RESEARCH ARTICLE
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Development and Evaluation of O-CaP Core Nanoparticles for Efficient Non-Viral Gene Delivery

ABSTRACT

Background: Nanoparticles have gained significant attention in biomedical applications, particularly in gene delivery, due to their tunable physicochemical properties. A new core nanoparticle, O-CaP-2 (Octadecylamine- Calcium Phosphate core nanoparticle), was synthesized and evaluated for its physicochemical properties, cytocompatibility, and gene transfer efficiency.

Methods: In this study, 2 different nanoparticles were synthesized and characterized, and their transfection efficiency was evaluated. A core-shell structure was formed by coating calcium phosphate nanoparticles (CaP NPs) with octadecylamine nanoparticles (OD NPs) to enhance stability and cellular uptake. The synthesized nanoparticles were characterized in terms of size and surface charge using a Zetasizer. Morphological properties were analyzed by scanning electron microscopy (SEM), while chemical interactions were investigated through Fourier transform infrared spectroscopy (FTIR). The cytotoxicity of the nanoparticles was assessed using MTT (2,5-diphenyltetrazolium bromide [MTT, Thiazolyl blue]) assay over a 24-hour period. Finally, nanoparticles exhibiting optimal physicochemical properties and biocompatibility were selected for transfection studies. The transfection efficiency of these nanoparticles was evaluated to determine their potential for gene delivery applications.

Results: The O-CaP-2 nanoparticle, with a zeta potential of +24.3 mV, an average size of 184 nm, and a PDI (polydispersity index) of 0.2, was selected for further evaluation. Scanning electron microscopy imaging showed dense, spherical particles, while FTIR analysis confirmed the presence of octadecylamine and calcium phosphate. MTT assays indicated no cytotoxic effects across tested concentrations. Gene transfer studies achieved a transfection efficiency of 78% ± 5.18%, demonstrating O-CaP-2's potential as a safe and effective gene delivery vector.

Conclusion: The O-CaP-2 core nanoparticle, characterized by its favorable physicochemical properties, biocompatibility, and high transfection efficiency, demonstrates strong potential as a safe and effective non-viral gene delivery vector.

Keywords: CCD1072-SK, core nanoparticle, gene delivery

What is already known on this topic?

- Nanoparticles are economical, highly biosecure tools preferred in gene and drug transfer as an alternative to viral vectors in recent years. Octadecylamine is a form of fatty acid also found in cell membranes and has no toxic effect on cells. Calcium phosphate is a mineral found in bone and tooth structure and is widely preferred especially in the field of biomaterials. Octadecylamine-based and calcium phosphate nanoparticles have been synthesized by scientists in the literature and used for the transfection of different cell lines, but there is no study in the literature that uses both components for a single type of nanoparticle.

What this study adds on this topic?

- O-CaP core nanoparticles synthesized by combining 2 different nanoparticles increase the transfection efficiency compared to 2 nanoparticles and strengthen the protection of the genetic material targeted for transfer from cellular and environmental factors. With the new type of O-CaP nanoparticles, positively charged, small-sized, and non-laborious-to-synthesize nanoparticles that can be used in gene transfer have been introduced to the literature.

INTRODUCTION

Nanotechnology has revolutionized various fields, including medicine, by enabling the development of novel drug delivery and gene therapy systems. Among these, nanoparticles have emerged as promising carriers for nucleic acids due to their ability to protect genetic material and facilitate cellular uptake.[1] Calcium phosphate nanoparticles (CaP NPs) have been widely investigated as gene delivery vectors due to their biodegradability, biocompatibility, and ability to enhance transfection efficiency. However, their stability and cellular uptake efficiency can be improved by surface modifications.[2]

Octadecylamine (OD) is a hydrophobic molecule that can enhance the stability and cellular interaction of nanoparticles when used as a coating material.[3] By forming a core-shell structure with CaP NPs as the core and OD NPs as the shell, the aim was to develop a nanoparticle system with improved physicochemical properties and gene delivery potential. The characterization of these nanoparticles, including size, surface charge, and chemical composition, is crucial for understanding their behavior in biological environments.[4]

