Self-assembling peptide P$_{11-4}$ presents potential of inducing odontoblast-like cells to biomineralization and cell migration.

Isaac Jordão de Souza Araújo$^1$, Gustavo Narvaes Guimarães$^2$, Renato Assis Machado$^{3,4}$, Luiz Eduardo Bertassoni$^5$, Robert Philip Wynn Davies$^6$, Regina Maria Puppin-Rontani$^7$.*

1 Department of Restorative Dentistry, Dental Materials Area, Piracicaba Dental School, State University of Campinas, Piracicaba, São Paulo, Brazil.; isaacjsouzaa@gmail.com
2 Department of Biosciences, Histology area, Piracicaba Dental School, State University of Campinas, Piracicaba, São Paulo, Brazil.; gu_narvae@yahoo.com.br
3 Hospital for Rehabilitation of Craniofacial Anomalies, University of São Paulo (HRAC/USP), Bauru, São Paulo, Brazil.
4 Department of Oral Diagnosis, School of Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, São Paulo, Brazil.; renatoassismachado@yahoo.com.br
5 Department of Biomaterials and Biomechanics, School of Dentistry Center for Regenerative Medicine, Oregon Health and Science University, Portland, Oregon, United States.; bertasso@ohsu.edu
6 Division of Oral Biology, School of Dentistry, University of Leeds, Leeds, United Kingdom.; R.P.W.Davies@leeds.ac.uk
7 Department of Health Sciences and Pediatric Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, São Paulo, Brazil.; rmpuppin@unicamp.br

* Correspondence: rmpuppin@unicamp.br

Abstract: Self-assembling peptide P$_{11-4}$ is amphiphilic and pH-triggered with demonstrated efficacy repairing early carious lesions in enamel. However, P$_{11-4}$ effects on dentin biomineralization and repair remain unexplored. Thus, cytocompatibility and effectiveness of P$_{11-4}$ inducing mineralization and migration of odontoblast-like cells (MDPC-23) were investigated. MDPC-23 were seeded in contact with P$_{11-4}$(0.5µg/ml and 1µg/ml), Dentin Matrix Protein 1 (DMP1 0.5µg/ml and 1 µg/ml) or Calcium hydroxide (Ca(OH)$_2$ 100µg/ml) solutions. Cytotoxicity was verified using MTT (n=6/group). Mineralization was tested using Alizarin Red (n=4/group). Cell migration was assessed by light microscopy (n=2/group). MTT and Alizarin Red data were compared using Kruskal-Wallis and Mann-Whitney (α=0.05). P$_{11-4}$ (0.5µg/ml and 1µg/ml) and DMP1 (0.5µg/ml and 1µg/ml) presented the highest cyto compatibility; Ca(OH)$_2$ presented the lowest. DMP1 1µg/ml exhibited the highest mineralization ability, with no difference to P$_{11-4}$ 1µg/ml. Ca(OH)$_2$ presented lower values than DMP1 1µg/ml (p<0.05), but similar to P$_{11-4}$ 1µg/ml. P$_{11-4}$ and DMP1 at 0.5 µg/ml showed induced less mineralization than P$_{11-4}$ and DMP1 at 1µg/ml (p<0.05), with no difference to Ca(OH)$_2$. All materials stimulated cell migration, however, lower concentrations of DMP1 and P$_{11-4}$ provided better results. P$_{11-4}$ is cytocompatible, induces mineralization and MDPC-23 migration like DMP1. P$_{11-4}$ could be an alternative for dentin mineralization and tooth repair.

Keywords: Self-assembling peptide; biomineralization; pulp-dentin complex; tooth repair.

1. Introduction

The response of the pulp-dentin complex to injuries is a natural defense mechanism to protect the dental organ from damage [1]. In order to maintain health and vitality, the pulp cells react against harmful stimuli synthesizing many proteins, which can mediate...
the formation of reactionary and reparative dentin [2] and participate in the biomineralization process [3].

