

SYNTHESIS OF FLUORENATED ANALOGUES OF SELF-ASSEMBLING PEPTIDE P_{11-4} AND STUDY ON P_{11-4} THERAPEUTIC EFFECT ON ARTIFICIAL INITIAL ENAMEL LESIONS

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ABSTRACT

Treatment of artificial initial enamel lesions is a key process in the dental practice. Unfortunately, traditional methods are often not accepted well by the patients, especially by the children. In this reason the search for new more acceptable approaches is a long-sought approach. The selection of an appropriate and accurate protocol to treat white spot lesions as well as reliably monitoring of the lesion dynamics is crucial to avoid the need for further operative treatment. Herein, we report synthesis of two fluorinated analogues of the peptide P_{11-4} and creation of a protocol to compare the effect of self-assembling peptide P_{11-4} (CURODONT Repair) on artificial enamel lesions on smooth surfaces after pH-cycling in a bioreactor system. The assessment of the effect is done using surface microhardness investigation. A well working protocol for studying of therapeutic effect of peptides on artificial initial enamel lesions was created. The obtained experimental data reveals that the treatment of artificial lesions with self-assembling peptide P_{11-4} using a newly created protocol and selected methodology resulted in improved surface microhardness in the experimental group.

Keywords: P_{11-4} , self-assembly fluorinated peptides, treatment of artificial initial enamel lesions

INTRODUCTION

Peptides are naturally existing molecules in human organism. They could play different roles, for example hormones such as insulin, vasopressin, oxytocin [1, 2] as well as neurotransmitter somatostatin [3, 4], which exerts multiple biological activities like inhibition of endocrine secretion and growth as well as cell proliferation. In addition, peptides have many advantages like small size, relatively simple synthesis, membrane penetration properties, self-assembly ability [5, 6] and good biocompatibility [7, 8]. Some small changes in the peptide structure could lead to

better pharmacodynamics and pharmacokinetic of the newly designed molecules and it is a useful strategy for creation of new compounds with better targeted properties and biological activity [9].

Caries lesions are the most popular dental problem. On the surface of the teeth a biofilm is formed during the time. The microorganisms contained in the biofilm produce acids as a result of their normal metabolism process, which leads to caries lesions formation [9]. In 2013 Brunton et al. reported the application of biomimetic self-assembling peptides in a treatment of early caries lesions [11]. Several years later Wierichs et al. and Alkilzy et al. described another aspect of the

carious lesion treatment with self-assembling peptide P_{11-4} (Ac-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH₂) [12, 13]. Taking into account very good results on the treatment of white spot lesions (WSLs) reported by these groups we made a design of analogues of the self-assembly peptide P_{11-4} containing fluorinated in the position 4 phenylalanine (Phe(4-F)). Herein, we report synthesis of two fluorinated analogues of the peptide P_{11-4} and creation of a protocol to study the effect of these peptides on artificial enamel lesions on smooth surfaces after pH-cycling in a bioreactor system using surface microhardness investigation.

EXPERIMENTAL

Chemical reagent and synthesis

The needed specifically protected amino acids $^{\alpha}$ N-Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Phe(4-F)-OH, Fmoc-Glu(Ot-Bu)-OH, Fmoc-Trp(Boc)-OH as well as solid phase carrier Fmoc-Rink Amide MBHA Resin, activation agents N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), N,N'-Diisopropylcarbodiimide (DIC) or Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), trifluoroacetic acid (TFA), N,N-Diisopropylethylamine (DIEA) and scavenger triisopropylsilane (TIS) were purchased from Iris Biotech (Germany). The solvents N, N'-dimethylformamide (DMF) and dichloromethane (DCM) are from Valerus (Bulgaria). Acetic anhydride is from Merck (Bulgaria). All reagents and solvents are used without any preliminary treatment.

The target compounds were synthesized using Fmoc(9-fluorenylmethoxycarbonyl)/Ot-Bu solid-phase peptide synthesis (SPPS) on Rink-amide MBHA resin. HBTU, DIC or PyBOP were used for amino acid activation. The coupling reactions were performed using amino acid/HBTU(or PyBOP)/DIEA/resin at a molar ratio 3/3/9/1 or amino acid/DIC/resin at a molar ratio 3/3/1. $^{\alpha}$ N-Fmoc-group of all amino acids during peptide chain synthesis was deprotected by treatment with 20 % piperidine in DMF. The coupling and deprotection reactions were monitored by means of the standard Kaiser test. The removing of the final peptides from the resin was done using a mixture of 95 % trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) and 2.5 % dH₂O. All peptides were obtained as a filtrate in TFA and

precipitated with cold dry diethyl ether. The precipitate was filtered and subjected to subsequent HPLC/MS analysis.

Acetylation of the target peptides on the resin were conducted as follow:

In a final step after removing of $^{\alpha}$ N-Fmoc protection group of aimed peptide a mixture of 10 mL acetic anhydride, 10 mL pyridine and 10 mL DCM is added to the peptide-resin in the reactor. Reaction mixture is stirred for 30 minutes and the solvents are removed from the reactor. The procedure is repeated twice. Further, peptide-resin is washed with 2xDMF, 2xDCM, 2x *i*-PrOH and 2 x diethyl ether and after drying is subjected to the deprotection from the resin.