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In this study, CaP NPs coated with ODA NPs were synthesized and characterized, and their suitability for gene delivery applications was assessed. The nanoparticles were analyzed using a Zetasizer for size and zeta potential, SEM for morphological properties, and FTIR for chemical composition. Furthermore, their cytotoxicity was evaluated using CC1072-SK cells, and their transfection efficiency was assessed. This research contributes to the development of an optimized nanoparticle system with potential applications in gene therapy and biomedical engineering.

MATERIALS AND METHODS

The CCD1072-SK human fibroblast cell line was sourced from the cell stocks at Yıldız Technical University Molecular Biology and Genetics Laboratory. These are not primary human cells. For this study, the following reagents were procured: Dulbecco's Modified Eagle Medium (DMEM) (high glucose, with glutamine), F-12 Nutrient Mixture (Ham's), Fetal Bovine Serum, Trypsin-EDTA Solution C (0.05%) with EDTA (0.02%) from Biological Industries (Beit-Haemek, Israel. The plasmid used to transfect CCD1072-SK human fibroblast cells was the human 4- in-1 iPSC PiggyBac Vector from System Biosciences (Palo Alto, CA, USA). This study used a commercially available cell line and no human tissue or cells were harvested, therefore no ethical approval and informed consent were required.

Synthesis of Nanoparticles

Calcium phosphate nanoparticles (CaP NPs) were synthesized using by the microemulsion method proposed in the paper by Yildirim et al[5] In this method, 2 different solutions were first prepared. The content of the first solution was formed with calcium chloride (0.05 M), sodium dodecyl sulfate (SDS, 1% w/v), and Tween80 (1% w/v) under continuous stirring for 24 hours. The other solution content was prepared with sodium citrate (0.025 M), disodium hydrogen phosphate (0.025 M), SDS (1% w/v) and Tween80 (1% w/v) dissolved with a magnetic stirrer for 24 hours.[5] The second solution was then added dropwise to the first solution, followed by centrifugation at 10,000 rpm to obtain a nanoparticle pellet. The mixture was stirred at 600 rpm for 72 hours and washed 4 times with ethanol at +4°C to remove volatiles. The obtained CaP NPs were dissolved in 2 μM CTAB solution and stored at −20°C for further lyophilization.

Octadecylamine nanoparticles (OD NPs) were made using an emulsion-solvent evaporation method. First, octadecylamine (0.2 M) was dissolved in 2 mL of chloroform to prepare the lipid solution. This solution was then slowly added to an aqueous solution containing 6% Tween 80 while stirring. To form the emulsion, the mixture was sonicated with a probe (30% amplitude, 50 seconds). After that, it was stirred for 4 hours to remove the solvent. The nanoparticles were collected by ultracentrifugation, freeze-dried, and stored at +4 °C for later use.[6]

In the nanoparticle synthesis stage, firstly 2 nanoparticles were synthesized separately and then O-CaP nanoparticles were obtained by forming a core-shell structure with 2 optimal particles. The synthesized nanoparticles were washed and collected via centrifugation, then resuspended in deionized water for further characterization.The obtained O-CaP nanoparticles zeta potential and size distributions were analyzed by Zetasizer.

Characterization of Nanoparticles

The size and zeta potential of the synthesized O-CaP nanoparticles were measured using a Malvern Nano ZS Zetasizer (Malvern Nano ZS Zetasizer is a brand name and model number) in liquid form with triplicate measurement. The morphological properties were observed using a Zeiss EVO LS 10 SEM. Prior to imaging, the samples were dripped onto SEM stabs and dried at room temperature. Since the samples are non-conductive, they were instrumented after Au/Pd coating. Chemical interactions and functional group analysis were performed using a Perkin Elmer Spectrum 100 FTIR system.