Biomineralization involves the formation of complex, well-organized structures through a synergistic action between a protein matrix and minerals, where the matrix offers a template for the nucleation, growth, and organization of inorganic phases over multiple length scales [4]. Among these proteins, Dentin Matrix Protein 1 (DMP1) is an acidic matrix phosphoprotein that belongs to the Small Integrin-Binding N-linked Glycoproteins (SIBLING) family, which are associated with the formation of mineralized tissues [5]. DMP1 is related to the biomineralization process in dentin and can induce the differentiation of odontoblasts [6]. Furthermore, DMP1 is expressed in all odontogenic stages (dental lamina, enamel organ, and dental papilla) with some variation in the protein expression levels between each stage [7].

DMP1 plays an important role throughout the odontogenesis and dentin biomineralization, and it was recently evidenced that this protein remains adjacent to the pulp cavity, in peritubular zones, and it is less deposited near the enamel-dentin junction [8]. Complementary to that, DMP1 is also involved with the reparative process in dentin and contributes to the nucleation of amorphous calcium phosphate (ACP) and in the organization of apatite crystallites in intrafibrillar collagen zones [9, 10].

Despite the recent literature that has clarified the role of these small proteins in tooth repair and regeneration, most of the procedures concerning the protection of pulp-dentin complex, to induce reparative processes, are based on the use of calcium silicate and calcium hydroxide (Ca(OH)$_2$) materials [11]. The widespread use of Ca(OH)$_2$ is mostly related to its high alkalinity (pH ~12.5-12.8), allied to calcium and hydroxyl dissociation that induces mineral deposition and dentin bridge formation [12]. Moreover, Ca(OH)$_2$ also induces partial solubilization of dentin and the release of small amounts of bioactive molecules involved with the formation of dentin bridges [13]. However, Ca(OH)$_2$ generates an inflammatory response of the subjacent pulp tissue, leading to coagulative necrosis, after that, the mineralized barrier formation isolates and protects the pulp against initial injuries [14]. Apart from Ca(OH)$_2$: protective effects, the production of dentin bridges are not homogeneous and may act solely as a plug that tries to block damage progression.

In this sense, it is important to develop novel alternatives to induce dentin biomineralization, mimicking the processes that occur during odontogenesis, once the mineral deposition is not naturally dependent on inflammatory and necrotic processes [10]. For this purpose, self-assembling peptide $\mathrm{P}_{11-4}$ is a promising candidate, which was inspired by the telopeptide at the C-terminal of the amelogenin. Amelogenin was previously thought to be involved only in enamel mineralization, but it has also been found to play an important role in the early stages of dentin formation, due to its transient expression by odontoblasts during pre-dentin deposition [15]. Therefore, it has been suggested that amelogenin may act as a signaling molecule during the initiation of hard matrix formation [15]. Moreover, it is proposed that amelogenin plays important roles not only on initial cytodifferentiation but also during tooth repair processes in humans since amelogenin is strongly re-expressed in newly differentiated odontoblasts and found to be distributed in the dentinal tubules under carious lesions in permanent teeth [16]. Thus, the use of a self-assembling peptide inspired by this protein could represent an interesting biomimetic alternative to repair and guide the biomineralization process in dentin.

$\mathrm{P}_{11-4}$ in its simplest terms can be described as a monomeric peptide that in response to specific external triggers forms hierarchical structures due to the formation of $\beta$-sheets [17]. This peptide is effective in reducing demineralization and attracting ions to remineralize enamel subsurface lesions [18, 19]. Besides the promising results on enamel, de Sousa et al., 2019 [20] demonstrated that $\mathrm{P}_{11-4}$ peptide is also capable of binding to type I collagen. Their findings indicated that $\mathrm{P}_{11-4}$ increases the collagen fibrils’ thickness,
which improves the resistance against the proteolytic activity. Furthermore, P11-4 has negatively charged domains mimicking those found in the native macromolecules involved in the mineralization process. The negatively charged domains enhance its organization and it can be viewed as analog protein [18, 21]. Moreover, because of its mineralization potential, de novo calcium and phosphate nucleation were also analyzed, and the crystal diameter and microstructure presented a significant modification in the presence of P11-4 [20].

