CHITOSAN MODIFIED POLY(LACTIC-CO-GLYCOLIC) ACID NANOPARTICLES INTERACTION WITH NORMAL, PRECANCEROUS KERATINOCYTES AND DENTAL PULP CELLS

Maria Justina Roxana Virlan[1a], Bogdan Calenic[1b], Cimpan Mihaela Roxana[2c], Daniela Elena Costea[3d], Maria Greabu[1e*]

[1]Department of Biochemistry, Faculty of Dentistry, University of Medicine and Pharmacy Carol Davila, Bucharest, Romania
[2]Department of Clinical Dentistry - Biomaterials, Faculty of Medicine and Dentistry, University of Bergen, Bergen, Norway
[3]The Gade Laboratory of Pathology, Department of Clinical Medicine, University of Bergen, Bergen, Norway

[a]DDS, MSc, PhD Student
[b]DDS, PhD, Lecturer
[c]DDS, PhD, Associate Professor
[d]DDS, PhD, Professor
[e]PhD, Professor, Head of Department

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ABSTRACT

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Introduction: Nanoparticles (NPs) can carry molecules to different body tissues. Due to their controlled delivery properties, chitosan covered poly-lacto-co-glycolic NPs (PLGAChi NPs) could be used to deliver drugs to oral tissues for the treatment of dental diseases or in anticancer therapy. The aim of this study was to determine the uptake and cytotoxicity of PLGAChi NPs on different types of cells found in the oral cavity.
Methodology: Normal oral keratinocytes (NOKs), precancerous keratinocytes (POE9i) and dental pulp cells (DPCs) were exposed for 12h and 24h to 20 g/mL and 200 g/mL PLGAChi NPs covalently tagged with fluorescein. 3D organotypic tissues of oral mucosa were grown in vitro and exposed to 200g/mL PLGAChi NPs for 24h.
Results: Both normal and premalignant oral mucosa cells (NOKs and POE9i) displayed uptake of PLGAChiNPs in a time and concentration-dependent manner, both in 2D and 3D models. A higher and more rapid uptake of PLGAChi NPs by precancerous cell line POE9i was observed when compared to NOKs. Interestingly, DPCs did not display internalized PLGAChi NPs, even at the highest concentration of 200 g/mL.
Conclusion: Chitosan-coated PLGAChi NPs proved to be able to cross the cellular membrane of oral keratinocytes, in 2D as well as in 3D cultures. The polymeric NPs used in the present study seem not to be suitable for applications that require NPs uptake by DPCs, as no evidence of uptake in these cells was found in this study. The finding that PLGAChi NPs showed significant internalization by human keratinocytes indicate that they could be used for drug delivery purposes to oral mucosa.
Keywords: chitosan, PLGAChi, nanoparticles, oral keratinocytes, dental pulp.

1. Introduction

Polymeric nanoparticles (NPs) have been considered as the most efficient vehicles for drug delivery due to their excellent pharmacokinetic properties such as particle size, surface charge, surface chemistry, hydrophobicity, degree of rigidity and degradation speed.[1-3] Specifically, poly-lacto-co-glycolic NPs (PLGA NPs) can transport molecules to different tissues in the body, facilitating intracellular uptake of various drugs.[4]
However, the overall negative charge of PLGA NPs has been reported to diminish their interaction with the negatively charged cell membrane.[5-6] PLGA NPs can be surface modified to carry a positive charge by the addition of a chitosan shell. PLGA-chitosan NPs combine the positive charge of chitosan and PLGA's ability to efficiently entrap hydrophobic and hydrophilic drugs.[7-8] Chitosan, the deacetylated derivative of chitin, is used as the coating polymer, because it is cationic, biocompatible and biodegradable.[28] Chitosan-modified NPs were develloped for the transport of active molecules through nasal, ocular, vaginal or intestinal mucosa.[6]

***Corresponding author:**

Prof. Dr. Maria Greabu, PhD, Professor, Department of Biochemistry, Faculty of Dentistry, „Carol Davila" University of Medicine and Pharmacy of Bucharest, Bucharest, Romania
8 Blvd. Eroii Sanitari, Sector 5, RO-050474 Bucharest, Romania
Tel/Fax: +40.721.274.932 / +40.213.110.984, e-mail: mariagreabu@yahoo.com

Chitosan nanocarriers could be used in future dental applications[9] such as in dentin pulp regeneration procedures,[10-11] in bone regeneration techniques,[12] in endodontics[13-15] or in periodontal therapy.[12, 16] Moreover, chitosan containing NPs were able to transport antitumour substances to different cancer cell lines,[17-19] including oral cancer cells.[18-19]

Despite numerous scientific reports regarding organic nanomaterials in medicine, more experiments are needed in order to asses the effects of organic NPs on the oral mucosa. It has been shown that the interactions between NPs and cells depends on the cell type, as well as on the size and surface charge of NPs.[20] NPs behave completely differently depending on their surface coverings and size, while the concentration and the exposure time to such NPs makes them cytotoxic or biocompatible. Moreover, the oral mucosa is composed of a variety of cells with different properties which may react differently to the same NPs. The pathologic conditions can also modify the response of human oral cells to NPs, due to changes in cell physiological status.