Cytotoxicity Assay

The cytotoxicity of the nanoparticles was evaluated using the MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with CC1072-SK human fibroblast cell line.[7] 10^5 cells were seeded into each well then incubated 24 hours for the cells to attach to the well. Second day each well treated with varying volumes of nanoparticles (10 μL, 20 μL, 30 μL) (1,1 ng/μL) for further 24 hours. On the last day, the medium was removed from the wells, MTT solution was added, and the plate was incubated for 4 hours. At the end of the time, each well was treated with dimethyl sulfoxide (DMSO) to dissolve the formazan crystals and the optical density was measured at 570 nm. Dimethyl sulfoxide was used as a positive control, and DMEM-F12 cell medium was used as a negative control.[8] All groups were performed in triplicate to ensure reproducibility.

Transfection Studies

Nanoparticles exhibiting optimal physicochemical properties and have no toxic effect on cells were selected for transfection. Transfection studies were performed on CCD1072-sk human fibroblast cells. 200 000 cells were seeded in each well using 24 well plates. The cells were allowed to attach and adapt to the wells by cultured for 24 hours in an incubator at 37°C and 5% CO_2. At the 23rd hour, the medium was replaced with serum-free medium to the cells were stressed and the make pores in the membrane structures opened. At 24 hours, DNA-O-CaP complex was added to the wells and the plate was placed in the incubator. Next day, transfection images were captured using a Zeiss Observer Z.1 inverted fluorescence microscope with GFP (Green Flourescent Protein) attachment at 24 hours and 48 hours. Transfection efficiency was calculated by comparing the number of transfected and non-transfected cells in 3 independent regions determined from the images.[9]

Table 1. Rates of O-CaP Nanoparticles Formation

OD Nanoparticles			CaP Nanoparticles		O-CaP Nanoparticles			
Zeta Potential	Size	Added Amount	Zeta Potential	Size	O-CaP No	Zeta Potential	Size	PDI
+36 mV	688 nm	25 µL	+15.7 mV	134.7 nm	O-CaP 1	19.8 mV	183 nm	0.3
+36 mV	688 nm	50 µL	+15.7 mV	134.7 nm	O-CaP 2	24.3 mV	184 nm	0.2
+36 mV	688 nm	75 µL	+15.7 mV	134.7 nm	O-CaP 3	28.1 mV	257 nm	0.2
+36 mV	688 nm	200 µL	+15.7 mV	134.7 nm	O-CaP 4	31.7 mV	426 nm	0.5
+36 mV	688 nm	300 µL	+15.7 mV	134.7 nm	O-CaP 5	33.1 mV	429 nm	0.3

CaP, calcium phosphate; OD, octadecylamine.

RESULTS

After the synthesis of OD and CaP nanoparticles, the nanoparticles with the optimum zeta potential and size for gene transfer were selected and used for core formation. In core formation, CaP nanoparticle was kept constant and OD nanoparticle was added at different ratios.Malvern Nano ZS Zetasizer device was used for the size and zeta potential analysis of the the new nanoparticle (O-CaP) in core form. All measurements were performed based on the DLS (dynamic light scattering) method with 3 repetitions.[10] The ratios in the formation process of O-CaP nanoparticles and the results are given in Table 1.