Apparatus and analytical methods

The peptide purity was monitored on a RP-HPLC Agilent Poroshell 120, 100 mm x 4.6 mm column using Shimadzu LC MS/MS 8045 system, at a flow rate mobile phase rate 0.30 mL min⁻¹, column temperature 40°C, and a linear binary gradient of two phases Mobile phase A: H₂O (10 % AcCN; 0.1% HCOOH) and Mobile phase B: AcCN (5 % H₂O; 0,1 % HCOOH) at the following gradient of both phases in time:

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.01	80	20
10.00	5	95
15.00	5	95
15.50	80	20
22.00	80	20

The compound structures were checked by electrospray ionization mass spectrometry in SCAN regime/ESI+ mode of ionization with the following parameters: nebulizing gas flow 3 L min⁻¹; heating gas flow 10 L min⁻¹; interface temperature 350°C; desolvation line temperature 200°C; heat block temperature 400°C; drying gas flow 10 L min⁻¹.

The optical rotation was measured with an automatic polarimeter Polamat A, Carl Zeiss, Jena at c = 1 in methanol. Melting points were monitored on a standard Kruss melting point meter M3000. All analytical data are summarized in Table 1.

During the study a pH-cycling model is used by

Table 1. Structure and analytical data for newly synthesized compounds.

Code	Structure	Molecular formula	MM _{exact}	[M+H] ⁺ Observed	t _R (min)	M.p. [°C]	α ₅₄₆ ^D [°]*
IU1	Ac-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe(4-F)-Glu-Gln-Gln-NH ₂	C ₇₂ H ₉₇ FN ₂₀ O ₂₂	1612.71	1613.75	2.011	225-226	+42
IU2	Ac-Gln-Gln-Arg-Phe(4-F)-Glu-Trp-Glu-Phe(4-F)-Glu-Gln-Gln-NH ₂	C ₇₂ H ₉₆ F ₂ N ₂₀ O ₂₂	1630.70	1631.70	2.020	224-225	-52

*(c = 1, methanol)

means of Bioreactor system CDC-Biofilm reactor (model CB90-1DH-Int.). All details about the model are described in details in the Results and Discussion section.

Biological study

Materials

Kits containing self-assembling peptides P₁₁₋₄ (CURODONT Repair) were purchased by Credentis AG, Windisch, Switzerland. Freshly human premolars extracted for orthodontic indications from patients in the children/adolescents age (ranged from 14 - 20 years of age), free from cracks, erosion, caries, or any structural defect was obtained. The clinical methodology of this study was approved by the Research Ethics committee of Medical University - Plovdiv (Protocol №3/20.05.2021).

Sample and solution preparation

The extracted teeth are cleaned of plaque, tartar and soft tissues from the tooth surface. The teeth are then separated 1 mm below the cement-enamel junction with a diamond disk at a slow speed, with crowns being reserved for examination. Samples are stored in a solution containing 0.1 % thymol until the start of the experiment.

The coronal part of each tooth is longitudinally sectioned in a mesiodistal direction into two sections using a high-speed diamond tipped disc, creating two enamel specimens and thus leading to a total of 24 enamel specimens. The specimens are randomly divided into two groups of 12 specimens each, according to Majithia et al. [14].

Samples are prepared by including tooth in

cylindrical blocks with the vestibular surface facing upwards and parallel to the horizontal plane. A window from 5 mm to 5 mm is provided for conducting the experiment. The proximal 2 mm from the surface is covered with acid-resistant nail polish for insulation and controllability in subsequent stages of the study.

Procedures and model

Initial surface microhardness by Vickers (B-VMH)

Specimens are analyzed in terms of baseline surface hardness to establish a parameter for the specimens' selection and for randomized distribution to the groups according to methodology described by Freitas et al. [15].

Initial laser fluorescence values by DIAGNOdent pen (B-LF pen).

Buccal or lingual surfaces of teeth are examined using device for laser fluorescence (DIAGNOdent pen 2190, KaVo, Biberach, Germany) [16]. Samples showing a moment value between 3 and 7 on the digital display are selected and values are recorded. Those showing a value greater than 7 are discarded and replaced by teeth having a moment value of 3 - 7 [17, 18]. After inclusion in resin blocks, a quarter of the surface of each sample is covered with a layer of acid-resistant nail polish (Top coat, Victoria beauty) as Healthy Control (SC). Further the creation of artificial caries lesion is done. After a quarter of the artificial lesion is coated with the same acid-resistant transparent nail polish for Demineralized Control (DC). The rest of the artificial lesion remains as uncovered area for treatment with the chosen agents (Treatment Area - TA) [19].