The aim of our study is to determine the uptake and effect of chitosan covered poly-lacto-co-glycolic NPs (PLGAChi NPs) on the cells found in the oral cavity, in normal and pathological conditions. NPs were tested on normal human oral keratinocytes (NOKs) and human dental pulp cells (DPCs), harvested from healthy human donors, as well as on POE9i cell line used as a model for precancerous oral keratinocytes. In the attempt to create a stronger resemblance to the natural 3D structure of the oral mucosal tissue, the PLGAChi NPs were also exposed to 3D organotypic (OT) oral mucosa tissues grown in vitro. The PLGAChi NPs tested in our study were previously fabricated and characterized by Navarro et al.[21- 22]

2. Materials and methods

2.1. Cell culture

NOKs, DPCs and normal oral fibroblasts (NOFs) were primary cells isolated from clinically healthy adult volunteers (n=5). Samples of gingival mucosa showing no sign of clinical inflammation at collection time were used to generate NOKs and NOFs. The protocol for the isolation of NOKs has previously been described by Costea et al.[23] DPCs were isolated following a protocol adapted from from Ishkitiev et al.[24] and Lee et al.[25]

POE9i cells are dysplastic, premalignant human immortalized oral keratinocytes. NOKs and POE9i keratinocytes were grown in Keratinocyte Serum-Free Growth Medium (KSFM) (from Sigma-Aldrich, St. Louis, MO) medium supplemented with 1 ng/mL epithelial growth factor, 25 µg/mL bovine pituitary extract, 20 µg/mL l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (all supplements were aquired from InVitrogen, Massachusetts, USA).

DPCs and NOFs were grown in DMEM medium (Sigma St Louis, Missouri) containing 10 % fetal bovine serum, 20 µg/mL l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (all supplements were aquired from InVitrogen, Massachusetts, USA). The protocol for growing normal human organotypics (OTs) was previously described by Costea et al.[23] The multilayered epithelium was elaborated using NOKs grown on top of collagen matrices populated with NOFs.

2.2. Viability Test

NOKs and POE9i cells were cultured in 6 well plates (150.000 cells/well) with 3.5 mL of culture medium. Cells were allowed to set for 24h into the incubator at 37°C and supplemented with 5% CO_2. Afterwards, the media was removed and the cells were washed with PBS. Then 3.5 mL media containing PLGAChi NPs at the tested concentrations was added in each well: 5 µg/mL, 20 µg/mL and 200 µg/mL. The viability was counted with trypan blue and an automatic cell counter (Sigma-Aldrich, St. Louis, MO). The counting was done in triplicates for every cell culture well.

2.3. Exposure of cells to PLGAChi NPs

The cells were seeded in two-well glass chambers (Thermo Fisher Scientific; Nunc™ Lab-Tek™) at a density of 75.000 cells/well. Every cell type was incubated with 1.5 mL of their own culture medium. The cells were kept for 48 h at 37°C till they became 70 % – 80 % confluent. Afterwards, the media were removed and washed twice with PBS. NOKs, DPCs and POE9i cells were exposed for 12h and 24h at the following concentrations of fluorescein marked PLGAChi NPs: 20 µg/mL and 200 µg/mL. 1.5 mL of media containing PLGAChi NPs at the mentioned concentrations was placed in every chamber slide: 20 µg/mL and 200 µg/mL. The solutions thus prepared were rotated for 30 minutes before exposure. The glass chambers were placed in the incubator in a humidified atmosphere at 37°C and supplemented with 5 % CO2 for 12 h or 24 h. At the end of the exposure time, the cells were washed three times with PBS in order to remove unattached particles, followed by fixation and staining. The controls were run in duplicate in each experiment and were placed into the incubator for one day.

The organotypic cultures were exposed to NPs after a total period of 10 days of coculture. The OTs were exposed to 200 µg/mL PLGAChi NP and let into the incubator for 24h. At the end of the exposure time, the OTs were washed three times with PBS in order to remove unattached particles, followed by fixation and staining.

Imaging and image analysis was performed using an optical microscope.

The fluorescent NPs uptaken by the cells were visualized by flourescence microscopy (AxioImager.M2 with ApoTome.2). The cells were mounted in Vectashield mounting medium with DAPI for nuclear staining and were visualized at a Zeiss up-right Axio Imager microscope with ApoTome slider module, using the 403 or 603 oil immersion objective lens.

Images were captured with Axi-oVision Rel 4.8 software controlled by AxioCam MRm camera (Carl-Zeiss, Germany). All images were representative of at least two independent experiments.
The quantification of NPs uptake was obtained with the help of the Icy software, using two plugins (HK-MEANS) : one developed by Dufour A.[26] and another one created by De Chaumont F.[27]

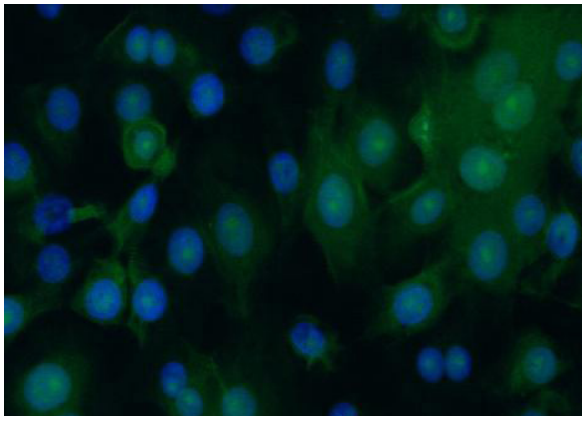
2.4. Statistical analysis

NPs uptake and cytotoxicity data were compared using Student's t-test. A p value < 0.05 was considered statistically significant.