For gene transfer, the nanoparticle is expected to have a positive zeta potential after forming a conjugate with negatively charged DNA. Thus, it is aimed to transfer the gene to be transferred to cells by protecting it from environmental factors. As shown in Table 1, the uncoated CaP nanoparticles exhibited a zeta potential of +15.7 mV, while OD nanoparticles had a significantly higher surface charge of +36 mV. Following the coating process, the resulting O-CaP nanoparticles showed a progressive increase in zeta potential values ranging from +19.8 mV to +33.1 mV, depending on the amount of OD used. This increase in surface charge indicates successful coating and enhances colloidal stability. More importantly, zeta potential plays a crucial role in cellular uptake, as nanoparticles with moderately positive charges tend to interact more readily with negatively charged cell membranes. The enhanced zeta potential of O-CaP-2 (+24.3 mV), which was selected for transfection studies, may have contributed to its effective cellular internalization and transfection performance. This explanation has been included in the revised manuscript to provide a clearer link between physicochemical properties and biological outcomes. According to this table, all of the synthesized O-CaP nanoparticles have positive zeta potential for gene transfer. The particle size is expected to be as small as possible and the PDI value as close to 0.1 as possible. Considering the particle sizes and PDI values, the most suitable synthesized nanoparticle for gene transfer was determined as O-CaP-2. Characterization studies of O-CaP-2 nanoparticles were completed with the following studies.

Characterization of Nanoparticles

Scanning electron microcope was used for morphological characterization of the synthesized O-CaP-2 nanoparticles. Before the analysis, the nanoparticle solution was dripped onto the SEM stab and dried at room conditions and Au/Pd was coated to give the samples conductive properties and to obtain better quality images.[11] The obtained images are given in Image 1. In the images, it is observed that the synthesized nanoparticles are densely dispersed and spherical.

Fourier transform infrared spectroscopy (FTIR) was performed to confirm the chemical structure and functional groups of octadecylamine (OD), octadecylamine-based calcium phosphate nanoparticles (CaP), and octadecylamine nanoparticles (O-CaP). The obtained spectra are given in Image 2.

In the FTIR analysis, characteristic peaks corresponding to phosphate groups (PO_4^{3-}) of CaP and functional groups from the OD polymer were observed. The presence of overlapping or shifted bands, particularly in the regions of –OH and C=O stretching vibrations, indicates potential hydrogen bonding and electrostatic interactions between OD and CaP surfaces. In the spectrum of pure octadecylamine (blue line), strong peaks around 2848-2920 cm^{-1} were observed, which are related to C–H stretching vibrations of methyl and methylene groups. A broad peak around 3300-3400 cm^{-1} shows the N–H stretching of primary amines, and the peak near 1600-1650 cm^{-1} is

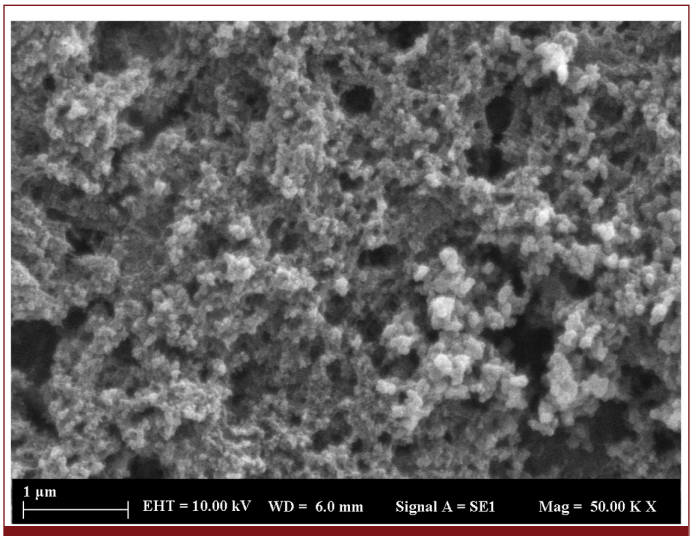

Image 1. Scanning electron microscopy images of O-CaP-2 nanoparticles.