Creation of artificial carious lesions

Early caries-like lesions are created on the exposed window on each tooth block by subjecting the blocks to 5 days demineralization in an acidified solution. Demineralization periods are chosen based on prior experience and to create lesions with comparable data [20]. Laboratory formation of artificial carious lesions in the enamel with demineralization solution including treatment for 5 days with a solution containing 1.5 mM CaCl_2 , 0.9 mM NaH_2PO_4 , 50 mM Acetate Buffer, pH 4.3 at 37°C, according to Ruan et al. [21]. Each specimen is immersed separately in a demineralizing solution, renewed daily, for 5 consecutive days, until a uniform WSL is created. Specimens are then rinsed carefully and stored in distilled deionized water [22].

Application method of CURODONT Repair

The established clinical protocol applies except for the etching process. Treatment of the surface with 2 % NaCl for 20 sec and 30 sec rinsing with water. Etching gel 37 % phosphoric acid is applied for 5 sec, then rinse with a water-air spray for 30 sec. Further the surface is dried with compressed oil-free air for 60 sec. A single dose of CURODONT Repair dissolved in 0.05 mL of distilled water is applied with a micro applicator and allowed to interact with the enamel surface for 5 min [23].

Surface microhardness (SMH) analysis

Surface microhardness is assessed at the baseline, after demineralization, and 21 days after pH-cycling in CDC Biofilm Reactor. The surface microhardness of the enamel specimens is measured using a digital Vickers microhardness tester (Tukon 1102, Wilson Hardness, Germany). The applied load is 100 g with an indentation time of 10 sec (Vickers pyramid: diamond right pyramid with a square base and an angle of $\alpha = 136^\circ$ between the opposite faces at the vertex and $\times 600$ magnification of microscope) [24]. The indentations are made for each specimen at three different locations ($\geq 100 \mu\text{m}$ from each other), and the average of three measurements is calculated and obtained as one reading. Indentation result can be seen at projector screen in the form of shadow shaping rhomb. The diagonal length is measured with micrometer. The three indentations, made for each specimen are independently averaged and reported in Vickers hardness number (VHN) [24]. The slabs with very low or very high surface hardness

values are excluded as well as those slabs with a great variability among the indentation values. So, selected slabs are within a range of 10 - 20 % above or below the average hardness value of all slabs and all slabs with a coefficient of variation of the 3 - 5 indentations greater than 10 % are excluded [21]. The percentage of microhardness recovery for each of the treatment groups is calculated using the formula presented by Majithia et al [14]:

$$\% \text{ recovery} = \frac{\text{VHN}_{\text{ramin}} - \text{VHN}_{\text{demin}}}{\text{VHN}_{\text{baseline}} - \text{VHN}_{\text{demin}}} \times 100$$

To assure the blinding of the study the two examiners, working at the Department of Pediatric Dentistry, Plovdiv, Bulgaria, performed the microhardness evaluation without knowledge of the procedures, performed to the samples [24].

Statistical methods

Measurements of central tendency and measurements of spread described the variables of interest. Shapiro-Wilk test checked if continuous variables follow normal distribution. The means of the normally distributed variables were compared by the independent-samples t-test for two unrelated groups. A repeated measures ANOVA with a Greenhouse-Geisser correction was applied to follow changes over time and post hoc analysis with a Bonferroni adjustment was used for pairwise comparisons. A 2-sided p-value of < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS Statistics v. 26 software (IBM Corp. Released 2019. Armonk, NY: USA).

