flow cytometry using a FACSCalibur system (Becton Dickinson Bioscience, San Jose, USA), as reported previously (Al Qtaish et al., 2022).

2.7. Cellular uptake

To analyse the cellular internalization process of nioplexes and nanodiaplexes, using freshly prepared and stored for 30 days at 4 °C formulations, niosomes and nanodiasomes were condensed with FITC-labelled pEGFP plasmid. Fluorescence microscopy and flow cytometry equipment were used to elaborate cellular uptake process in a qualitative and quantitative way, respectively (Al Qtaish et al., 2022).

2.8. Animals and anesthetics

Procedures were performed following the RD 53/2013 Spanish and 2010/63/EU European Union regulations, as well as the Association for Research in Vision and Ophthalmology (ARVO), once obtained the approval of the Miguel Hernandez University Standing Committee for Animal Use in the Laboratory.

2.9. Transfection studies in rat primary central nervous system cell cultures and immunocytochemistry assays

E17-E18 rat embryos (Sprague Dawley) were employed for the extraction of primary central nervous system (CNS) cells, from the brain cortex and retinal tissue. Cells were removed and cultured onto pre-coated glass coverslips in 24 well plates.

Cortical and retinal cells were transfected with nanodiaplexes from freshly prepared and 30 days stored nanodiasomes. Lipofectamine™ 2000 (ThermoFisher Scientific) was used as a positive control. Transfections experiments were repeated three times for each condition and GFP expression was analyzed at 96 h after transfection.

Cell fixation was carried out with 4% paraformaldehyde for 25 min and permeabilized using 0.5% Triton X-100 during 5 min. After blocking with a solution of 10% BSA (v/v) in PBS for 1 h at RT, cells were incubated with primary antibody chicken anti-EGFP (ThermoFisher Scientific) overnight at 4 °C. Secondary antibody Alexa Fluor 555 goat anti-chicken IgG (ThermoFisher Scientific) and Hoechst 33,342 (Sigma-Aldrich, Spain) were applied for 1 h at 4 °C. Coverslips were analyzed by a Zeiss AxioObserver Z1 (Carl Zeiss) microscope equipped with an ApoTome system and Leica TCS SPE spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.10. Intravitreal and subretinal administration of formulations

In vivo transfections of nanodiaplexes were carried in C57BL/6J mice with freshly prepared (n = 10) and 30 days stored nanodiasomes (n = 10). Animals were anesthetized, and intravitreal (n = 5) or subretinal (n = 5) injections were administered under microscope (Zeiss OPMI® pico; Carl Zeiss Meditec GmbH, Jena, Germany) using a Hamilton microsyringe with a blunt 34-gauge needle (Hamilton Co., Reno, NV). The nanodiaplexes solution injected was 0.5 μL which contained 100 ng of EGFP plasmid. As negative controls, the untreated right eyes were used.

EGFP expression was analyzed qualitatively one week after the injection of complexes from freshly or 30 days stored nanodiasomes in frozen sections of the retina, as previously described (Mashal et al., 2017). Cryosections were incubated with the primary antibodies chicken anti-EGFP (ThermoFisher Scientific) and rabbit anti-Iba1 (Abcam) overnight at 4 °C. Secondary antibodies Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 555 goat anti-chicken (both ThermoFisher Scientific) were applied for 1 h at 4 °C. Nuclei were stained with Hoechst 33,342 (Thermo Fisher Scientific). The samples were analyzed and photographed using a Leica TCS SPE spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.11. Statistical analysis

Data were analyzed using SPSS 15.0 software. Normality and homogeneity of variances were evaluated with the Shapiro-Wilk test and the Levene test, respectively. Student́s *t* test or ANOVA followed by post-

hoc HSD Tukey test were employed under parametric conditions. On the contrary, Kruskal-Wallis test and/or Mann-Whitney *U* test were used under non-parametric conditions. In all cases, *P* value $\leq$ 0.05 was considered statistically significant. Data were represented as mean $\pm$ standard deviation (SD).

3. Results

3.1. Physicochemical characterization of formulations

In general, formulations containing NDs presented higher mean particle size values than their counterparts in all conditions (Fig. 2A). At day 0, freshly prepared nanodiasome formulations showed a mean particle size of 128.7 $\pm$ 4.2 nm, which maintained stable over time and was significantly increased ($P < 0.05$) only after 30 days of storage at 25 °C. Regarding the niosome formulation, the mean particle size at day 0 was 90.5 $\pm$ 10.3 nm and presented significant oscillations ($P < 0.05$) at day 15 of storage at 25 °C and at day 30 of storage at 4 °C.

The measurement of zeta potential of nanodiasomes and niosomes at different days and temperatures of storage revealed more oscillations in the case of niosome formulations than their counterparts (Fig. 2B). The mean zeta potential value of freshly prepared nanodiasomes at day 0 was 35.2 $\pm$ 0.3 mV and presented a statistically relevant increase ($P < 0.05$) after 30 days of storage at both 4 °C and 25 °C. On the other hand, niosome formulations showed a mean zeta potential value of 20.2 $\pm$ 2.5 mV at day 0, which significantly increased after 15 days of storage at 25 °C ($P < 0.05$) and decreased after 30 days of storage at 4 °C ($P < 0.05$) and 25 °C ($P < 0.01$).