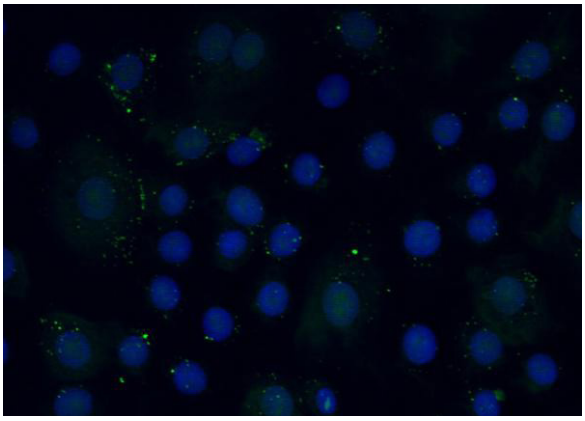
3. Results

3.1. PLGAChiNPs uptake by NOK, POE9 and DPC cells

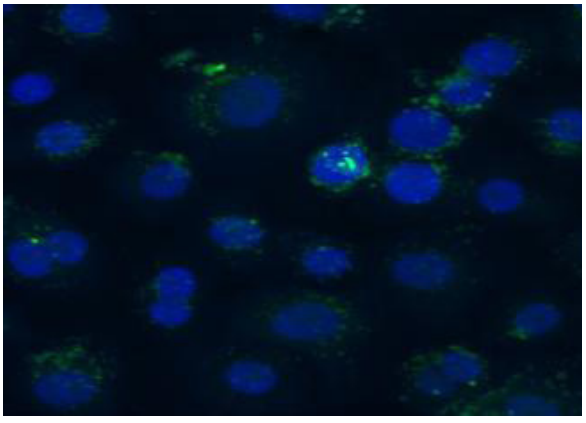
The PLGAChi NPs uptake by NOKs and POE9i cells was determined by fluorescence microscopy (Fig. 1, Fig. 2), as well as by confocal imaging (Fig. 4, Fig. 5). The quantification of NPs uptake revealed a significant amount of NPs inside the cells, both in normal human oral keratinocytes NOKs, as well as in premalignant oral keratinocytes POE9i (Fig. 6). Interestingly, the data obtained revealed a penetration of PLGAChi NPs in almost all the keratinocytes exposed to the NPs at the tested concentrations and time points (Fig. 6). The uptake of NPs inside NOKs varied from 91,83 % +/- 7, 37 to 100 % for NOKs, meaning that a significant amount of keratinocytes incorporated the PLGAChi NPs. In NOKs the percentage of cells which showed incorporation of PLGAChi NPs was 91,83% +/- 7,37 after 12h exposure at a concentration of 20 µg/mL PLGAChi NPs. 92,39+/- 1,34 of the exposed NOKs showed NPs uptake after 24h exposure to 20 µg/mL PLGAChi NPs. PLGAChi NPs entered 98,55% +/-1,95 of the tested NOKs after incubation for 24h with 200 µg/mL PLGAChi NPs.
The total uptake inside NOKs was observed after one day of incubation with 200 µg/mL PLGAChi NPs. In POE9i cell line, all tested samples showed a 100% uptake of PLGAChi NPs at all the tested concentrations (20 µg/mL PLGAChi NPs and 200 µg/mL PLGAChi NPs) and exposure times (12h and 24h) (Fig. 6) .
The fluorescence microscopic images showing DPCs presented no relevant differences between control images and images of DPCs exposed to PLGAChi NPs (Fig. 3). No NPs were observed inside the cells exposed to PLGAChi NPs, even at the highest concentration 200 µg/mL and at the longest exposure time, 24h.



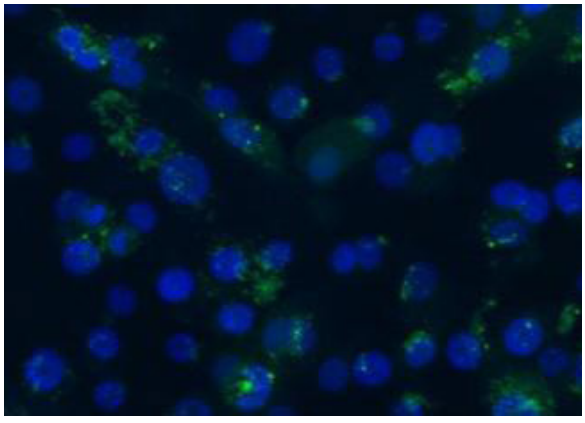
A. NOKs control.



B. NOKs 20 µg/mL PLGAChi NPs12h.



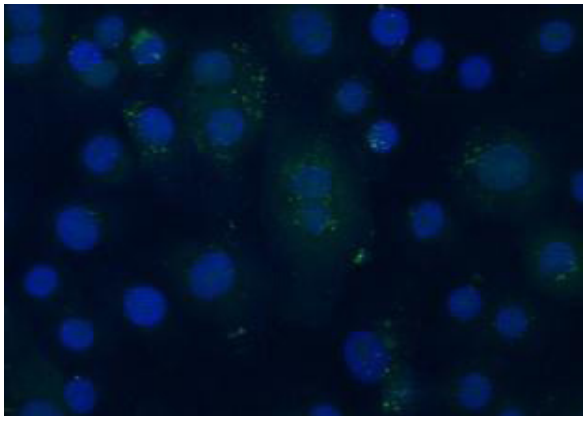
C. NOKs 20 µg/mL PLGAChi NPs 24h.



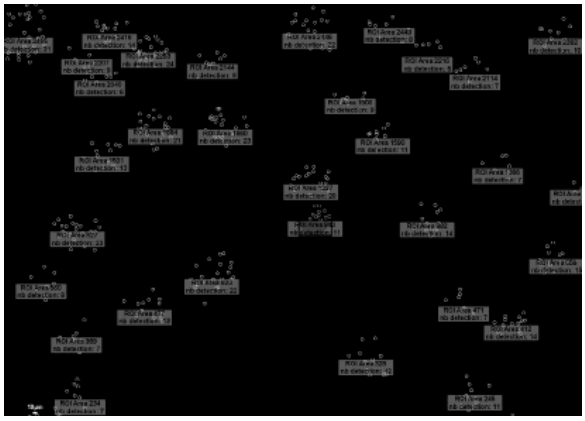
D. NOKs 200 µg/mL PLGAChiNPs12h.

3.2. PLGAChi NPs uptake in 3D organotypic cell cultures of normal human mucosa in 3D cell cultures

Intracellular uptake of the PLGAChi NPs was visible in the epithelial compartment of the reconstituted human oral mucosa grown in vitro. The images obtained by fluorescence microscopy revealed that PLGAChi NPs were able to penetrate the superficial layers of the reconstituted epithelial mucosa after 24h exposure at a concentration of 200 µg/mL.