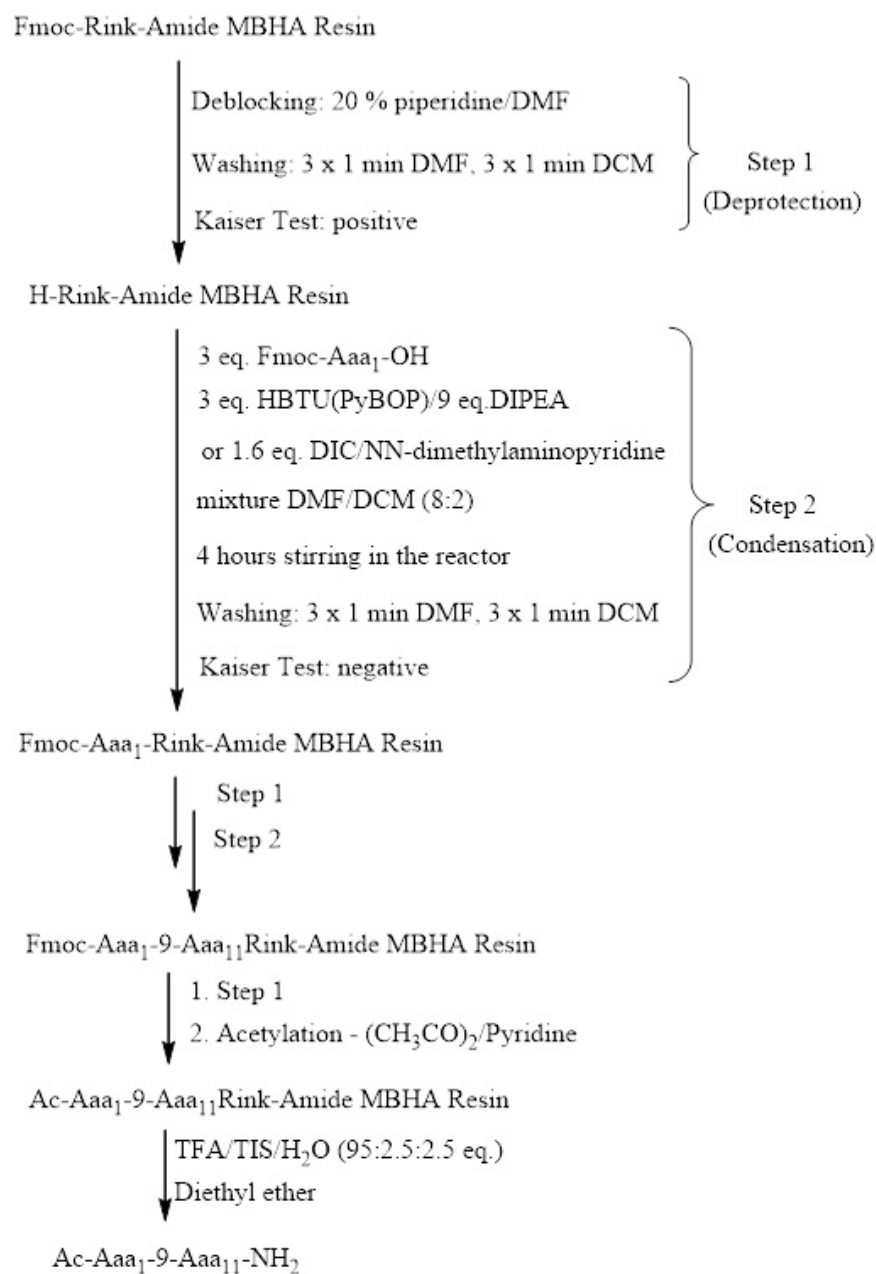
RESULTS AND DISCUSSION

Synthesis of fluorinated analogues of P_{11-4}

The fluorinated analogues of P_{11-4} named Ac-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe(4-F)-Glu-Gln-Gln- NH_2 (IU1) and Ac-Gln-Gln-Arg-Phe(4-F)-Glu-Trp-Glu-Phe(4-F)-Glu-Gln-Gln- NH_2 (IU2) were synthesized using Fmoc/Ot-Bu SPPS on Rink-amide MBHA resin according to Scheme 1.

Newly synthesized peptides were fully characterized and the obtained data are summarized in Table 1.

Herein, the influence of commercially available self-assembly peptide P_{11-4} (Ac-Gln-Gln-Arg-Phe-



Scheme 1. Summarized protocol for synthesis of aimed peptides, where Aaa₁ and Aaa₁₁ are both Gln - the first amino acid in the N-terminus and the last amino acid in the C-terminus of aimed peptides.

Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH₂) on the process of artificial enamel lesions repairmen is investigated. Further, the found effects of P₁₁₋₄ will be compared to those of newly designed fluorinated peptide analogues of P₁₁₋₄ but preliminary results show better repairing properties of fluorinated analogues than those of the target P₁₁₋₄ peptide.

Characterization of the created lesion

Characterization of samples after demineralization is achieved by measurement of surface microhardness (D-SMH) and by DIAGNOdent pen analysis to record baseline values of demineralized surfaces (D-LF pen). The teeth are scanned with Diagnodent and the samples showing a moment value of 9 and above on the digital display are taken for further evaluation.

A total of 24 samples are randomly divided into two groups of 12 samples each.

Group 1: Control group, no treatment - Group NT.

Group 2: Subjected to treatment with Curodont Repair - Group CR. CURODONT Repair™ (Credentis AG, Windisch, Switzerland) that incorporates the self-assembling peptide (P₁₁₋₄) based Curolox™ technology. During the following treatment, the nail varnish was carefully and totally removed with acetone from two group. Artificial caries lesion is assessed by enamel surface microhardness evaluation at baseline level, after demineralization, and 21 days after pH-cycling model in bioreactor.

Details about the model (remin, demin, pH cycling)

During the study a pH-cycling model in bioreactor was used. First, all 24 samples are randomly divided into 2 groups of 12 samples each one. After treatment of the samples with the indicated means, according to their protocol of application, a model of pH-cycling in a bioreactor system is applied. For the purpose of the study the CDC Biofilm Reactor was used to recreate as realistically as possible the conditions of the oral environment typical of childhood. We believe that this will give optimal results compared to the standard pH cycling protocols applied so far.