Due to the inherent interaction of P11-4 with type I collagen, its similarities to the macromolecules involved in the mineralization process, and calcium-phosphate nucleation, it is necessary to compare the effects of this peptide with compounds capable of inducing dental tissue mineralization to demonstrate its efficacy. One under-reported area is P11-4's interaction with specific cell types. Therefore, this study focuses on the interaction of the self-assembling peptide with odontoblast-like cells. Here we assess the cytocompatibility, the mineral deposition, and potential of inducing cell migration, comparing its performance to Ca(OH)₂ and to DMP1, a well-known bioactive molecule involved in biomineralization. Based on the current literature we hypothesize that the self-assembling peptide is cytocompatible and induces mineralization, therefore it is potentially an appropriate material as an alternative therapy to replace calcium hydroxide in tooth repair and dentin regeneration as a pulp capping agent.

2. Materials and Methods

Cell culture

Immortalized odontoblast-like cell MDPC-23 were cultured in Petri dishes with DMEM (GIBCO, Auckland, NY, USA) medium, supplemented with 10% FBS (GIBCO) and 1% penicillin/streptomycin antibiotic (GIBCO) and incubated at 37 °C and 5% CO₂ until the pre-confluent stage was reached. Then, the cells were detached, counted, seeded (at a density of 7.5x10⁴ cells/ml) into cell culture plates and kept at 37 °C, 5% CO₂, before use. To compare the effects of the different biomineralization agents, the cells were cultured in the presence of Ca(OH)₂ (Sigma Aldrich, St. Louis, MO, USA) (100µg/ml), recombinant DMP1 (R&D Systems, Inc. Minneapolis, MN, USA) (0.5µg/ml and 1.0µg/ml) and, P11-4 peptide (Curodont™ Repair, Credentis AG, Windisch, SWI) (0.5µg/ml and 1.0µg/ml). Also, a negative control, with no specific treatment was included in each assay.

Cell viability assay (MTT)

The cell viability was verified using 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). The cells were cultured into 96-wells plates, treated concerning the material and concentration (n=7), and incubated (37 °C and CO₂ 5%) at 24h and 72h. After each period of incubation, the medium was aspirated, and the cells were washed with sterile phosphate buffer solution (PBS). Then, 150 µl of MTT solution was added to each well and incubated at 37 °C and 5% CO₂ for 4h. Subsequently, the MTT solution was aspirated and the formazan crystals precipitated were diluted with 150 µl of isopropyl alcohol. After the complete dissolution of formazan crystals, the plates were carried to a microplate reader (Biochrom Asys UMV340, Biochrom Ltd. Cambridge, UK). The absorbance was measured at 570 nm and the values of cell viability were expressed as a percentage.

Mineral deposition (Alizarin Red S)

The mineral deposition was evaluated using Alizarin Red S. The cells were cultured in an osteogenic medium with 2 mM β-glycerophosphate, 50 µg/ml ascorbic acid, and 1 µl/ml
Dexamethasone in a 24-well cell culture plate. A group with only osteogenic medium and a separate group containing only DMEM supplemented with 10% FBS was used as the positive and negative controls, respectively. The test groups were calcium hydroxide, DMP1, and P11-4 at the same concentrations previously mentioned (n=4). The medium was changed every 3 days and the mineral deposition was evaluated after 21 days of culture. Alizarin Red S staining protocol was followed to measure mineralization [22, 23]. Briefly, after the culture period, the cells were washed with PBS and fixed with 4% paraformaldehyde for 1h at room temperature. The monolayer was washed twice with PBS, and then 1 ml of 40 mM Alizarin Red S solution (pH=4.1) was added to each well, left at room temperature for 20 minutes under gentle shaking. After, the solution was aspirated, and the wells were washed with distilled water 4 times to remove the unincorporated dye. Then, the wells were photographed with a camera (Nikon 3200, Nikon, Tokyo, Japan) to make the qualitative analysis.

To quantify the staining, 800 µl of 10% acetic acid was dispensed into each well and left for 30 minutes at room temperature with shaking. Next, the monolayers poorly attached to the well were aspirated and transferred to 1.5 ml microtubes and vortexed for 30 seconds. Then, 500 µl was added to the slurry, heated to 85 °C for 10 minutes, and kept in ice for 5 minutes and the slurry was centrifuged at 20,000 g for 15 min. After centrifugation, 500 µl of supernatant was transferred to a new 1.5 ml microtube. Then, 200 µl of 10% ammonium hydroxide was added to neutralize acetic acid. The absorbance was read in duplicate using 150 µl of the supernatant in a 96-well plate at 405 nm in a microplate reader.