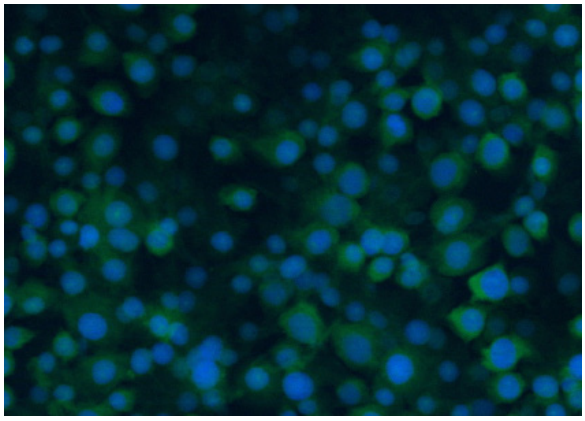


E. NOKs 200 µg/mL PLGAChi NPs 24h.

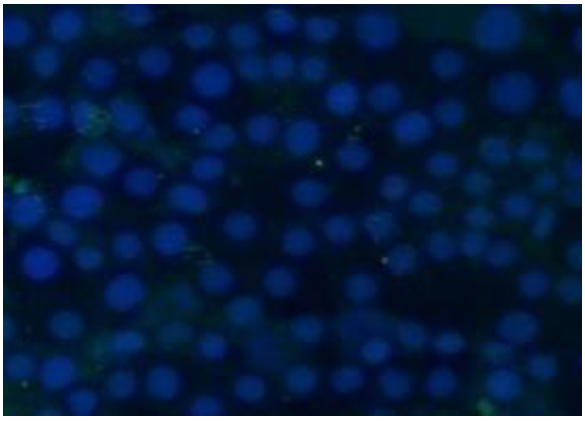


F. PLGAChi NPs detection (NOKs 200 µg/mL PLGAChi NPs 24h).

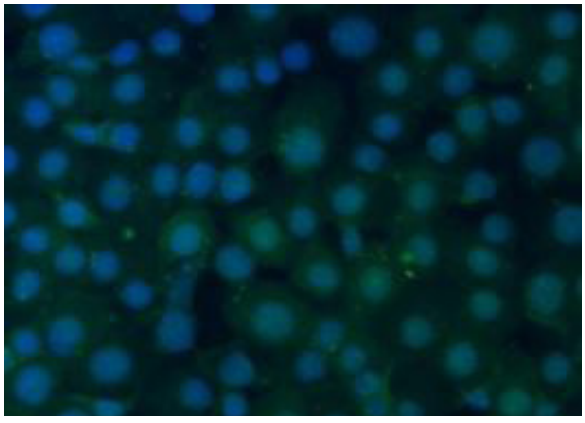
Figure 1. Fluorescence images showing uptake of PLGAChi NPs byNOKs.



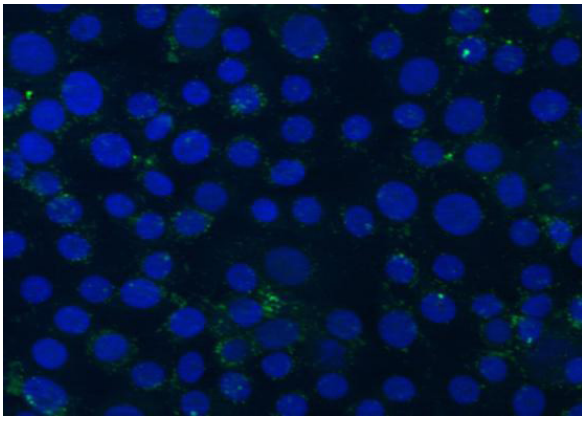
A. POE9i Control.



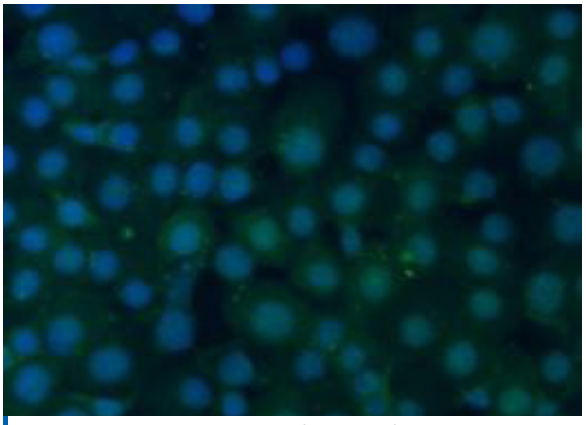
B. POE9i 20 µg/mL PLGAChi NPs12h.



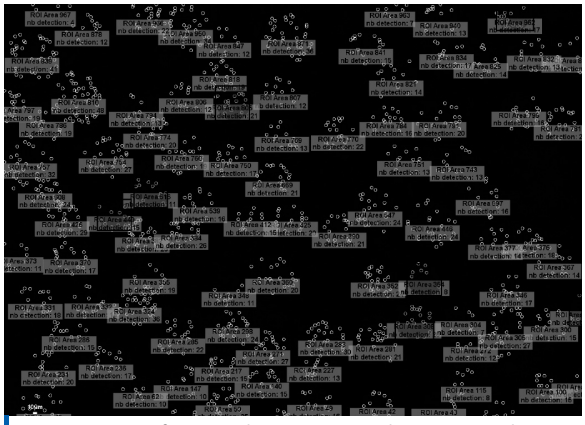
C. POE9i 20 µg/mL PLGAChi NPs 24h.