The pH cycling is carried out for a period of 7 days with demineralization attacks being 5 per day (20 minutes each, in total), corresponding to 3 main meals and 2 snacks, while at rest during the day and at night, the solution is maintained alkaline by means of

a remineralizing solution, i.e., artificial saliva with a pH of 7.2. Demineralization attacks are performed by introducing 10 % sucrose for 20 minutes in the bioreactor system according to Filipov et al. and Rudnev et al. [25, 26]. The planned 5 demineralization attacks daily for 20 min each with 10 % sucrose are chosen in order to mimic the maximum pH dynamics of the oral environment in situations with a high risk of caries, typical in childhood. During the day we apply active incubation with a constant flow of fresh artificial saliva at speed 11 mL min⁻¹ in the bioreactor system and during 8h “night” period the specimens are subjected to a remineralizing solution (passive incubation). After the 7-day period, the bioreactor is left for passive incubation (constant temperature and stirring for 14 days).

The pH cycling model is adopted to simulate the changes occurring in the oral cavity [21], where the artificial saliva solution are prepared using analytical grade chemicals and deionized water [14, 27]. The amounts of solution are large enough to prevent the solutions from becoming extremely supersaturated with or depleted of mineral ions, meaning that a constant pH 7.2 is achieved [28 - 30]. The artificial saliva described by Schmidlin et al. prepared by diluting of 2.4 g KCl, 1.7 g NaCl, 0.1 g MgCl₂×6H₂O, 0.2 g CaCl₂×2 H₂O, 0.2 g KSCN, 0.7 g KH₂PO₄, and 0.1 g H₃BO₃ till total amount of 1 L with distilled water [31].

Justification of the suitability of the model (lengths of remin/demin phases) for the representation of real-world conditions

The preparation for this *in vitro* study is organized in two stages: clinical and laboratory. This includes the process of collecting biological material from participants (volunteers) and laboratory work with the collected material. Thus, an experimental protocol is formed, where the goal is cultivating oral inoculum biofilms *in vitro*.

To perform the clinical stage of the task, we enroll pre-selected donor patients who meet the inclusion criteria: age 8-18 years, informed parental consent to participate in the experiment, absence of acute or chronic disease with oral manifestation and normal quantitative as well as qualitative composition of saliva [25]. The clinical methodology of this study was approved by the Research Ethics committee of Medical University, Plovdiv (Protocol №3/20.05.2021).

Table 2. Descriptive statistics for the SMH variables by groups - measurements of central tendency and measurement of spread.

		mean	SD	Median	IQR	min-max
CR Group	B-SMH	286.80	51.04	294.45	99.40	216.20 - 359.90
	D-SMH	117.34	49.48	105.50	68.20	61.80 - 213.70
	C-SMH	160.16	51.15	174.10	94.40	88.60 - 236.20
NT Group	B-SMH	290.48	47.25	290.25	70.60	230.00 - 375.90
	D-SMH	105.10	106.90	106.90	42.10	73.00 - 151.60
	C-SMH	113.25	37.48	102.80	71.20	73.20 - 173.80

The results from descriptive statistics for calculated microhardness is summarized in Table 2.

Statistically significantly higher mean value of C-SMH was observed in CR group (160.16), compared to the mean value registered in NT group (113.25) ($t = 2.56$, $p = 0.018$). We were not able to reject the Null hypothesis for no difference between groups (CR group vs. NT group) in mean values calculated for the first (baseline – B-SMH) time point and respectively for the second (after demineralization – D-SMH) time point.

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that mean SMH scores, collected at CR group, differed statistically significantly between the three points of measurement ($F(1.212, 13.334) = 39.50$, $p = 0.000$). Post hoc tests using the Bonferroni correction revealed that there is statistically significant difference between SMH means for all pairwise comparisons (Fig. 1). A repeated measures ANOVA with a Greenhouse-Geisser correction determined that mean SMH scores, collected at NT group, differed statistically significantly between the three points of measurement ($F(1.471, 16.179) = 105.411$, $p = 0.000$). Post hoc tests using the Bonferroni correction revealed that there is statistically significant difference in SMH means measured in baseline and after demineralization ($p < 0.05$), same observation is valid for baseline and after pH-cycling in the Bioreactor, comparisons of the mean measurements ($p < 0.05$). No difference was recorded in SMH means measured in after demineralization and after pH-cycling ($p > 0.05$) (Fig. 1).

The methodology of investigation the treatment effects in the majority of studies is focused on the mechanical or radio-graphical changes of caries lesion, such as microhardness testing and microradiography where the results mainly reflect the changes in mineral

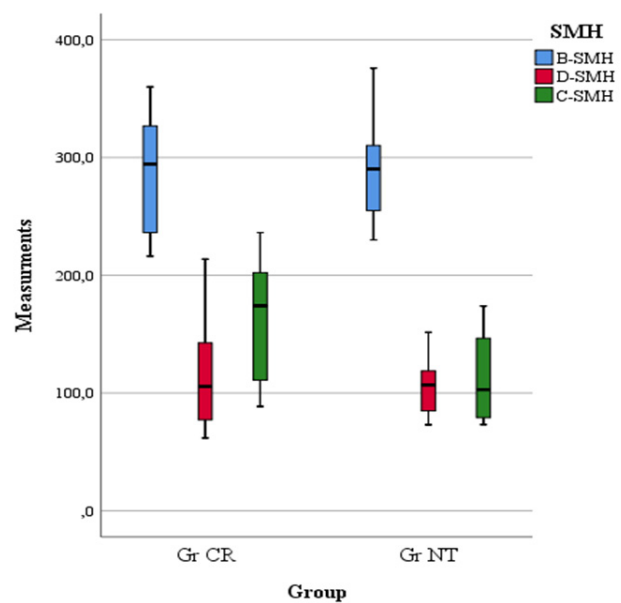


Fig. 1. Repeated Measures ANOVA of the SMH measurements by groups.