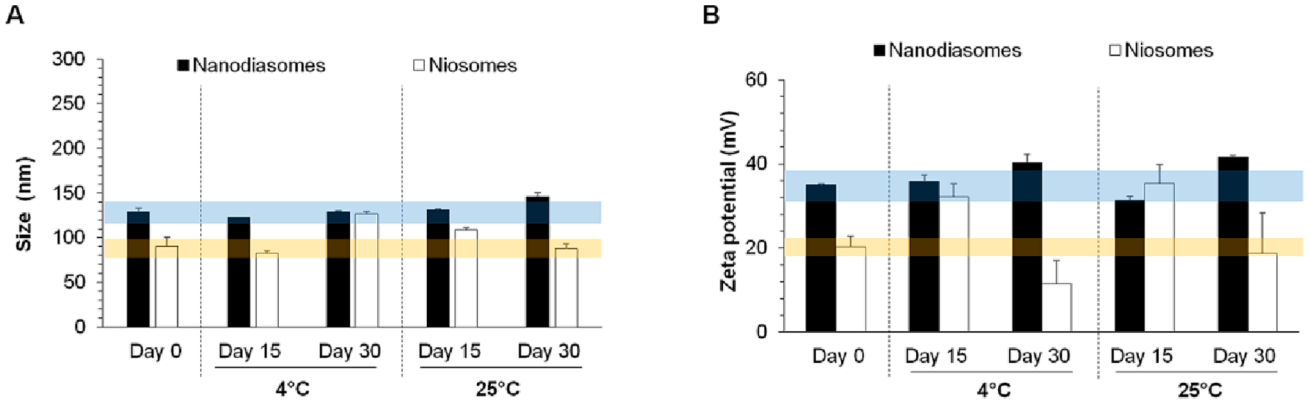
Dispersity values of nanodiasomes were lower than niosomes and remained stable with little oscillations at all conditions tested, while niosome formulations showed higher values and more variations, especially after being stored during 15 days at 25 °C (Fig. 2C).

Nanodiasomes under electron cryo-tomography microscopy (Fig. 2D) showed a spherical shape, with the NDs integrated in the lipid layer (Fig. 2D, white arrow).

3.2. Gene delivery efficiency and toxicity of nioplexes and nanodiaplexes

The comparative evaluation of cell viability and gene delivery efficiency in cells between nanodiaplexes and nioplexes, prepared with fresh formulations or with formulations stored for 15 and 30 days at different temperatures, showed that nanodiaplexes were better tolerated by cells and achieved significantly higher transfection rates at all conditions. Data were normalized to Lipofectamine 2000^{TM} which reported 39.5 $\pm$ 12.2% of EGFP expression in live cells, and 79.7 $\pm$ 14.6% cell viability in HEK-293 cells (data not shown). The mean percentage of live cells exposed to freshly prepared nanodiaplexes was 90.79 $\pm$ 2.5%, while this value was significantly lower ($P < 0.001$) for nioplexes which presented a mean percentage of live cells of 78.8 $\pm$ 5.8% (Fig. 3A, lines). These values remained relatively stable over time and different storage temperatures, with little oscillations but no statistically relevant differences compared to the values of day 0 in both formulations.

Regarding transfection efficiency, the percentage of EGFP expressing live cells exposed to freshly prepared nanodiaplexes and nioplexes were, respectively, 89.8 $\pm$ 3.4% and 23.3 $\pm$ 1.1% (Fig. 3A, bars). These values remained stable for both formulations over time and storage conditions, always maintaining significantly higher transfection percentages in cells treated with nanodiaplexes than with nioplexes ($P < 0.001$). In addition, the MFI data (Fig. 3B) corroborated the advantage of nanodiaplexes over nioplexes, with significantly higher MFI values obtained in cells exposed to nanodiaplexes prepared with nanodiasome formulations at all days and storage conditions tested ($P < 0.001$). Fig. 3C and D show representative fluorescence microscopy images of HEK-239 transfected cells with both formulations at day 0 and after 30 days of storage at 4 °C, respectively.



C

	Day 0	Day 15		Day 30	
		4°C	25°C	4°C	25°C
Nanodiasomes	0.28 ± 0.03	0.20 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	0.34 ± 0.03
Niosomes	0.46 ± 0.02	0.48 ± 0.11	0.67 ± 0.09	0.42 ± 0.14	0.47 ± 0.01

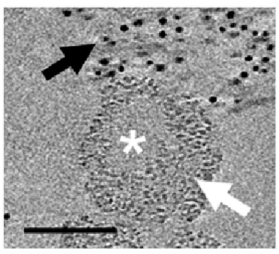


Fig. 2. Physicochemical characterization and stability of formulations at different days and storage temperature. **A.** Mean particle size. **B.** Zeta potential. **C.** Dispersity. Each value shows the mean $\pm$ SD of 3 readings. Blue and orange stripes represent $\pm$ 10% deviation respect to nanodiasomes and niosomes parameters at day 0, respectively. **D.** Cryo-electron tomogram slice of a nanodiasome; asterisk indicates the aqueous phase; white arrow indicates the lipid layer of the nanodiasome with nanodiamonds integrated in the lipid structure; black arrow indicates higher densities of the tomogram (more electron-dense material), which correspond to gold nanoparticles added to the sample for tilt series alignment. Scale bar: 100 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

