Analysis of the microhardness of artificial demineralized enamel and remineralization with polymer-controlled remineralization systems

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Анализ на промените в микротвърдостта при реминерализация на изкуствен емайлов кариес с полимер-контролирана реминерализационна система

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Summary

Introduction: *Dental caries is a microbial disease of the mineralized dental structures, characterized by demineralization of the inorganic portion and destruction of the organic matrix of the tooth. Hence, demineralization is considered to be the fundamental mechanism for caries development. The extensive studies on remineralization have led to the development of newer technologies that promote enamel remineralization and prevent enamel demineralization providing promising oral health assistance.*

Aim: *The purpose of this study is to evaluate the potential for remineralization of the poly-2-dimethylaminoethyl metacrylate (PDMAEMA) with **in situ** formed calcium phosphates on artificial enamel caries lesions evaluating the surface micro hardness*.

Materials and methods: *Intact buccal surfaces of 30 extracted third molars were demineralized in 0.1M lactic acid for 6 days, pH = 4.5. The remineralization was done using PDMAEMA with calcium phoshpates for 7 days. Vickers microhardness test was done in five different points per sample. Based on the results obtained, the average Vickers Hardness Number (VHN) and the standart deviation for each sample was calculated as follows: for intact enamel, after demineralization and after remineralization.*

Results: *At baseline, samples mean VHN value was 330.64 ± 12.3, after demineralization the mean value was – 125.64± 2.41 and after remineralization the mean value was 231.62 ± 8.35.*

Conclusion*: In the limitations of this in vitro study enamel microhardness increased after applying polymer controlled mineralising system of PDMAEMA/calcium phosphates on artificial enamel lesions. It was found that system has the potential to promote the recristalisation processes of enamel lesions.*

Key words: *artificial enamel caries, remineralization, PDMAEMA*

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Резюме

Въведение*: Кариесът е заболяване на минерализираните зъбни структури, при което се наблюдава деминерализация на неорганичната съставка и деструкция на органичния матрикс. Деминерализацията има основно значение за развитието на кариеса. Проучването на механизмите на реминерализацията е определящо за развитието на нови материали, които стимулират емайловата реминерализация.*

Цел*: Целта на настоящото проучване е да оцени реминерализационния потенциал на система от поли-2-диметиламинотеил метакрилат (ПДМАЕМА) с in situ формирани калциеви фосфати върху изкуствени емайлови лезии, като се анализират промените на повърхностната микротвърдост.*

Материали и методи: *Интактната букална повърхност на 30 екстрахирани молари е деминерализирана в 0.1 М млечна киселина за 6 дни, pH=4.5. Реминерализацията се извършва с разтвор на ПДМАЕМА и калциеви фосфати за 7 дни. Изследва се емайловата микротвърдост по Викерс в пет различни точки за образец. За всеки образец се изследва средната стойност на Vickers Hardness Number (VHN) със стандартните отклонения както следва: при интактна емайлова повърхност, след деминерализация и след реминерализация.*

Резултати*: За интактните повърхности средната стойност на VHN е 330.64 ± 12.3, след деминерализацията - 125.64± 2.41, след реминерализацията -231.62 ± 8.35.*

Заключение*: В ограниченията, които съществуват в in vitro проучването, стойностите за емайловата микротвърдост се повишават след апликацията на полимер-контролираната реминерализационна система PDMAEMA/калциеви фосфати върху изкуствени кариозни лезии. Установява се, че системата има потенциал да стимулира рекристализационни процеси върху емайловата повърхност.*

Ключови думи*: изкуствен емайлов кариес, реминерализация, ПДМАЕМА.*

Introduction

Dental caries is a major public health problem [1]. Enamel caries pathophysiology is not only a cumulative loss of enamel minerals, but also a dynamic process characterized by discontinuous periods of demineralization and remineralization. The balance between de/remineralization in concurrence of favoring pathological factors (cariogenic bacteria, fermentable carbohydrates, salivary dysfunction) and the protective factors (sufficient saliva, antibacterial agents, re-mineralizing ions) determines the lesion progression or reversal of the carious process [2]. Despite the considerable reduction of caries presence in many countries, it still remains a major public health problem affecting people of all ages. A better understanding of regenerative, physiochemical mechanisms of remineralization process should be a relevant scientific pathway to develop new innovative remineralization technologies, beyond fluoride-mediated remineralization.

Fluoride-containing oral care products are effective in remineralizing enamel but they do not have the potential to promote formation of organized apatite crystals [3]. Presently, there is an attempt to shift from reparative to regenerative biomineralization therapies or a so called biomimetic remineralization approach [4].