content of caries lesion [32 - 34]. One of the most common and reliable methods for this purpose is the assessment of change in surface microhardness using Vickers microhardness tester. In the protocol of our study, microhardness values (SMH) are measured after demineralization and remineralization of specimens. SMH analyses have been broadly used to assess the demineralization and remineralization changes that occur in the enamel. SMH evaluations are considered as simple, fast, and easy to measure in a nondestructive manner. However, changes of caries in microstructure are usually associated with variation in both mineral and organic content. Electrochemical methods are solid methods to detect any changes in mineral constituents which reflect the microstructure of hard tissue [35].

Some *in vitro* studies compare the remineralization potential of modern agents without recreating real conditions of the oral environment (pH cycling), but store the samples in de- and remineralizing solutions for a certain amount of time (so-called simplified chemical model) [28, 36]. These investigative methods are easy to implement, relatively cheap and provide stability and reproducibility of results. Therefore, they continue to be preferred for experimental research. Recently, most *in vitro* studies have been performed by recreating the conditions of the oral environment through a pH-cycling regime. The rationale behind the pH-cycling model is based on mimicking the *in vivo* periodic alternation of pH, occurring in the oral cavity when sugars are metabolized, leading to caries formation. The duration of pH cycling ranges from a few days to several weeks or months, often framing a certain time period (5, 7, 10, 28, 30 days, etc.) [14, 16, 27, 37]. Very few studies use biofilm models with cryogenic bacteria. Even fewer studies use an oral biofilm reactor with active incubation (open system) to mimic the physiological and microbiological properties of natural dental plaque.

Unfortunately, chemical experimental models, such as pH cycling and immersion using demineralising agents, do not simulate the demineralisation process that occurs in the oral cavity, owing to the absence of microorganisms. Therefore, they reflect only the physical-chemical aspects of enamel dissolution. An *in vitro* model that uses bacterial biofilm would be more representative than the chemical experimental models since dental caries is a multifactorial disease [38].

CONCLUSIONS

During this study a well working protocol for studying of therapeutic effect of peptides on artificial initial enamel lesions was created. The obtained experimental data reveals that treatment of artificial lesions with self-assembling peptide P₁₁₋₄ using newly created protocol and selected methodology resulted in improved surface microhardness in the CURODONT Repair group. Taking into account the last mentioned above in our future study using already created protocol we will compare the obtained data from treatment with CURODONT Repair with those obtained after treatment with newly synthesized fluorinated analogues of P₁₁₋₄. In addition, the microbiological model is planned to be

used in order also to compare the results with the pH-cycling models for the evaluation of caries-like lesions.

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REFERENCES

1. P. Sonksen, J. Sonksen, Insulin: understanding its action in health and disease, Br. J. Anaesth., 85, 1, 2000, 69-79, doi:10.1093/bja/85.1.69
2. H.K. Caldwell, W.S. Young, Oxytocin and Vasopressin: Genetics and Behavioral Implications" (PDF). In Lajtha A, Lim R (eds.). Handbook of Neurochemistry and Molecular Neurobiology: Neuroactive Proteins and Peptides (3rd ed.). Berlin: Springer. 2006, pp. 573-607, ISBN 978-0-387-30348-2
3. A. Costoff, Structure, Synthesis, and Secretion of Somatostatin". Endocrinology: The Endocrine Pancreas. Medical College of Georgia, Section 5, Chapter 4, 2008, p. 16
4. Y. Liu, D. Lu, Y. Zhang, S. Li, X. Liu, H. Lin, The evolution of somatostatin in vertebrates, Gene, 463, 1-2, 2010, 21-8, doi:10.1016/j.gene.2010.04.016
5. K.H. Chan, B. Xue, R.C. Robinson, C.A.E. Hauser, Systematic single moiety variations of ultrashort peptides produce profound effects on self-assembly, nanostructure formation, hydrogelation, and phase transition, Scientific Reports, 7, 2017, 12897
6. K.H. Chan, W.H. Lee, M. Ni, Y. Loo, C.A.E. Hauser, C-Terminal residue of ultrashort peptides impacts on molecular self-assembly, hydrogelation, and interaction with small-molecule drugs, Scientific Reports, 8, 2018, 17127
7. M. Thayer, Improving peptides, Chemical and Engineering News, 89, 2011, 13-20
8. C. Borghouts, C. Kunz, B. Groner, Current strategies for the development of peptide-based anti-cancer therapeutics, J. Pept. Sci., 11, 11, 2005, 713-726