D. POE9i 200 µg/mL PLGAChi NPs 12h.

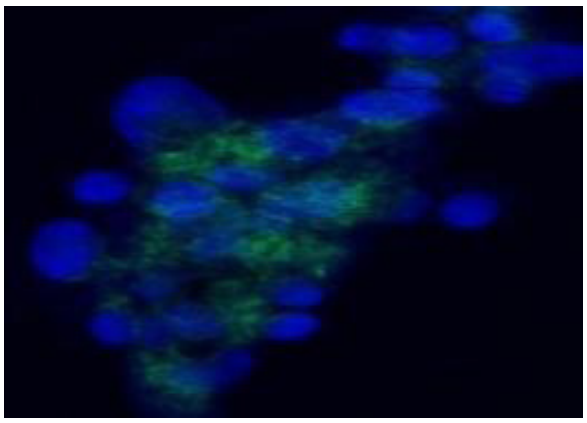


E. POE9i 200 µg/mL PLGAChiNPs 24h.

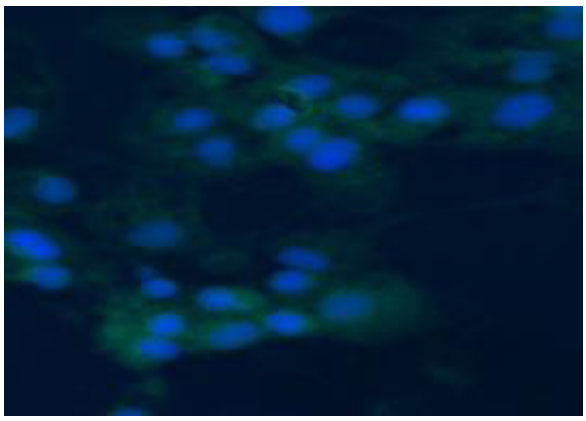


F. Detection of PLGAChi NPs internalization inside POE9i (image of POE9i 200 µg/mL PLGAChi NPs 12h).

Figure 2. Fluorescence images showing uptake of PLGAChi NPs inside POE9i. The fluorescent green dots are the internalized PLGAChi NPs.



A. DPCs Control.



B. DPCs 200 µg/mL PLGAChi NPs 24h.

Figure 3. Fluorescence microscopy images showing no signs of PLGAChi NPs uptake by DPCs cells.

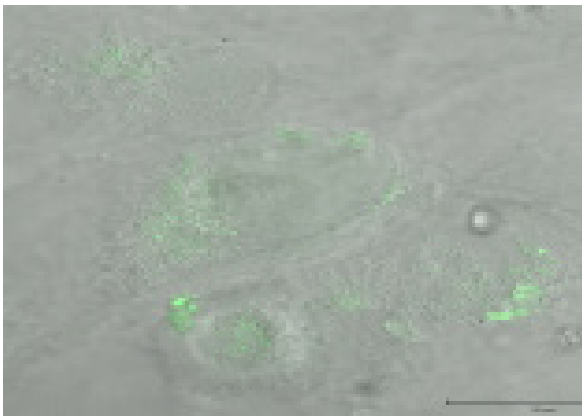


Figure 4. Confocal microscopy image demonstrating PLGAChi NPs uptake inside NOKs. The green fluorescent PLGAChi NPs are observed inside cells.

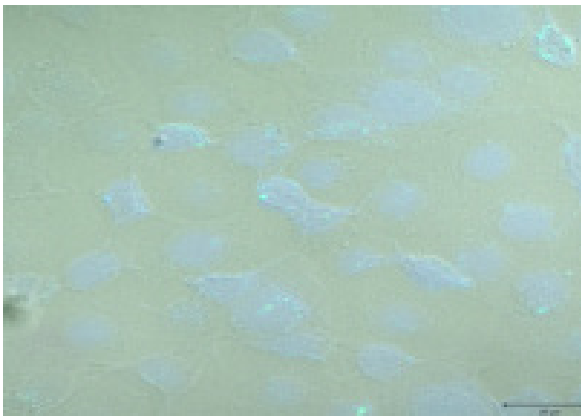


Figure 5. Confoccal Images of POE9i exposed to 200 µg / mL PLGAChi NPs for 12H. The fluorescent PLGAChi NPs green NPs are observed inside the cells.

3.3. Cytotoxicity evaluation of PLGAChi NPs in NOKs and POE9i cells

After 24h exposure to PLGAChi NPs, NOKs demonstrated no significant difference in the viability values at all tested concentrations: 5µg/mL, 20 µg/mL and 200 µg/mL PLGAChi NPs. A slight decrease in viability was observed inthe POE9i cell line exposed to 20 µg/mL PLGAChi NPs. However, the POE9i samples exposed to 20 µg/mL PLGAChi NPs showed 81% percentage of viable cells, as compared to 88 % live cells, in the control sample.

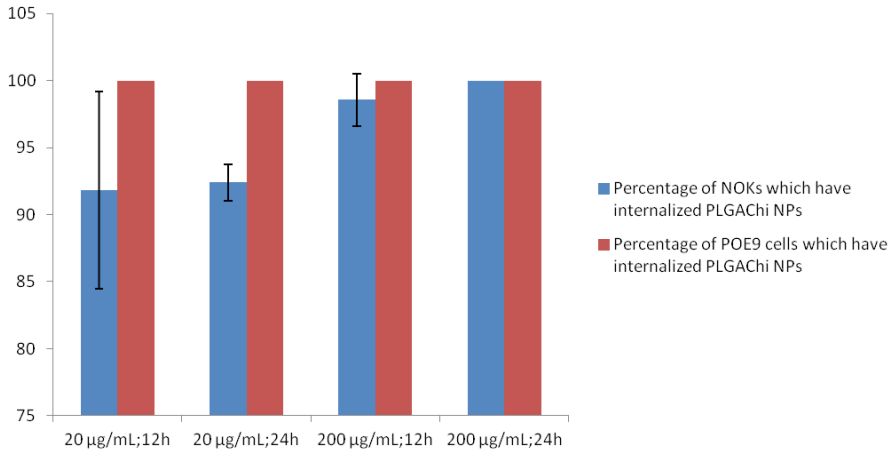


Figure 6. Percentage of cells which have internalized PLGAChi NPs from the total amount of exposed cells. NOKs and POE9i cells (average values).