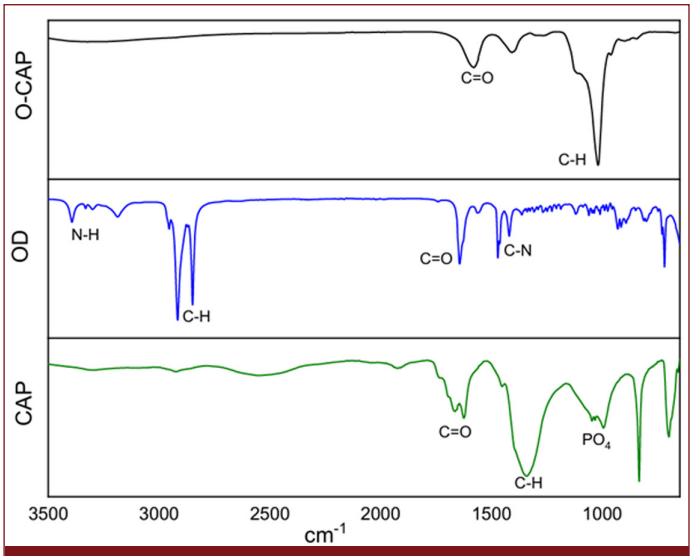

Image 2. Fourier transform infrared spectroscopy spectrum of O-CaP nanoparticles, octadecylamine and calcium phosphate.

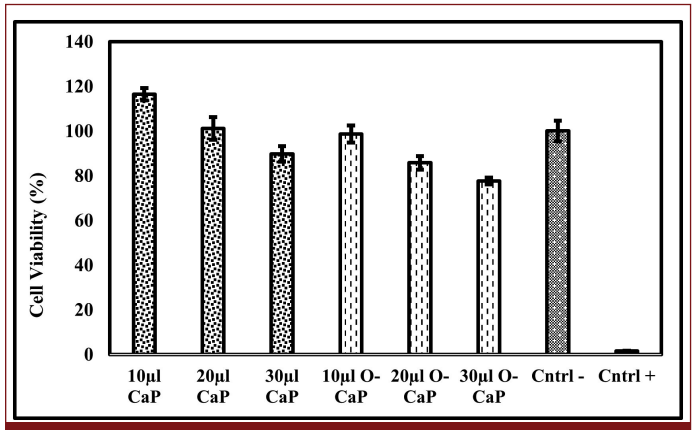

Image 3. Cytotoxicity analysis of calcium phosphate and O-CaP nanoparticles on CCD1072-SK cells.

due to N–H bending. In the O-CaP spectrum (black line), these peaks are still present but with lower intensity, possibly due to partial interaction or structural changes during nanoparticle formation.[12] In the CaP spectrum (green line), 1624 cm^{-1} shows carbonyl group (C=O) or amide I bands, 1340 cm^{-1} indicate C-H stretching, and a clear peak between 1030 and 1100 cm^{-1} appears, which corresponds to the asymmetric stretching of phosphate groups (PO_4^{3-}), indicating the formation of calcium phosphate.[13] The presence of C–H stretching bands in all samples shows that the alkyl chain structure is still present.[14] Slight shifts in the peaks indicate chemical interaction or complex formation between CaP and OD. These results confirm that octadecylamine and calcium phosphate were successfully incorporated into the O-CaP-2 structure and that there was a successful coating and stable integration of OD onto the nanoparticle surface.

Cytotoxicity analysis was performed with human fibroblast cells (CCD1072-SK) to examine the effect of nanoparticles on cells after morphological and chemical bond characterization studies were completed. Cytotoxicity levels were determined by the survival rate in the experimental groups compared to the control groups via MTT assay.[15] The analysis was performed during 24 hours of incubation. Survival rates in the experimental groups were determined by assuming 100% viability of the negative control group. The results are given in Image 3.

Accordingly those results, 10 µL and 20 µL CaP nanoparticles induced cell proliferation with 116.5% (±2.8) and 101.2% (±5.0), respectively. 30 µL CaP nanoparticles with 89.8% (±3.4), 10 µL O-CaP nanoparticles with 98.6% (±3.8), 20 µL O-CaP nanoparticles with 85.7% (±2.9), and 30 µL O-CaP nanoparticles with 77.6% (±1.4) showed no toxic effect on cells. All ratios of the synthesized nanoparticles were found to be suitable for gene transfer into cells. Studies have shown that calcium phosphate is a non-toxic, bioabsorbable component.[16]