9. C. Cabrele, T.A. Martinek, O. Reiser, Ł. Berlicki, Peptides Containing β -Amino Acid Patterns: Challenges and Successes in Medicinal Chemistry, *J. Med. Chem.*, 57, 2014, 9718-9739, dx.doi.org/10.1021/jm5010896
10. H.M. Ayoub, R.L. Gregory, Q. Tang, F. Lippert, The anti-caries efficacy of three fluoride compounds at increasing maturation of a microcosm biofilm, *Archives of Oral Biology*, 117, 2020, 104781, <https://doi.org/10.1016/j.archoralbio.2020.104781>
11. P.A. Brunton, R.P.W. Davies, J.L. Burke, A. Smith, A. Aggeli, S.J. Brookes, J. Kirkham, Treatment of early caries lesions using biomimetic self-assembly peptides - a clinical safety trial, *British Dental Journal*, 215, 2013, E6, DOI: 10.1038/sj.bdj.2013.741
12. R.J. Wierichs, J. Kogel, J. Lausch, M. Esteves-Oliveira, H. Meyer-Lueckel, Effects of Self-Assembling Peptide P₁₁₋₄, Fluorides, and Caries Infiltration on Artificial Enamel Caries Lesions in vitro, *Caries Res.*, 51, 2017, 451-459, DOI: 10.1159/000477215
13. M. Alkilzy, R.M. Santamaria, J. Schmoeckel, C.H. Splieth, Treatment of carious lesion using self-assembling peptides, *Advances in Dental Research*, 29, 1, 2018, 42-47, DOI: 10.1177/0022034517737025
14. U. Majithia, K. Venkataraghavan, P. Choudhary, K. Trivedi, S. Shah, M. Virida, Comparative evaluation of application of different fluoride varnishes on artificial early enamel lesion: An in vitro study, *Indian J. Dent. Res.*, 27, 5, 2016, 521-527, doi:10.4103/0970-9290.195642
15. M.C.C.de A. Freitas, L.V. Nunes, L.P. Comar, D. Rios, A.C. Magalhães, H.M. Honório, L. Wang, In vitro effect of a resin infiltrant on different artificial caries-like enamel lesions, *Arch Oral Biol.*, 95, 2018, 118-124, doi:10.1016/j.archoralbio.2018.07.011
16. Comparison Between Fluoride and Nano-Hydroxyapatite in Remineralizing Initial Enamel Lesion: In-Vitro Study, Accessed April 25, 2021. <http://repository.bau.edu.lb:8080/xmlui/handle/1080/6983>
17. V. Varma, H.K. Sundeep, S.S. Bhat, S.S. Sargod, A. Rao, Comparative Evaluation of Remineralization Potential of Two Varnishes Containing CPP-ACP and Tricalcium Phosphate: An In Vitro Study, *Int. J. Clin. Pediatr. Dent.*, 12, 3, 2019, 233-236, doi:10.5005/jp-journals-10005-1629
18. J. Jayarajan, P. Janardhanam, P. Jayakumar, Deepika, Efficacy of CPP-ACP and CPP-ACPF on enamel remineralization - An in vitro study using scanning electron microscope and DIAGNOdent®, *Indian J Dent Res.*, 22, 1, 2011, 77, doi:10.4103/0970-9290.80001
19. P. Schmidlin, K. Zobrist, T. Attin, F. Wegehaupt, In vitro re-hardening of artificial enamel caries lesions using enamel matrix proteins or self-assembling peptides, *J. Appl. Oral Sci.*, 24(1), 2016, 31-36, doi:10.1590/1678-775720150352
20. B.T. Amaechi, Protocols to study dental caries in vitro: pH cycling models. In: *Methods in Molecular Biology*, Humana Press Inc., 1922, 2019, 379-392, doi:10.1007/978-1-4939-9012-2_34
21. Q. Ruan, D. Liberman, R. Bapat, K.B. Chandrababu, J.-H. Phark, J. Moradian-Oldak, Efficacy of amelogenin-chitosan hydrogel in biomimetic repair of human enamel in pH-cycling systems, *J. Biomed. Eng. Informatics*, 2, 1, 2016, 119-128, doi:10.5430/jbei.v2n1p119
22. D. Kamal, H. Hassanein, D. Elkassas, H. Hamza, Complementary remineralizing effect of self-assembling peptide (P11-4) with CPP-ACPF or fluoride: An in vitro study, *J. Clin. Exp. Dent.*, 12, 2, 2020, e161-e168, doi:10.4317/JCED.56295
23. A. Belcheva, R.E. Feghali, T. Nihtianova, S. Parker, Effect of the carbon dioxide 10,600-nm laser and topical fluoride gel application on enamel microstructure and microhardness after acid challenge: an in vitro study, doi:10.1007/s10103-018-2446-4
24. J.D. Silvertown, B.P.Y. Wong, K.S. Sivagurunathan, S.H. Abrams, J. Kirkham, B.T. Amaechi, Remineralization of natural early caries lesions in vitro by P₁₁₋₄ monitored with photothermal radiometry and luminescence, *J. Investig. Clin. Dent.*, 8, 4, 2017, 1-8. doi:10.1111/jicd.12257
25. I. Filipov, In Vivo Collection and SEM Identification of Oral Biofilm Using Indirect Composite Prototype Restorations. *Clinical and Laboratory Study*, 60, 2, 2018, 300-307, doi:10.1515/folmed-2017-0092
26. J.D. Rudney, R. Chen, P. Lenton, J. Li, Y. Li, R.S. Jones, C. Reilly, A.S. Fok, C. Aparicio, A reproducible oral microcosm biofilm model for testing dental materials, *J. Appl. Microbiol.*, 113, 6, 2012, 1540-1553, doi:10.1111/j.1365-2672.2012.05439.x