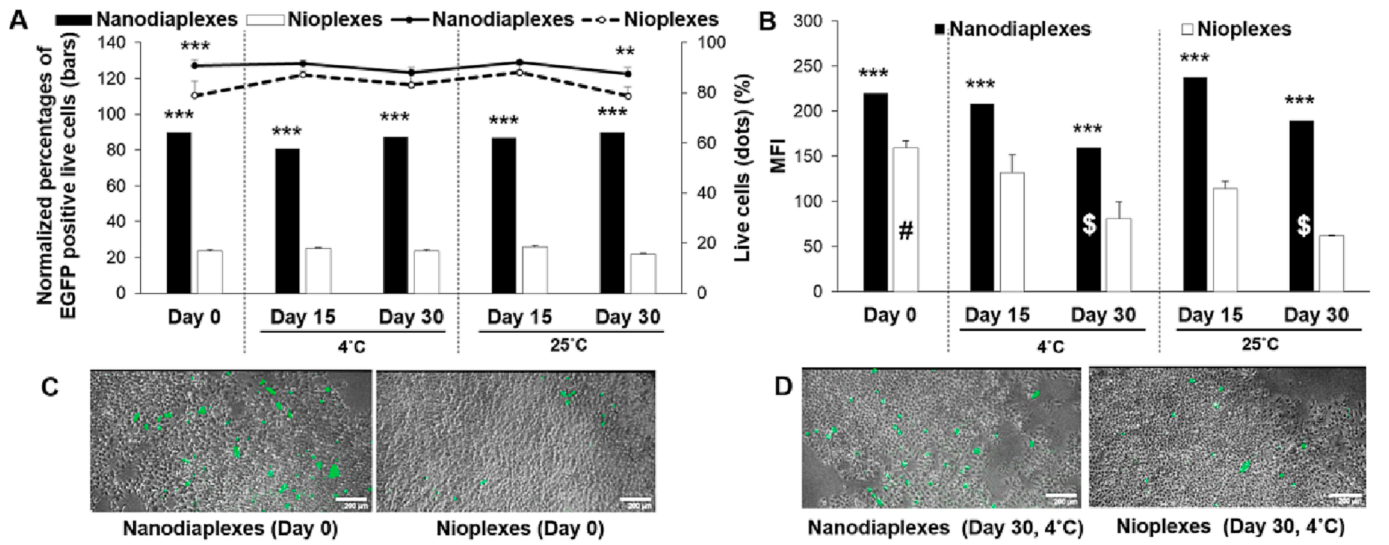


Fig. 3. Gene delivery efficiency and toxicity of formulations in HEK-293 cells 48 h after transfection with nanodiaplexes and nioplexes at 5/1 cationic lipid/DNA ratio (w/w) over time at 4℃ and 25℃. **A.** Normalized percentages of EGFP positive live cells (bars) and cell viability (dots) obtained by flow cytometry. **B.** Mean fluorescence intensity values obtained by flow cytometry. Each value represents the mean ± SD of 3 measurements. **C-D.** Merged images showing EGFP signal in HEK-293 transfected cells with both complexes at 5/1 lipid/DNA ratio (w/w) at day 0 (C) and after 30 days of storage at 4 °C (D). Scale bars: 200 μm. *** $P < 0.001$; ** $P < 0.01$ for nanodiaplexes vs nioplexes, no negative significant differences in term of live cells (%) for nioplexes between day 0 and the rest of days and temperatures; # $P < 0.05$ for nioplexes between day 0 and the rest of days and temperatures; $ $P < 0.05$ for nanodiaplexes at day 30 compared with the rest of days and temperatures.

3.3. Cellular uptake of nioplexes and nanodiaplexes

The analysis of cellular uptake in HEK-293 cells 4 h after exposure to complexes prepared with fresh and 30 days at 4 °C stored nanodiasomes and niosomes, revealed significantly higher ($P < 0.05$) cell internalization percentages for ND based formulations, at both conditions (Fig. 4A). These cell uptake percentages remained stable over time for both formulations, with statistically relevant differences. Such values were normalized to Lipofectamine 2000™ which reported 43.5 ± 2.7% of FITC-pEGFP positive cells 4 h after transfection (data not shown). Fig. 4B shows representative images of cellular uptake in HEK-293 cells exposed to both formulations at days 0 and 30.

3.4. Gene delivery efficiency of nanodiaplexes in rat primary cell cultures

The transfection assay of nanodiaplexes in rat primary retinal cells with freshly prepared (Fig. 5A) and 30 days stored nanodiasomes at 4 °C (Fig. 5B) showed similar EGFP expression, indicating that the transfection efficiency of that formulation maintained stable over a month. Additionally, the transfection efficiency of fresh and 30 days stored nanodiasomes was also evaluated in another CNS cell type, specifically in rat primary neuronal cell culture, which clearly corroborated the high gene delivery capacity, by means of EGFP expression, of stored formulations over a month and even 3 months (Supplementary Fig. S1).