Hardness testing is an important method in de- and remineralization experiments The hardness of the human tooth has been determined by a variety of methods, including abrasion, scratch and indentation techniques. This methods are easy, quick, and require a very small area of specimen surface for testing. Using Vickers hardnes testing, the specimen surfaces were impressed with a diamond indenter at a certain load for a certain period of time [5].

The aim of the present study was to evaluate the remineralization potential of poly-2-dimethylaminoethyl metacrylate (PDMAEMA) and *in situ* formed calcium phosphates suspension.

Materials and methods

Sample collection

Thirty non-carious erupted third molars were extracted for orthodontic purposes. The exclusion criteria are carious, fractured, restored, hypomineralized, fluorotic lesions and any other visible defects. The contaminants and surface debris were removed by manually cleaning the tooth using a periodontal curette, rinsed with distilled water and then stored in 0.1% thymol solution during the sample preparation.

Sample preparation

The radicular part of the teeth was removed. Then each crown was cut longitudinally, to separate the buccal and lingual halves, using a diamond blade saw while under water cooling. The buccal half was covered with two layers of acid-resistant varnish (*Clearance, France*) excluding the middle region grounded to provide a flat surface approximately, window of 3x3 mm^2 using SiC paper with grit sizes 320, 600 and 1200 (*Shofu, Super-Snap Rainbow Technique Kit, Japan*) and polished using 1 μm diamond suspension. Then the buccal part was cut into two halves – right and left 1/ one for demineralization observation with SEM, 2/and the other – for SEM evaluation of remineralization. Each specimen was then embedded in a self-cure acrylic resin block with the buccal surface leveled on top and lying flat and parallel to the horizontal plane.

Demineralization of the samples

After obtaining baseline data by testing microhardness of the initially prepared enamel sample windows, samples were demineralized using demineralization solution of $CaCl_2$ (2.2 mM), NaH_2PO_4 (2.2 mM), lactic acid (0.1 M), fluoride (0.2 ppm); the demineralizing solution was adjusted to a pH of 4.5. The samples were stored in this solution for 6 days, and the solution was changed every 24 hours.

Remineralization of the samples

The solution of PDMAEMA charged with *in situ* formed calcium phosphates (delivered by the Laboratory for polymers, Faculty of Chemistry and Pharmacy, Sofia Univerisity, Bulgaria) was used as remineralizing system.

After each treatment, the samples were cleaned with distilled water and immersed in artificial saliva which was composed of KH_2PO_4 - 0.33 g, Na_2HPO_4 - 0.34 g, KCl – 1.27 g, NaSCN – 0.16 g, NaCl- 0.58 g, $CaCl_2$ – 0.17 g, NH_4Cl – 0.16 g, urea – 0,2 g, glucose – 0,03 g, mucin – 2,7 g in 1000 ml distilled water, pH-6.5. The samples were left immersed in the artificial saliva at room temperature, and the solution was replenished every 24 h for 7 days.

Hardness testing

Vickers hardness measurements were made with a micro-hardness tester Leica VMHT apparatus, Leica Mikrosysteme GmbH, Wien, Austria