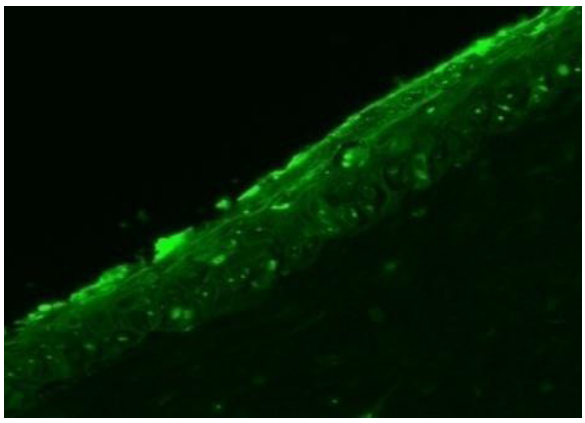


Figure 7. Penetration of PLGAChi NPs in the 3D organotypic models of human mucosa. The green fluorescent dots represent the PLGAChi NPs which were able to enter the reconstituted OT of normal human mucosa. The sample was exposed to 200µg/mL PLGAChi NPs for 24h.

In the NOKs and POE9i cell there was no statistical difference between the control and treated cells (Fig. 8).

4. Discussion

Polymeric NPs are still viewed as the first option for drug delivery and also widely used in the research of other diseases.[1-3] Recently, a wide variety of studies has been undertaken leading the way for possible future applications of PLGA NPs in a high number of dental fields, from periodontology and endodontics to tissue regeneration of skin, bone or cartilage.[28] Biocompatibility, biodegradability, flexibility, and minimal side effects are the main advantages when using PLGA for biomedical applications.[4] However, the overall negative charge of these NPs has been reported to diminish their interaction with the negatively charged cell membrane, while the rapid opsonization of hydrophobic PLGA NPs is a major limitation

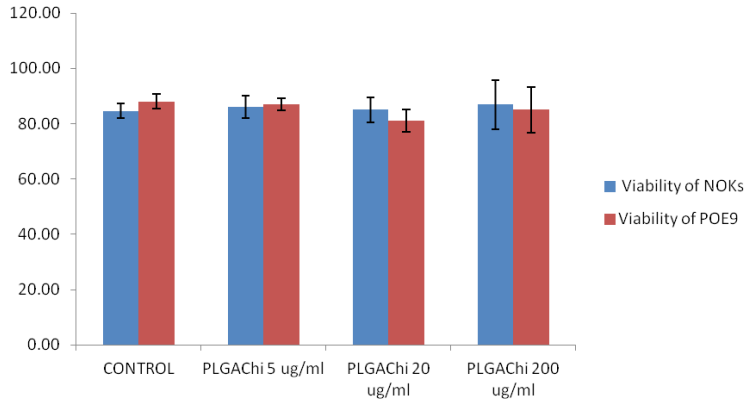


Figure 8. Viability of NOKs and POE9i after exposure to PLGAChi NPs.

that hinders their employment for biomedical applications.[5-6]

In order to improve PLGA NP proprieties, several research groups have tried to cover the polymers with a chitosan coating. Chitosan is obtained from chitin which is a positively charged polysaccahride found in crustaceans.[28] Chitosan contains many amino and hydroxyl groups, thus it can bind effectively to negatively charged substances (such as the cells membranes) via electrostatic interactions or hydrogen bonding, thus improving the intracellular uptake.[9] Chitosan by itself is known to strongly adhere to negatively charged surfaces due to its high charge density at pH< 6.5.[29] The advantages of modifying the surface of PLGA NPs with a mucoadhesive polymer, such as chitosan, may potentially include the inversion of zeta potential, the ability to promote cellular adhesion and retention of the delivery system at the target site.[30]

In order to fabricate polymeric NPs for future dental applications, we have tested the positive charged chitosan coated PLGA NPs (PLGAChi NPs) on oral cavity cells. NPs interaction with the oral epithelial cells was assessed by cytotoxicity measurements. After 24h of incubation with PLGAChi NPs (20 µg/mL NPs and 200 µg/mL NPs) no significant statistic differences were observed in the viability of samples and controls (Fig. 8). PLGA Chi NPs were biocompatible to all the tested cell lines: NOKs and POE9i cells. We found no association between the significant increase in the cell uptake of chitosan containing NPs and cell toxicity, at the tested concentrations. Due to PLGA and chitosan's well known biodegradability and biocompatibility, it was expected that NPs made of chitosan and PLGA would be well tolerated by the cells. Our results are in agreement with other comparable studies. Following a 2015 experiment, S. Alqahtani stated that chitosan covered PLGANPs did not affect the viability of Caco-2 cells.[31] Caco-2 cells displayed a viability above 95%, even after incubation for one day with a higher concentration of NPs than it was used in our study: 500 µg/mL PLGAChi NPs.[31] Moreover, chitosan covered NPs did not contribute additional toxicity to colorectal cancer cells after 3 days of exposure at a 75 µM solution of NPs.[32] The viability of the cells incubated with chitosan NPs remained at about 90 % relative to the untreated cells on day 1 and at about 89 % on day 3.[32] Another recent study also found that the surface modification of PLGA NPs with chitosan did not show any significant diffference in cytotoxicity of PLGA NPs.[6] The results indicated that A549 cell lung carcinoma cells exhibited around 80 % cell survival as compared to positive controls (10 % cytotoxic) for the following concentrations of PLGAChi NPs: 0.25, 0.5, 0.75, 1,1.25, 1.5, 1.75 and 2 mg/mL.[6] Other investigators have reported PLGAChi NPs to be safe even at much higher concentrations of 20 mg/mL.[33-35] In addition, other research groups revealed interesting cytotoxicity results of chitosan NPs effect on human skin keratinocytes HaCaT cells. Tretinoin containing chitosan solid lipid NPs were not cytotoxic to HaCaT cells even at the highest concentration 500 µg/mL used, which led to around 5 % less viability compared with the control.[36] Also lecithin/chitosan NPs can be applied to skin cells at concentrations up to 200 µg/mL without inducing plasma membrane damage or cell viability decrease.[37] Similarly, in a recent study, HaCaT cells exposed to melatonin containing lecithin/chitosan NPs in a concentration of chitosan of 1.25-20 µg/mL for 2 hours showed no relevant cytotoxicity.[38] However, a significant reduction in the cell viability of HaCaT cells was observed in the case of cells treated with NPs at a chitosan concentration of 20 µg/mL.[38] Chitosan-alginate NPs did not have a toxic effect on human monocytes but there was mild toxicity to skin keratinocytes at higher concentration of NPs.[29] Moreover, chitosan and PLGA NPs loaded with chlorexidine dihydrochloride in vitro toxicity evaluation on human gingival fibroblasts was between 20 % and 60 % in all experimental conditions.[9] Poly-γ-glutamic acid/glycol chitosan NPs incorporating p-phenylenediamine (PDA) showed lower cytotoxicity against HaCaT human skin keratinocyte cells than PDA alone.[39] Interestingly, PDA-incorporated NPs showed reduced apoptosis and necrosis reaction in HaCaT cells.[39]