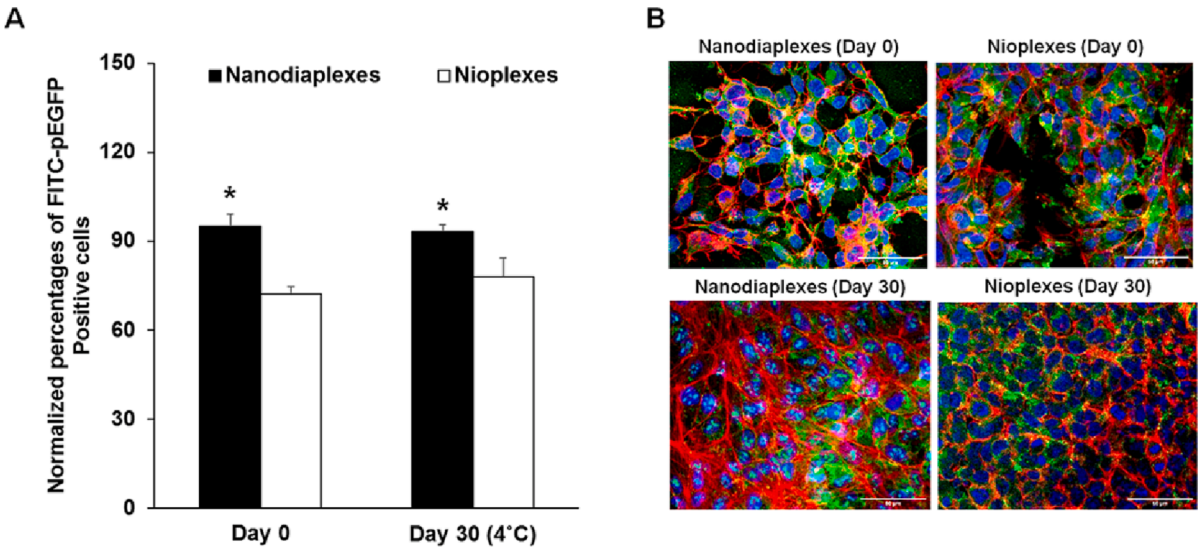


Fig. 4. Cellular uptake in HEK-293 cells 4 h after exposure to nanodiasomes and niosomes at 5/1 lipid/DNA ratio (w/w) at day 0 and after 30 days of formulations storage at 4℃. **A.** Normalized percentages of FITC-pEGFP positive live cells after the exposure to these complexes. Each value represents the mean ± SD of 3 measurements. **B.** Confocal microscopy images. Cell nuclei were colored in blue (DAPI); F-actin in red (Phalloidin); nanodiaplexes and nioplexes in green (FITC). Scale bars: 50 μm.* $P < 0.05$ for nanodiaplexes vs nioplexes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

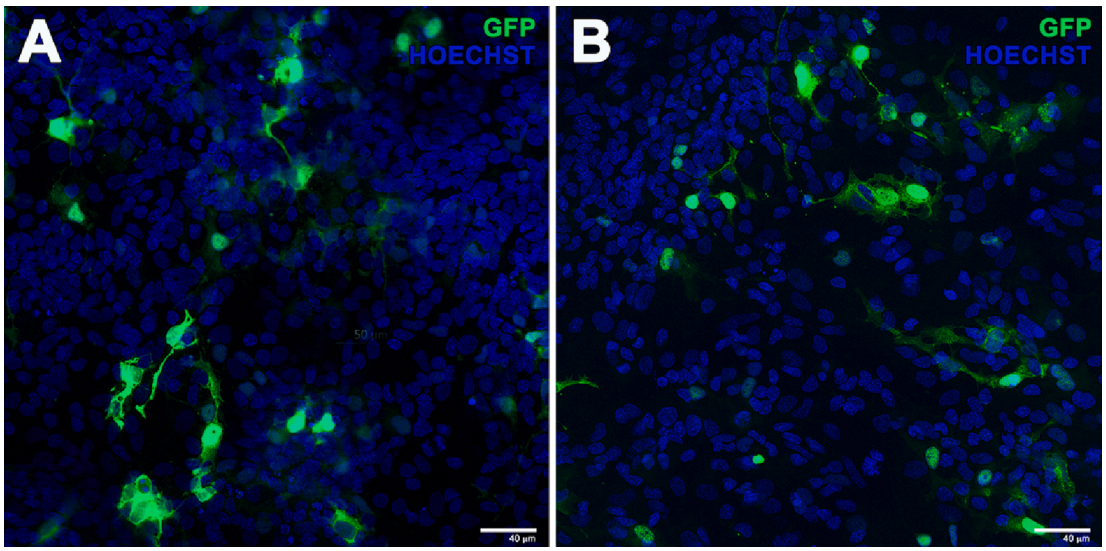


Fig. 5. EGFP signal in primary culture of rat retinal transfected cells with freshly prepared **(A)** and 30 days stored at 4 °C **(B)** nanodiasomes at 5/1 lipid/DNA ratio (w/w). Scale bar: 40 µm. Blue: Hoechst 33,342 (cell nuclei); Green: EGFP. Scale bars: 40 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. *In vivo transfection efficiency of nanodiaplexes*

Nanodiaplexes, prepared with fresh and stored nanodiasomes, were administered to the mouse eye through intravitreal (Fig. 6A and C) and subretinal injections (Fig. 6B and D), and fluorescence signal was detected in different retinal cell layers after one week. Both subretinally and intravitreally administered nanodiaplexes showed that EGFP expression colocalized mainly with microglial marker Iba-1 and was also located in the ganglion cell layer (GCL), as well as in the inner nuclear layer (INL) with some diffused fluorescence signal in the outer nuclear layer (ONL), and even the retinal pigment epithelial cell layer (RPE) after subretinal injections (Fig. 6B and D). Results also showed that the intensity of the fluorescence signal was comparable in both transfections with freshly prepared and 30 days stored formulations. Additionally, mouse retinal cells tolerated well the exposition to nanodiaplexes, in terms of cell viability, considering the results reported in the qualitative analysis.