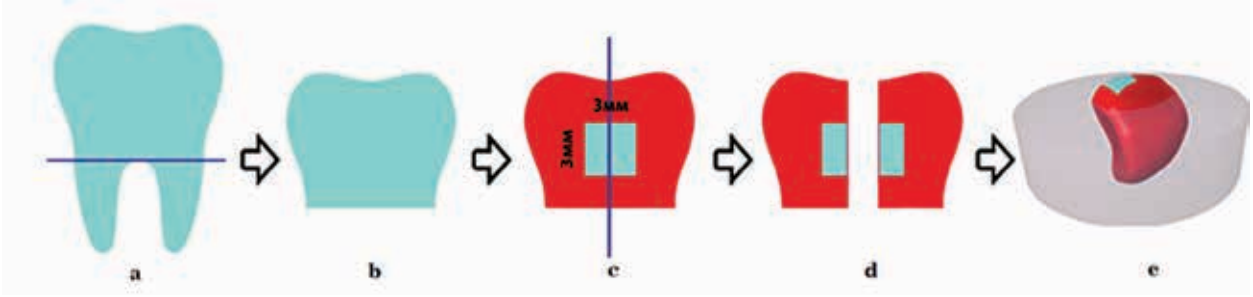


***Fig**.1. Sample preparation technique:*

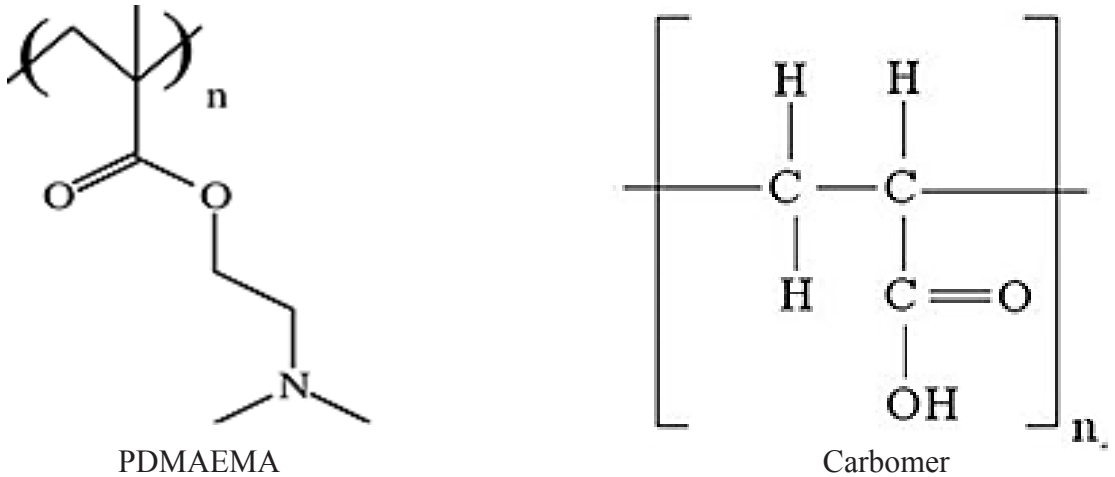
a). The radicular part of the tooth is removed, and the crown is separated in two halves and the vestibular half is preserved;

b). The vestibular half of the crown is polished

c). Two coats of acid-resistant varnish are applied, except for a square window with dimensions 3mm / 3mm;

d). Longitudinally separated into two halves /left, right/ vestibular part, the cut surface is also varnished;

e). Each specimen is embedded in a self-cure acrylic resin block.



PDMAEMA Carbomer

Fig. *2. Chemical formulas of the compounds in the suspension*

with a square based diamond indenter with 136° angle. This tester has a light microscope of high resolution and contrast with magnification of 400 ×. A load of 300 mg was applied for 15 seconds at 5 different points (each 120 μm apart) and the mean was measured. The rhomboid indent was measured for length and depth digitally and the hardness value was calculated in Vickers Hardness Number (VHN). After load removal, diagonals of the indentation were measured with an optical microscope. The hardness number was defined by the ratio between the indentation load and the area of the residual impression, which depended on the indenter shape. Then the hardness of the materials was calculated using this equation:

HV = 1854 (F/d2) for Vickers microhardness.

Table *1. Values with standard deviation of the enamel's microhardness at baseline, after demineralization and remineralization.*

	N of Samples	Mean VHN	Standard Deviation	Standard Error Mean
Baseline	30	330.64	12.32	1.03
After Demineralization	30	125.64	2.41	0.23
After Remineralization with PDMAEMA and calcium phosphates	30	231.62	8.35	0.86

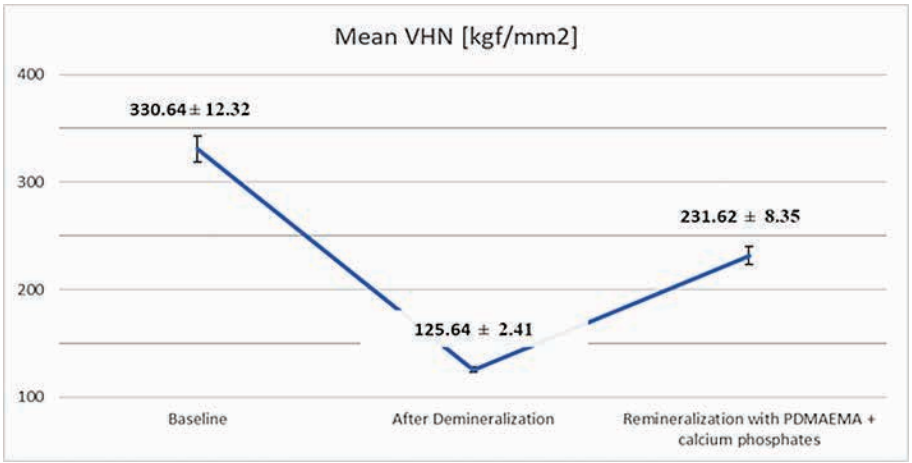


Fig*. 3. Graphics with the microhardness values and standart deviations at baseline, after demineralization and remineralization.*

Results

The microhardness of the enamel in VHN, measured at baseline, before the demineralization, was found to be 330.64 ± 12.32 standard deviation (SD). The hardness value fell considerably after demineralization (125.64± 2.41 SD). The mean hardness value after remineralization with PDMAEMA for 7 days, 6 hours per day increased to 231.62 ± 8.35 SD.