A possible explanation for the chitosan NPs high biocompatibilty could be that chitosan is much more cytotoxic in a free soluble form than when it is incorporated into NPs, due to the fact that in the case of NPs, a significant portion of the positive amino groups of chitosan are engaged in electrostatic interractions.[38, 40]

To confirm PLGAChi NPs efficiency in intracellular penetration, the cellular internalization of PLGAChi NPs conjugated with fluorescein was investigated by fluorescence microscopy. The results indicated significant differences in NPs uptake between the different cell lines used in this study.

Fluorescence microscopy experiments conducted after 12h and 24h of incubation revealed a rate of inglobation influenced by the cell type. DPCs did not internalize PLGAChi NPs, even at the highest concentration (200 µg/mL PLGAChi NPs) and the longest incubation time (24h) (Fig. 3). But the microscopic results showed supporting evidence of incorporation of the tested NPs in oral keratinocytes, both in normal and in pathologic precancerous conditions (Fig. 1, Fig. 2, Fig. 4, Fig. 5).

The data obtained revealed a significant uptake of PLGAChi NPs into oral keratinocyte cells after 12h exposure with 20 µg/mL NPs: 91,83 % in NOKs and 100 % in POE9i cells (Fig. 6). The percentage of NOKs which have internalized the chitosan covered PLGA NPs increased gradually with the incubation time and concentration of the solution of polymeric NPs. The highest uptake of PLGAChi NPs inside both keratinocyte types was observed after 24h exposure to 200 µg/mL

PLGAChi NPs, when all cells were penetrated by the NPs (Fig. 6). What is mostly important is the fact that the PLGAChi NPs entered in a higher amount in the precancerous cells than in the normal oral keratinocytes. Chitosan covered NPs demonstrated a 100 % uptake at all tested concentrations and time points in POE9i precancerous cells. Moreover, the PLGAChi NPs were able to get internalized into the epithelial cells in reasonable amounts and in a time and concentration dependant manner. The percentage of keratinocyte cells with internalized NPs increased over incubation time, demonstrating a growing and highly efficient process of internalization of PLGAChi NPs by human NOKs and POE9i keratinocytes. The results obtained in 3D studies confirm the fact that PLGAChi NPs can enter the oral keratinocytes (Fig. 7).

Interestingly, what was observed was a higher and more rapid uptakeof PLGAChi NPs in precancerous keratinocytes compared to NOKs (Fig. 6). Based on these data, we hypothesise a preference of chitosan covered NPs for uptake byprecancerous keratinocytes over normal keratinocytes. This has been also hypothesised previously by[41] who showed that epithelial cell cultures forming tight junctions did not internalize NPs, while those lacking tight junctions, i.e., the cancer cells, did internalize. Although the NPs used in that study differ from our study, this could provide a possible molecular explanation. Interestingly, previous research articles have also found a preference of chitosan covered NPs for uptake by cancer stem cells. A doxorubicin-encapsulated polymeric nanoparticle surface-decorated with chitosan was able to target and eliminate tumor reinitiating cancer stem-like cells.[42] Moreover, hyaluronic acid-decorated dual responsive nanoparticles of Pluronic F127, PLGA, and chitosan were developed recently for targeted co-delivery of doxorubicin and irinotecan to eliminate cancer stem-like cells.[43] Also, chitosan-coated hyaluronic acid, docetaxel containing NPs were more effective against CD44+ cells than free docetaxel.[44] We have not investigated this aspect in our study, but this could be further investigated. The findings of this study provide evidence for the penetration of PLGAChi NPs not only in single cells, but also in oral mucosal cells assembled in 3D tissus, as shown by the results on the human 3D organotypic reconstructed human mucosa models grown in vitro (Fig. 7). Altough the 3D organotypic models replicate only to a certain extend the structure of the human tissue, in this case the human oral mucosa, they better resemble the oral microenvironment of the oral keratinocytes than the 2D models.

To our knowledge this is the first study that assesed the penetration of PLGAChi NPs in the reconstructed oral human mucosa. The NPs crossed the superficial epithelial layers, reaching the underlying conective tissue (Fig. 7).