27. R. Soares, Assessment of Enamel Remineralisation After Treatment with Four Different Remineralising Agents: A Scanning Electron Microscopy (SEM) Study, *J. Clin. Diagnostic Res.*, 11, 4, 2017, ZC136, doi:10.7860/jcdr/2017/23594.9758
28. S. Savas, F. Kavrik, E. Kucukyilmaz, Evaluation of the remineralization capacity of CPP-ACP containing fluoride varnish by different quantitative methods, *J. Appl. Oral Sci.*, 24, 3, 2016, 198-203, doi:10.1590/1678-775720150583
29. P.R.A. Oliveira, A.B.M. Fonseca, E.M. Silva, T.C.L. Coutinho, M.A. Tostes, Remineralizing potential of CPP-ACP creams with and without fluoride in artificial enamel lesions, *Aust. Dent. J.*, 61, 1, 2016, 45-52, doi:10.1111/adj.12305
30. R.J. Wierichs, J. Kogel, J. Lausch, M. Esteves-Oliveira, H. Meyer-Lueckel, Effects of Self-Assembling Peptide P11-4, Fluorides, and Caries Infiltration on Artificial Enamel Caries Lesions in vitro, *Caries Res.*, 51, 5, 2017, 451-459, <https://doi.org/10.1159/000477215>
31. P. Schmidlin, K. Zobrist, T. Attin, F. Wegehaupt, In vitro re-hardening of artificial enamel caries lesions using enamel matrix proteins or selfassembling peptides, *J. Appl. Oral Sci.*, 24, 1, 2016, 31-36, doi:10.1590/1678-775720150352
32. R. Talaei, Z. Hoseini, R. Ghorbani, N. Ameli, Mineral content identification of white spot lesions around orthodontic brackets following the use of different concentrations of nano-hydroxyapatite mouthwash and fluoride gel, *Middle East. J. Rehabil. Heal.*, 7, 1, 2020, 1-7, doi:10.5812/mejrh.96516
33. S. Tahmasbi, M. Adhami, A. Valian, R. Hamed, Effect of Three Different Remin-eralizing Agents on White Spot Lesions; An In Vitro Comparative Study, *J. Islam. Dent. Assoc. Iran*, 28, 3, 2016, 98-103, doi:10.30699/jidai.29.3.98
34. Z.A. Shaik, T. Rambabu, G. Sajjan, M. Varma, K. Satish, V.B. Raju, S. Ganguru, N. Ventrapati, Quantitative analysis of remineralization of artificial carious lesions with commercially available newer remineralizing agents using SEM-EDX- in vitro study, *J. Clin. Diagnostic Res.*, 11, 4, 2017, ZC20-ZC23, doi:10.7860/JCDR/2017/22270.9642
35. M.-L. Mei, C.-H. Chu, K.-H. Low, C.-M. Che, E.-C.-M. Lo, Caries arresting effect of silver diamine fluoride on dentine carious lesion with *S. mutans* and *L. acidophilus* dual-species cariogenic biofilm, *Med. Oral Patol. Oral Cir. Bucal*, 18, 6, 2013, 824-855, doi:10.4317/medoral.18831
36. R. Talaei, Z. Hoseini, R. Ghorbani, N. Ameli, Mineral Content Identification of White Spot Lesions Around Orthodontic Brackets Following the Use of Different Concentrations of Nano-Hydroxyapatite Mouthwash And Fluoride Gel, *Middle East Journal of Rehabilitation and Health Studies*, 7, 1, 2020, e96516, doi:10.5812/mejrh.96516
37. R. Alkattan, F. Lippert, Q. Tang, G.J. Eckert, M. Ando, The influence of hardness and chemical composition on enamel demineralization and subsequent remineralization, 75, 2018, 34-40, doi:10.1016/j.jdent.2018.05.002
38. J.A. Rodrigues, C.S. Sarti, C.M. Assunção, R.A. Arthur, A. Lussi, M.B. Diniz, Evaluation of laser fluorescence in monitoring non-cavitated caries lesion progression on smooth surfaces in vitro, *Lasers Med. Sci.*, 32, 8, 2017, 1793-1800. doi:10.1007/S10103-017-2262-2