In order to test the hypothesis for the equality of the arithmetic mean between the values of the studied parameters at Baseline, After Demineralization and After Remineralization with PDMAEMA and calcium phosphates a Student's t-test for paired-samples was performed.

The correlation dependence was measured for the different experimental groups and the analysis shows a moderate proportional relationship between the measured parameter at Remineralization and Demineralization / R = 0.3132; 0.3 <R <0.5.

The correlation analysis clearly recognizes a statistically significant relationship between the values measured during demineralization and the values measured after remineralization with PDMAEMA and calcium phosphates by measuring a moderate proportional relationship.

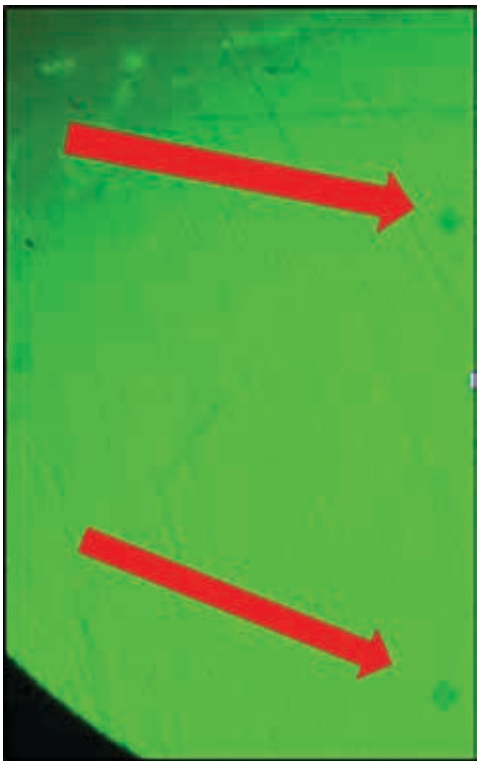


Fig.4. *Light microscope image of well-shaped indentations in enamel before demineralization. It presents good shapes to obtain the VHN values of human tooth because both diagonals have the same length and they are straightly reading*

Table 2. *Student's t-test for paired samples*

		Mean	N	Std. Deviation	
Pair 1	Baseline	330,64	30	12,32	1,03
	After Demineralization	125,64	30	2,41	0,23
Pair 2	Baseline	330,64	30	12,32	1,03
	After Remineralization	231,62	30	8,35	0,86
Pair 3	After Demineralization	125,64	30	2,41	0,23
	After Remineralization	231,62	30	8,35	0,86

Table 3*. Student's t-test for paired-samples*

		N	df	T	Correlation
Pair 1	Baseline & After Demineralization	30	29	-8,3707	-0,0585
Pair 2	Baseline & After Remineralization	30	29	-2,3032	-0,3537
Pair 3	After Demineralization & After Remineralization	30	29	8,2931	0,3132

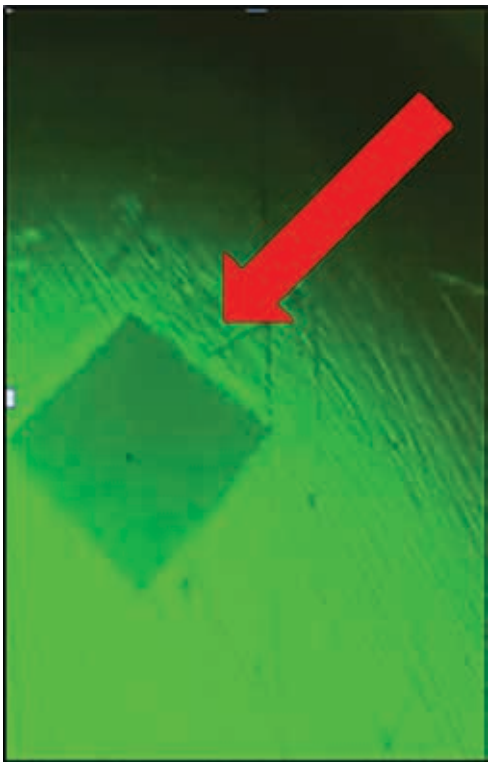


***Fig**.5. Light microscope image of an indentation after demineralization of the enamel with lactic acid solution.The magnification is the same as in the previous figure, the size of the indentation is bigger.*

Discussion

Since the surface layer of the enamel has a crucial role in the caries process, evaluation of changes in this area has great importance. The calculation of surface's micro hardness is a suitable method, which was carried out using Vickers Hardness Measuring method.