Our results were in agreement with previous studies which showed a high uptake of polymeric NPs when fortified with chitosan, and that the uptake of chitosan coated NPs was much higher that that of uncoated NPs.[6, 30-31, 45-46] In a study from 2015, S.Alqahtani showed a significantly higher 3.5 fold cellular uptake of chitosan coated PLGAChi NPs compared to PLGA NPs in Caco-2 cells.[31]

In another study, positively charged chitosan covered PLGA NPs exhibited enhanced mucoadhesion, compared to negatively charged PLGA NPs and enhanced intracellular uptake in A549 cell line human lung carcinoma cells.[6] PLGAChi NPs managed to get internalized into Caco-2 cells with reasonable amounts after just 1h.[31] Another research group stated that PLGAChi NPs are internalized by hepatocytes 3A and fibroblasts 3T6 in a few minutes.[30]

Also, Chronopoulos reported that the uptake of PLGAChi NPs appears faster than with PLGA NPs, with major amounts of cytoplasmatic NPs found after only 5 minutes.[30]

Interestingly, uptake saturation is reached after 2-3h of incubation with PLGAChi NPs in human 3A hepatocytes and 3T6 fibroblasts although the uptake of PLGA NPs still appears less extensive than for PLAChi NPs.[30]

The performance of a delivery system depends on the polymeric composition, the size and surface charge.[38,47] Therefore, smaller sizes of NPs and a positive zeta potential lead to a better internalisation inside cells due to the attractive interaction with the negatively charged cell membranes.[48,49]

Hence, the size and zeta potential of the cureent NPs fabricated in our study are in favour of particle internalisation. Our data demonstrated that PLGAChi NPs exhibited a significant internalisation into the human oral keratinocytes .

However, the experiment presents a series of limitations. As other studies showed the rapid internalisation of chitosan covered NPs in minutes or hours,[30,31] the exposure time used in our study (12h and 24h) might have been too long. Further investigations using a wider variety of concentrations and time points are needed in order to asses the differences in uptake of PLGAChi NPs between normal and patologic conditions. The use of organotypic models of reconstructed human mucosa in vitro resemble much more the natural conditions in vivo than the usual tests on monolayer cell cultures. But the organotypic models cannot substitute the in vivo experiments as they are composed only of a collagen biomatrix and epithelial cells, without other components of the natural mucosa, such as the imune cells and vascular components.[23,50] Moreover, saliva might interfere with NPs penetration in the oral mucosa and hinder the uptake inside the oral epithelium[50] As the reconstructed oral mucosa samples did not have a protective mucus layer, it is hard to predict the influence of saliva on the NPs penetration inside human mucosa in vivo. Future in vivo experiments should clarify and add significant data to the potential uses of PLGAChi NPs in oral medicine.

5. Conclusions

This study offers new insight on NPs uptake within human oral cells. PLGAChi NPs are not suitable in applications regarding DPCs, as they do not enter these cells. But, PLGAChi NPs are internalised by both human keratinocytes and fibroblasts. Chitosan-coated PLGA NPs have proved to be potent in crossing the cellular membrane of epithelial cells. Therefore, PLGAChi NPs are highly recommended for being used in drug delivery systems to the oral mucosa. This promising results suggest the need for further studies regarding PLGAChi NPs, and its uses in oral mucosa diseases or anticancer therapy. In conclusion, more research is needed to fully explore the underlying mechanisms of celllular uptake of PLGA with chitosan surface modification.

Author Contributions

MJRV contributed to the concept of the article, data gathering, data analysis and interpretation. BC's contribution was very important in the concept, interpretation and critical revision of the manuscript. DEC contributed to establishing of protocols, data gathering, interpretation of the results and critical revision of the manuscript. MRC critically revised the manuscript. MG contributed to all stages of the article from the concept of the article, protocols, interpretation and critical revision of the manuscript. All authors approved the final version of the article.

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Maria Justina Roxana VIRLAN
DDS, MSc, PhD Student
Department of Biochemistry
Faculty of Dental Medicine, U.M.F."Carol Davila "Bucharest, Romania

CV

Dr Justina Virlan graduated from the Faculty of Dental Medicine in 2012 and completed an Endodontics residency. In 2015 she finished a master programme at the Faculty of Medical Engineering. A laureate of the National Chemistry Competition and a PhD student at the Biochemistry Department of the Dental Medicine Faculty, she has a high interest in nanomaterials and their applications in dentistry.

Questions

Chitosan modified poly(lactic-co-glycolic) acid nanoparticles (PLGAChi NPs) could be used to deliver drugs:

- ❑ a. to the dental pulp cells;
- ❑ b. to the oral mucosa;
- ❑ c. to the dental pulp cells and oral mucosa;
- ❑ d. none of the above.

The PLGAChi NPs (used in this study) can enter :

- ❑ a. normal oral keratinocytes (NOKs);
- ❑ b. precancerous oral keratinocytes (POE9i);
- ❑ c. dental pulp cells (DPC);
- ❑ d. normal oral keratinocytes (NOKs) and precancerous oral keratinoctes (POE9i).

The multilayered epihelia of oral mucosa was grown in vitro using:

- ❑ a. collagen matrix;
- ❑ b. collagen matrix; normal oral fibroblasts (NOFs);
- ❑ c. collagen matrix; normal oral fibroblasts (NOFs); normal oral keratinocytes (NOKs);
- ❑ d. collagen and matrigel matrix; normal oral fibroblasts (NOFs); normal oral keratinocytes (NOKs).

In the cell lines that have internalized PLGAChi NPs, the maximum uptake of NPs was observed after exposure to:

- ❑ a. 200 g/mL PLGAChi NPs for 24 h;
- ❑ b. 200 g/mL PLGAChi NPs for 12 h;
- ❑ c. 20 g/mL PLGAChi NPs for 12 h;
- ❑ d. 20 g/mL PLGAChi NPs for 24 h.