Cell migration assay

The cells were cultured in 24-well plates and stored at 37 °C and 5% CO2 until they reached the confluent stage. After confluence, a 1000 µl pipette tip was used to make a scratch in the cell monolayer. The cells were washed with PBS to remove debris and cells not attached to the plate. After debris removal, 500 µl of culture medium were added to each well, containing concentrations of each material, according to the groups previously described, including a control group with no treatment. Then, the plate was put into a 37 °C and 5% CO2 chamber positioned in a 4X magnification at light microscope (Carl Zeiss, Oberkochen GER) adjusted to capture images of the wells for 72h with a digital camera (Axio Cam MRc, Carl Zeiss). Each group was tested in duplicate and the images (baseline and 72h) were visually analyzed to compare cell migration behavior regarding the treatments.

Statistical analysis

All data obtained from MTT and Alizarin Red S assays were submitted to the Shapiro-Wilk test to evaluate data distribution. Non-normal distribution was evidenced in both experiments and the data were analyzed by Kruskal-Wallis test and Mann-Whitney to compare groups (α=0.05), using the statistical software SPSS, version 21 (IBM statistics, Akron, NY, USA).

3. Results

3.1 MTT assay

The cell viability percentage for groups tested for 24 h and 72 h are shown in Figure 1. At 24 h, Ca(OH)2 exhibited significant cytotoxicity. Meanwhile, the concentrations of DMP1 and P11-4, appeared not to affect the cells, indicating more than 80% cell viability. After 72 h,
h, Ca(OH)$_2$ presented similar values compared to the control group (untreated cells). On the other hand, P$_{11}$-4 and DMP1 at 1 µg/ml exhibited significantly higher cell viability rather than Ca(OH)$_2$ and even the control. P$_{11}$-4 and DMP1 at 0.5 µg/ml presented slightly higher cell viability than Ca(OH)$_2$ but with no statistical significance.

**3.2 Mineral deposition**

After 21 days of culture in osteogenic media, DMP1 (1µg/ml) exhibited the highest mineralization potential, while P$_{11}$-4 at the same concentration had demonstrated similar behavior. Although Ca(OH)$_2$ had lower mineralization values compared to DMP1 and P$_{11}$-4 (1µg/ml), it was higher than P$_{11}$-4 and DMP1 (0.5µg/ml). P$_{11}$-4 and DMP1 (0.5µg/ml) presented values even lower than the osteogenic control; however, no difference was found (Figure 2).
Figure 2. Mean, standard deviation (top), and representative images (bottom) of mineral deposition after 21 days of culture in osteogenic media. Different capital letters represent statistical differences (p<0.05). DMP1 at 1.0 µg/ml presented the highest mineralization potential (p<0.05), which was not significantly different from P11-4 1.0 µg/ml. P11-4 1.0 µg/ml induced more mineral deposition than the control group and DMP1 and P11-4 at 0.5 µg/ml (p<0.05), but with no difference to Ca(OH)\(_2\). DMP1 and P11-4 at 0.5 µg/ml had the lowest mineralization potential.

3.3 Cell Migration

All the experimental groups exhibited some level of cell migration directed towards the gap resulting from the removal of the cells. There was less visible migration of the control group compared to all different treatments. No completion in closing the wound was evidenced even with a remarkable migration exhibited by DMP1 0.5 µg/ml (Figure 3).
Figure 3. Representative SEM images for wound and healing experiments at baseline and after 3 days of migration. Control, with no treatment, (A-B); Ca(OH)\(_2\) (C-D); DMP1 0.5 µg/ml (E-F); DMP1 1 µg/ml (G-H); P\(_{11}-4\) 0.5 µg/ml (I-J); P\(_{11}-4\) 1 µg/ml (K-L).

4. Discussion

Regenerative endodontic therapies have been widely discussed in the last years regarding pulp disinfection [24, 25], revascularization [26], and immune response [27] through different materials. However, the biomimetic approach is still an emerging alternative in pulp-capping procedures for deep cavities or pulp micro exposures.