Microhardness test was selected in this study as an effective method to evaluate and compare the demineralization and remineralization changes, but also as a simple and inexpensive one [6]. The square shape of indent obtained in Vickers hardness testing is easy and accurate to measure. Even the minute changes in the square shape indent obtained after the test can be easily detected [7].

Selection of testing conditions depended on the researcher's decision, because there is no standard condition for enamel microhardness testing. Results at different indentation loads and times were reported by a lot of microhardness studies. A high load is chosen for the reason that it produces a large impression, and it is thus easy to measure the indentation diagonal [8,9]. The parameters for the present study were set to time 15 seconds and load 300 g.

The development of novel enamel remineralization systems has significantly progressed in recent years with many of them already in clinical use, while others are in various stages of development. Mature enamel is acellular structure and does not resorb or remodel itself, therefore, enamel regeneration is particularly challenging. Advances in tissue engineering methods have yielded biomimetic methods that have demonstrated a strong potential for regenerating the hierarchical enamel microstructure [10].

Amongst the biomimetic agents, polymer-controlled systems play an important role. Some polymers can act as "artificial proteins" so they can mimic the functions of organic matrices in modulating the biomineralization of tooth enamel [11].

Poly-2-dimethylaminoethyl metacrylate (PDMAEMA) is a mucoadhesive polymer, that is cationic if dissolved into acidified media or if quaternised by using an alkylating agent [12, 13]. PDMAEMA has numerous potential uses, which include using it as a non-viral gene delivery vector [14, 15] in water purification [16, 17] and in drug delivery [18]. PDMAEMA has an efficient antimicrobial effect. The mode of action of cationic biocides has been suggested to progress as follows: (1) adsorption onto the bacterial cell surface, (2) diffusion through the cell wall, (3) binding to the cytoplasmic membrane, (4) disruption of the cytoplasmic mem-

brane, (5) release of cell cytoplasmic constituents and (6) cell death. PDMAEMA may work in a similar manner by adsorbing to the cell surface through electrostatic interactions and disrupting the cytoplasmic membrane through hydrophobic interactions.

The combination of PDMAEMA and calcium phosphates in enamel's remineralization is being investigated for the first time, so there is no information available in the literature for the remineralization effect, estimated with microhardness testing. However, studies investigating the effect of other non-fluoride remineralization systems on microhardness values are present. They found that agents such as casein phosphopeptide – amorphous calcium phosphate (CPP-ACP), functionalized tricalcium phosphate (fTCP), nanohydroxylapatite (nHAp) demontstrate an increase in enamel microhardness [20, 21]. Within the limitations of this *in vitro* study it was found that the surface enamel microhardness decreased to 125.64± 2.41 SD after demineralization with lactic acid, and after treating with PDMAEMA and calcium phospahtes, microhardness increased significantly (231.62 ± 8.35) compared to the baseline microhardness values.

In this study, the average VHN value of normal enamel was found to be 330.6 ± 12.32 SD, which was in agreement with earlier published data [22, 23]. *Cuy et al*. found that enamel hardness varies depending on the degree of mineralization of enamel, local variations from enamel rods and tufts and increased porosity near the dentino-enamel junction [24]. The present study was performed on polished enamel surface, which was similar to the studies done by *Wongkhantee et al and Sukasame et al.*

The purpose for investigating of PDMAEMA and calcium phosphate suspension is the possibility to mimic the function of organic matrices in modulating the biomineralization of tooth enamel. Also it can serve as a "reservoir" for calcium and phosphates ions and in this way supply the demineralized enamel with adequate amount of ions, necessary for its biomimetic mineralization.

Conclusion

Within the limitations of this *in vitro* study, treating the enamel with PDMAEMA and calcium phosphates, an increase in VHN values was observed. It is supposed that the newly synthesized product used for artificial carious lesion treating, may induce recrystallization processes on the enamel surface. These results indicate that further studies are needed to confirm the remineralizing effect of the system.

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