Başak et al[17] evaluated the biochemical effects, cytotoxicity, and genotoxicity potentials of Nano-Calcium Phosphate Nanoparticles on the hFOB cell line, reporting that cell viability remained above 80% for both β-TCP and BCP (biphasic tricalcium phosphate) nanoparticles at concentrations up to 80 ppm. However, significant cytotoxic effects were observed at concentrations of 160 ppm

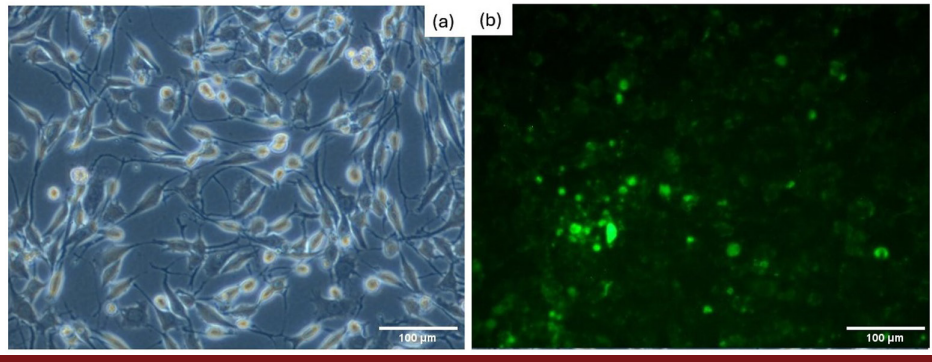

Image 4. Transfection images of CCD1072-SK cells at 24 hours: (a) Phase Contrast 20×, (b) GFP 20×.

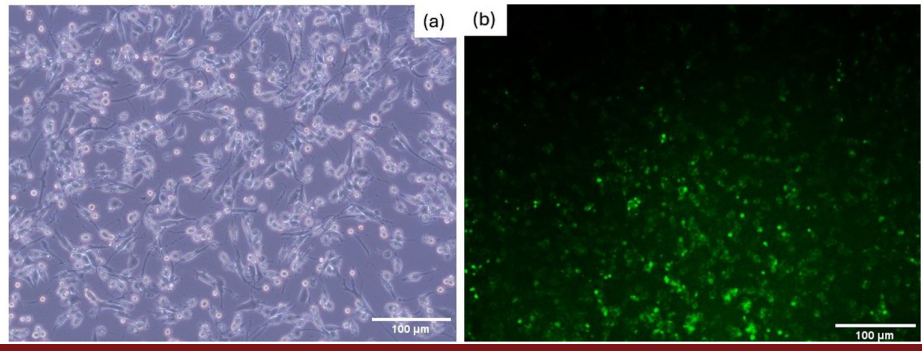

Image 5. Transfection images of CCD1072-SK cells at 48 hours (a) Phase Contrast 10× (b) GFP 10×.

and higher for BCP, and at 640 ppm for β-TCP, as evidenced by both reduced cell viability and increased LDH (lactate dehydrogenase) release ($P < .05$). The findings showed that the cytotoxicity of the particles was strongly concentration dependent. It was found that there are threshold values for the toxicity of each particle, and the safety of the particles is related to these specific concentration ranges.[17]

When the results of the cytotoxicity analysis were analyzed, the highest concentration of O-CaP nanoparticles was obtained with 10 µL O-CaP nanoparticles, and this concentration was preferred in transfection studies. Cells seeded in 24-well plates were exposed to plasmid DNA and O-CaP conjugate for 24 hours. After 4 hours of interaction, the conjugates were removed from the wells and the plate was left to incubate for 24 hours. At 24 and 48 hours after transfection, the cells were examined under a fluorescent microscope and images were taken. Image 4 shows the images obtained at 24 hours and Image 5 shows the images obtained at 48 hours.