4. Discussion

The high versatility of non-viral vectors relies on the large variety of available nanomaterials and preparation methods that can be employed. Among the wide plethora, NDs have been recognized as powerful tools to increase the transfection efficiency of many non-viral vector systems due to their unique physicochemical properties, including versatile surface chemistry and ease of functionalization, together with their high biocompatibility and low toxicity (Al Qtaish et al., 2022). In addition, NDs show a favourable particle distribution, being almost spherical in shape. Interestingly, they can also be easily functionalized with many chemical compounds, show a high surface area-to-volume ratio, and their production process can be easily scalable (Krüger et al., 2006; Liu et al., 2007). As NDs present low stability in suspension, their combination with niosome formulations could be necessary to provide enhanced stability, which is necessary for gene delivery applications. Therefore, in this work we combined NDs with a niosome formulation, based on a cationic lipid and non-ionic surfactant, obtaining a final formulation named nanodiasome in order to assess over time at several storage temperatures the stability of nanodiasomes compared with niosomes devoid of NDs. To evaluate the stability of the formulations, relevant physicochemical parameters that affect to the transfection process, along with biocompatibility and transfection efficiency studies were performed in *in vitro* and *in vivo* conditions.

The physicochemical characteristics constitute key parameters that determine the biological behaviour of the formulations, including their cellular internalization process, gene delivery efficiency and biocompatibility. In the present work, nanodiasomes showed a slightly higher mean particle size than niosomes at day 0, probably due to the incorporation of NDs as additional elements, which might have affected to the packing of the formulation. The lower dispersity values observed with nanodiasomes, indicated a more homogeneous particle size distribution for that formulation compared to the niosome formulation. Both formulations presented statistically relevant oscillations in their physicochemical parameters, especially after 30 days being stored at 25 °C, suggesting that these parameters are better preserved if formulations are kept at 4 °C rather than at higher temperatures. Hence, in general terms, it can be said that nanodiasomes are physicochemically more stable over time than niosomes. Therefore, NDs integration in the lipid structure of niosomes is involved in supplying higher stability to the formulation, probably providing more rigidity, by affecting the arrangement of the lipid membrane and modifying the rheological and packing behaviour of the formulation (Sainz-Ramos et al., 2021).

After the evaluation of physicochemical properties, biological *in vitro* transfection studies were performed in HEK-293 cells. We found that transfected cells with nanodiaplexes presented higher cell viability values than the ones transfected with nioplexes, which suggests that the formulations based on nanodiasomes are better tolerated by these cells. These results are in accordance with the previously reported high biocompatibility and low toxicity of NDs (Krüger et al., 2006; Liu et al., 2007; Zhang et al., 2009). In addition, the gene delivery efficiency of nanodiaplexes was approximately 4-fold superior than the one of nioplexes, and this difference was maintained over time. The MFI values refer to the quantity of the expressed GFP protein, and also indicated a higher transfection capacity of nanodiaplexes over nioplexes. In this sense, it is noteworthy that the number of DNA copies per cell decreased progressively for nioplexes from day 15, while nanodiaplexes did not suffer any alteration in this parameter until day 30. Taken all together, this could suggest that the combination of NDs with niosomes enhances the stability of the formulation, achieving more consistent and successful transfection results over time. To better understand the differences observed between both formulations, we studied their cellular uptake at 0 and 30 days after being stored at 4 °C. We found statistically relevant differences in the percentage of cellular uptake between both