The self-assembling peptide, P\(_{11}-4\), effects on odontoblast-like cells were evaluated in this study, and the material presented cytocompatible characteristics. The cell response is a determinant factor for regenerative therapies [24, 28] and, in contrast to calcium hydroxide, which exhibited low cell viability at 24h but then recovered significantly after 72
hours, P11-4 demonstrated high biocompatibility at all time points, which suggests a non-inflammatory response and proliferative stimuli. Regarding cell migration and proliferation experiments, all the materials supported cell migration. However, the lowest concentration of DMP1 (0.5µg/ml) induced faster cell migration compared to other groups (Figure 3E-F).

P11-4 at the highest concentration (1.0µg/ml) was effective in inducing mineral deposition in a similar way to DMP, both of which indicated superior results compared to calcium hydroxide. This inductive response and the cytotoxicity data bring insights to support the hypothesis that the self-assembling peptide could be used as a cell-friendly substitute for calcium hydroxide in the future. However, from the best of our knowledge, this is the first study using P11-4 to stimulate odontoblast-like cells in dentin regeneration, then more studies to compare other parameters regarding the properties of the materials and the in vivo response are necessary.

Apart from that, the similarities in the results for P11-4 and DMP1 could be associated with the negatively charged domains of the peptide amino acids sequence as a consequence of its three glutamic acids [29]. In addition, these findings could also be associated with the fact that the amino acid sequence of P11-4 is similar to the residues of the amelogenin C-terminal, which plays an important role in the mineralization activity of that protein [30]. Carboxyl domains in peptides and proteins contribute to crystal nucleation by their repetitive organization, which can direct mineral arrangement [31]. On the other hand, the N-terminal helps in the stabilization of the amorphous mineral phase is important during crystal growth [32].

In the same way, DMP1 also has negatively charged domains and C- and N-terminals, which are, in fact, associated with the role of that protein on biomineralization [9,33]. Thus, the similarities effects of both peptide and protein were evidenced in this study and could be investigated in further studies. Moreover, the entire mechanism of the peptide, including its morphology and the role of biomineralization in this concentration regime should be clarified. However, it is known that the negatively charged domains, on the self-assembled surface, act as a heterogeneous nucleating template, dehydrating the calcium ion, producing nucleating centers [34].

P11-4 can bind type I collagen to form thicker fibers, reduce proteolysis, and induce mineral nucleation in other in vitro models [20]. In addition, P11-4 presented high levels of β-sheet formation [29] and had demonstrated a pH-responsive state transition that contributes to the kind of structure to form [17]. Besides, self-assembling peptides have stimulated mineralization biomarkers like alkaline phosphatase and osteocalcin in pre-osteoblast cells (MC3T3) [35] but, for P11-4, no previous evidence of a modulatory effect for the odontoblast-like cell has been described. Thus, the possibility that the self-assembling peptide may behave as a synthetic analogous protein and plays a role in the biomineralization process, guiding the mineralization of the pre-dentin matrix, prospects future investigations in pulp-capping procedures and dentin regeneration strategies.

Although this is an in vitro study and P11-4 was tested as a solution, which is hard to keep acting in a clinical situation, our data bring initial evidence to purpose P11-4 as a synthetic biomimetic alternative in tertiary dentin formation. To confirm that, further studies are necessary to measure the effects of P11-4 on molecular regulatory response. Besides, we are looking for carrying mechanisms to keep the peptide in contact with the damaged dentin or micro pulp exposures while it induces gradual biomineralization.

5. Conclusions

P11-4 a self-assembling peptide showed no cytotoxicity effects, induced cell migration, and mineral deposition in odontoblast-like cells after 21 days. P11-4 exhibited similar
values in all experiments compared to DMP1 at 1µg/ml and induced more mineral deposition than calcium hydroxide.

**Author Contributions:** Conceptualization, methodology, formal analysis, and validation I.J.S.A. and R.M.P.-R.; investigation, I.J.S.A, G.N.G, and R.A.M; resources, R.M.P-R; data curation, I.J.S.A and R.M.P-R.; writing—original draft preparation, I.J.S.A.; writing—review and editing, I.J.S.A., G.N.G, R.A.M., L.E.B., R.P.W.D, and R.M.P-R.; visualization, supervision and project administration R.M.P.-R.. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Coordination of Superior Level Staff Improvement (CAPES) Grant number: AUX/CAPES/PROEX:0878/2018.

**Acknowledgments:** The authors thank Dr. Marcelo Rocha Marques from the Histology Laboratory of Piracicaba Dental School for the collaboration.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**