To calculate transfection efficiency, 3 independent regions were selected from the transfection images taken under a fluorescence microscope and the cells within the region were counted. alculate transfection efficiency, 3 independent regions were selected on transfection images and cells within the region were counted. Transfection experiments conducted on CCD1072-SK human fibroblast cells resulted in an observed transfection efficiency of 78 ± 5.18%.

Chernousova et al[18] compared Lipofectamine, a commercial transfection agent, with calcium phosphate nanoparticles and reported that cells proliferated much more easily in the presence of nanoparticles. Although the nanoparticles showed a lower efficiency than Lipofectamine when gene silencing efficiency was considered, the nanoparticles showed a steady increase in gene silencing starting ~5 hours after incubation with the cells. After 72 hours of incubation with the nanoparticles, eGFP was silenced in ~240% of the original cell number, accompanied by ~460% of non-silenced cells.[18]

DISCUSSION

The aim of this study is to create a new core nanoparticle with O-CaP and CaP nanoparticles and to evaluate the gene transfer efficiency of these particles to cells after the completion of characterization studies. The core particle O-CaP formed with the synthesized nanoparticles was subjected to an electrical field and size determination with the Zetasizer device. The results showed that all formed particles had sufficient electrical field and appropriate size for gene transfer. The O-CaP-2 structure, with a PDI value closest to 0.1, size as small as possible, and positively charged formulations,[19] was found to be suitable for characterization and gene transfer studies to be used in further studies with a positive zeta potential of 24.3 mV, a size of 184 nm, and a PDI value of 0.2.

The characterization of O-CaP-2 nanoparticles was carried out by SEM and FTIR analysis. Scanning electron microscopy analysis showed that the particles were dense and spherical under the microscope. The chemical bonds of the particles were evaluated by FTIR analysis, and it was determined that the core nanoparticle carries the structures of octadecylamine and calcium phosphate nanoparticles.

O-Cap-2 nanoparticles were examined by MTT cytotoxicity assay to evaluate the effect on cells before the transfection study and to determine the optimum nanoparticle ratio that can be used. The results showed that all O-CaP-2 concentrations examined did not have any toxic effect on the cells. Since the viability obtained with the lowest concentration of 10 µL was the highest at 98.6% (±3.8%), 10 µL O-CaP-2 was preferred for transfection studies. As a result of transfection studies performed with CCD1072-SK human fibroblast cells, a transfection efficiency of 78% ± 5.18% was obtained.

With the results obtained within the scope of the study, it was revealed that O-CaP-2 core nanoparticle is an effective tool for gene transfer to CCD1072-SK cells.
In conclusion, the newly synthesized O-CaP-2 core nanoparticle, composed of octadecylamine and calcium phosphate, demonstrates excellent potential for gene

delivery applications. The nanoparticle exhibited favorable size, charge, morphology, and chemical composition for efficient cellular uptake and gene transfer. It also showed no cytotoxic effects on CCD1072-SK human fibroblast cells and achieved a high transfection efficiency of 78%. These findings highlight O-CaP-2 as a promising, safe, and effective non-viral vector for gene therapy and warrant further investigation in more complex biological systems. Despite the promising in vitro results, the current study is limited by the lack of in vivo evaluation, which is essential to fully assess the biocompatibility, biodistribution, and gene transfection efficiency of O-CaP nanoparticles in a physiological environment. Future studies should focus on optimizing targeting strategies to enhance cellular uptake specificity and explore the therapeutic potential of these nanoparticles in relevant animal models.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: N/A.

Informed Consent: Peer-review: Externally peer-reviewed.

Author Contributions: Concept – H.S.V.; Design – H.S.V.; Supervision – H.S.V.; Resource – H.SV.; Materials – H.S.V.; Data Collection and/or Processing – H.S.V.; Analysis and/or Interpretation – H.S.V.; Literature Search – H.S.V.; Writing – H.S.V.; Critical Reviews – H.S.V.

Declaration of Interests: The authors have no conflicts of interest to declare.

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