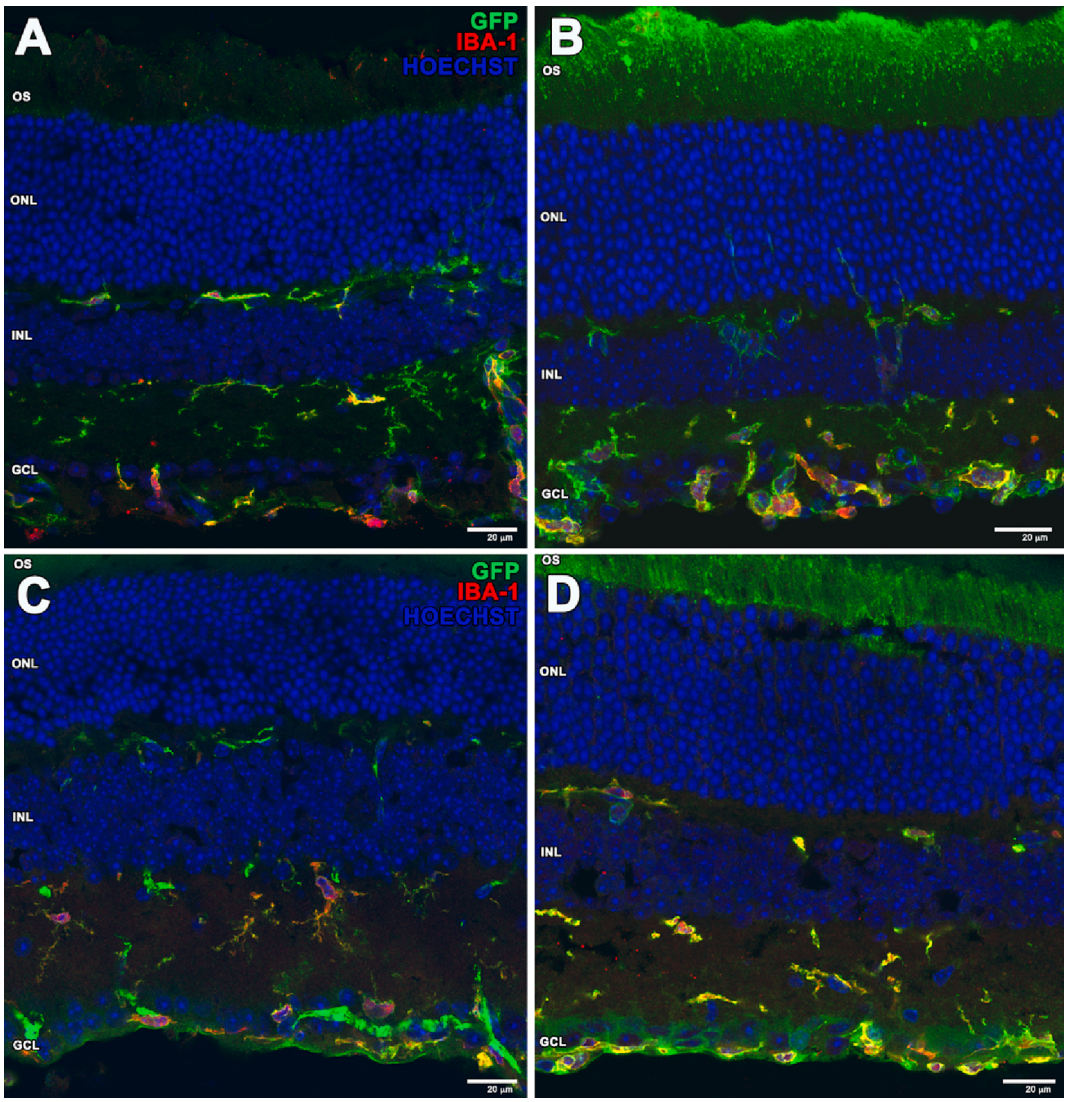


Fig. 6. *In vivo* assays showing EGFP signal in mouse retina after intravitreal (IV) **(A-C)** and subretinal (SR) **(B-D)** administration of freshly prepared **(A-B)** and 30 days stored **(C-D)** nanodiasomes vectoring EGFP plasmid at 5/1 lipid/DNA ratio (w/w). Blue: Hoechst 33,342 (cell nuclei); Green: EGFP; Red: Iba-1. OS: outer segments; ONL: outer nuclear layer; INL; inner nuclear layer; GCL: ganglion cell layer. Scale bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

formulations, obtaining almost 100% normalized values of cellular uptake with nanodiasomes and around 70% with niosomes at both storage conditions, which could support the idea that NDs increase the rigidity of the niosome formulation, enhancing the cellular entry (Manzanares and Cena. 2020). This higher cellular uptake could in part explain the differences in transfection efficiency between the two formulations, but further aspects need also to be taken into account. Traditionally, cellular endocytosis of non-viral vectors is mediated through the endosomal pathway, which eventually leads to endosomal vesicles with an acidic environment and digestive enzymes (Agirre et al., 2015). In these vesicles, the DNA risks of being degraded before reaching the nucleus. Therefore, DNA endosomal escape becomes a key step in order to achieve successful gene delivery. In this sense, it has been reported that NDs are able to escape the endosome confinement by rupturing the vesicle membrane shortly after their cellular uptake (Chu et al., 2015), which would also contribute to justify the higher gene delivery efficiency of nanodiaplexes compared to nioplexes counterparts. In addition, further transfection assays in CNS cells, both retinal and neuronal primary cells, confirmed effective transgene expression after transfection with both freshly prepared and 30 days stored nanodiaplexes.

Therefore, based on these results, we performed an *in vivo* assay in order to determine the gene delivery efficiency of nanodiaplexes from fresh and 30 days stored nanodiasomes formulation in mouse retina. Formulations were injected through intravitreal and subretinal routes, which are widely used into the clinic for the treatment of genetically based retinal disorders (Conley and Naash. 2010). In most cases, after intravitreal injection, ganglion cell layer of the retina shows high transgene expression levels (Farjo et al., 2006), which, for instance, could be interesting for the treatment of glaucoma, a highly prevalent inherited retinal disorder that causes blindness (Almasieh et al., 2012; Kachi et al., 2005). On the other hand, the more invasive subretinal route is useful for transfecting the outer layer of the retina (Almasieh et al., 2012; Kachi et al., 2005), which would be interesting to face retinal diseases related to mutations at the photoreceptors and the retinal pigment epithelium level, such as Leber's congenital amaurosis, Stagardt's disease or retinitis pigmentosa (Lipinski et al., 2013). In the present study, EGFP signal was found mainly in microglial cells. Such expression was also located in both, the inner and outer layers of the retina both after intravitreal and subretinal injection of nanodiaplexes, which suggest that this formulation is able to efficiently diffuse along the different retinal layers achieving high transgene expression at different levels, which would be relevant from the therapeutic point of view. In

addition, results revealed high EGFP expression *in vivo* after the administration of 30 days stored formulation, indicating that the storage of the formulation at 4 °C for 30 days does not affect its transfection efficiency.

5. Conclusions

Taken together, the main conclusions of the present work are that (i) nanodiasomes present higher mean particle size, lower dispersity and higher zeta potential values than niosomes, (ii) nanodiasomes preserve more constant their physicochemical parameters over time than niosomes and both formulations prefer low temperatures for storage, (iii) nanodiaplexes present an around 4-fold superior transfection efficiency than nioplexes, in terms of percentage of live transfected cells, although both maintain their transfection efficiency over time, (iv) nanodiaplexes are more efficiently uptaken by HEK-293 cells than nioplexes, (v) high gene delivery efficiency of nanodiaplexes is maintained over time in rat central nervous primary cell cultures and (vi) also *in vivo* after subretinal and intravitreal injection of nanodiaplexes in mouse retina.

CRediT authorship contribution statement

Nuseibah H. AL Qtaish: Investigation, Methodology, Visualization, Writing – original draft. **Ilia Villate-Beitia:** Formal analysis, Visualization, Writing – original draft. **Idoia Gallego:** Formal analysis, Visualization, Writing – review & editing. **Gema Martínez-Navarrete:** Investigation, Visualization, Writing – review & editing. **Cristina Soto-Sánchez:** Investigation, Visualization, Writing – review & editing. **Myriam Sainz-Ramos:** Investigation, Writing – review & editing. **Tania B Lopez-Mendez:** . **Alejandro J. Paredes:** . **Francisco Javier Chichón:** Formal analysis, Data curation. **Noelia Zamarreño:** Methodology. **Eduardo Fernández:** Supervision, Writing – review & editing. **Gustavo Puras:** Conceptualization, Supervision, Project administration, Writing – review & editing. **José Luis Pedraz:** Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.122968.

References

Agirre, M., Ojeda, E., Zarate, J., Puras, G., Grijalvo, S., Eritja, R., et al., 2015. New insights into gene delivery to human neuronal precursor NT2 cells: a comparative study between lipoplexes, nioplexes, and polyplexes. Molecular Pharmaceutics. 12 (11), 4056–4066.

Al Qtaish, N., Gallego, I., Villate-Beitia, I., Sainz-Ramos, M., Lopez-Mendez, T.B., Grijalvo, S., et al., 2020. Niosome-based approach for in situ gene delivery to retina and brain cortex as immune-privileged tissues. Pharmaceutics 12 (3), 198.

Al Qtaish, N., Gallego, I., Villate-Beitia, I., Sainz-Ramos, M., Martinez-Navarrete, G., Soto-Sanchez, C., et al., 2021. Sphingolipid extracts enhance gene delivery of cationic lipid vesicles into retina and brain. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V 169, 103–112.

Al Qtaish, N., Gallego, I., Paredes, A.J., Villate-Beitia, I., Soto-Sanchez, C., Martinez-Navarrete, G., et al., 2022. Nanodiamond integration into niosomes as an emerging and efficient gene therapy nanoplatform for central nervous system diseases. ACS Applied Materials & Interfaces 2022 14 (11), 13665-13677.

Alhaddad, A., Adam, M.P., Botsoa, J., Dantelle, G., Perruchas, S., Gacoin, T., et al., 2011. Nanodiamond as a vector for siRNA delivery to Ewing sarcoma cells. Small (Weinheim an der Bergstrasse, Germany) 7 (21), 3087–3095.

Almasieh, M., Wilson, A.M., Morquette, B., Cueva Vargas, J.L., Di Polo, A., 2012. The molecular basis of retinal ganglion cell death in glaucoma. Progress in retinal and eye research 31 (2), 152–181.

Alwani, S., Kaur, R., Michel, D., Chitanda, J.M., Verrall, R.E., Karunakaran, C., et al., 2016. Lysine-functionalized nanodiamonds as gene carriers: development of stable colloidal dispersion for in vitro cellular uptake studies and siRNA delivery application. International Journal of Nanomedicine 11, 687–702.

Bartelds, R., Nematollahi, M.H., Pols, T., Stuart, M.C.A., Pardakhty, A., Asadikaram, G., et al., 2018. Niosomes, an alternative for liposomal delivery. PLoS One 13, e0194179.

Bi, Y., Zhang, Y., Cui, C., Ren, L., Jiang, X., 2016. Gene-silencing effects of anti-survivin siRNA delivered by RGDV-functionalized nanodiamond carrier in the breast carcinoma cell line MCF-7. International Journal of Nanomedicine 11, 5771–5787.

Chauhan, S., Jain, N., Nagaich, U., 2020. Nanodiamonds with powerful ability for drug delivery and biomedical applications: recent updates on in vivo study and patents. Journal of Pharmaceutical Analysis 10, 1–12.

Chen, M., Zhang, X., Man, H., Lam, R., Chow, E., Ho, D., 2021. Nanodiamond vectors functionalized with polyethylenimine for siRNA delivery. The Journal of Physical Chemistry Letters 1 (21), 3167–3171.

Chu, Z., Miu, K., Lung, P., Zhang, S., Zhao, S., Chang, H.C., et al., 2015. Rapid endosomal escape of prickly nanodiamonds: implications for gene delivery.Scientific reports 5, 11661.

Conley, S.M., Naash, M.I., 2010. Nanoparticles for retinal gene therapy. Progress in retinal and eye research 29 (5), 376–397.

Do, H.D., Couillaud, B.M., Doan, B.T., Corvis, Y., Mignet, N., 2019. Advances on non-invasive physically triggered nucleic acid delivery from nanocarriers. Advanced drug delivery reviews 138, 3–17.

Edgington, R, Spillane, KM, Papageorgiou, G, Wray, W, Ishiwata, H, Labarca, M, et al., 2018. Functionalisation of detonation nanodiamond for monodispersed, soluble DNA-nanodiamond conjugates using mixed silane bead-assisted sonication disintegration. Scientific reports 8 (1), 728.

Farjo, R., Skaggs, J., Quiambao, A.B., Cooper, M.J., Naash, M.I., 2006. Efficient non-viral ocular gene transfer with compacted DNA nanoparticles. PLoS One 1, e38.

Ginn, S.L., Amaya, A.K., Alexander, I.E., Edelstein, M., Abedi, M.R., 2018. Gene therapy clinical trials worldwide to 2017: an update. The Journal of Gene Medicine 20 (5), e3015.

Grijalvo, S., Puras, G., Zarate, J., Sainz-Ramos, M., Qtaish, N.A.L., Lopez, T., et al., 2019. Cationic niosomes as non-viral vehicles for nucleic acids: challenges and opportunities in gene delivery. Pharmaceutics 11 (2), 50.

Ibraheem, D., Elaissari, A., Fessi, H., 2014. Gene therapy and DNA delivery systems. International Journal of Pharmaceutics 459 (1-2), 70–83.

Kachi, S., Oshima, Y., Esumi, N., Kachi, M., Rogers, B., Zack, D.J., et al., 2005. Nonviral ocular gene transfer. Gene therapy 12 (10), 843–851.

Krüger, A., Liang, Y., Jarrea, G., Stegka, J., 2006. Surface functionalisation of detonation diamond suitable for biological applications. Journal of Materials Chemistry 16, 2322–2328.

Lim, D.G., Prim, R.E., Kim, K.H., Kang, E., Park, K., Jeong, S.H., 2016. Combinatorial nanodiamond in pharmaceutical and biomedical applications. International journal of pharmaceutics 514 (1), 41–51.

Lim, D.G., Rajasekaran, N., Lee, D., Kim, N.A., Jung, H.S., Hong, S., et al., 2017. Polyamidoamine-decorated nanodiamonds as a hybrid gene delivery vector and siRNA structural characterization at the charged interfaces. ACS applied materials & interfaces 9 (37), 31543–31556.

Lipinski, D.M., Thake, M., MacLaren, R.E., 2013. Clinical applications of retinal gene therapy. Progress in retinal and eye research 32, 22–47.

Liu, K.K., Cheng, C.L., Chang, C.C., Chao, J.I., 2007. Biocompatible and detectable carboxylated nanodiamond on human cell. Nanotechnology 18 (32), 325102.

Manzanares, D., Cena, V., 2020. Endocytosis: the nanoparticle and submicron nanocompounds gateway into the cell. Pharmaceutics 12 (4), 371.
Mashal, M., Attia, N., Puras, G., Martinez-Navarrete, G., Fernandez, E., Pedraz, J.L., 2017. Retinal gene delivery enhancement by lycopene incorporation into cationic niosomes based on DOTMA and polysorbate 60. Journal of controlled release : official journal of the Controlled Release Society 254, 55–64.
Mashal, M., Attia, N., Soto-Sanchez, C., Martinez-Navarrete, G., Fernandez, E., Puras, G., et al., 2018. Non-viral vectors based on cationic niosomes as efficient gene delivery vehicles to central nervous system cells into the brain. International journal of pharmaceutics 552 (1–2), 48–55.
Mashal, M., Attia, N., Martinez-Navarrete, G., Soto-Sanchez, C., Fernandez, E., Grijalvo, S., et al., 2019. Gene delivery to the rat retina by non-viral vectors based on chloroquine-containing cationic niosomes. Journal of controlled release : official journal of the Controlled Release Society, 304, 181–190.
Ojeda, E., Puras, G., Agirre, M., Zarate, J., Grijalvo, S., Eritja, R., et al., 2016a. The influence of the polar head-group of synthetic cationic lipids on the transfection efficiency mediated by niosomes in rat retina and brain. Biomaterials 77, 267–279.
Ojeda, E., Puras, G., Agirre, M., Zarate, J., Grijalvo, S., Eritja, R., et al., 2016b. The role of helper lipids in the intracellular disposition and transfection efficiency of niosome formulations for gene delivery to retinal pigment epithelial cells. Int. J. Pharm. 503, 115–126.
Puras, G., Martinez-Navarrete, G., Mashal, M., Zarate, J., Agirre, M., Ojeda, E., et al., 2015. Protamine/DNA/niosome ternary nonviral vectors for gene delivery to the retina: the role of protamine. Molecular pharmaceutics, 12(10), 3658–3671.
Riley, M.K., Vermerris, W., 2017. Recent advances in nanomaterials for gene delivery-a review. Nanomaterials (Basel, Switzerland) 7 (5), 94.
Sainz-Ramos, M., Villate-Beitia, I., Gallego, I., Al Qtaish, N., Menendez, M., Lagartera, L., et al., 2021. Correlation between biophysical properties of niosomes elaborated with chloroquine and different tensioactives and their transfection efficiency. Pharmaceutics 13 (11), 1787.
Shahryari, A., Saghaeian Jazi, M., Mohammadi, S., Razavi Nikoo, H., Nazari, Z., Hosseini, E.S., et al., 2019. Development and clinical translation of approved gene therapy products for genetic disorders. Frontiers in genetics, 10, 868.
Suzuki, Y., Hyodo, K., Tanaka, Y., Ishihara, H., 2015. siRNA-lipid nanoparticles with long-term storage stability facilitate potent gene-silencing in vivo. Journal of controlled release : official journal of the Controlled Release Society, 220(Pt A), 44–50.
Zhang, X.Q., Chen, M., Lam, R., Xu, X., Osawa, E., Ho, D., 2009. Polymer-functionalized nanodiamond platforms as vehicles for gene delivery. ACS Nano 3 (9), 2609–2616.