

Activity report for the project:
A temporary occlusion system designed on biomimetic principles for dental applications

PN-II-RU-PD-2012-3-0073

Implementation period: May 2013 – October 2015

The main objective of the project was to obtain a temporary occlusion system for dental applications based on two components: a *soft component*, designed to ensure the protection and remineralization of the dentin or even more dentin-pulp complex regeneration, and a *hard component*, which can act as a sealant film, having appropriate mechanical and physicochemical properties to interface the soft component with the mouth environment. The soft component can be regarded as a direct capping material, while the hard component can be compared with a sealant dental product, usually used in the dental practice.

The project *objectives* were the following:

- Obtaining of a temporary occlusion system, based on *soft* and *hard* components
- Study of the interactions at the interface of injectable substitute / photopolymerizable resin

The *soft component* of the system was produced by direct nucleation of HAp precursors onto premineralized atelocollagen microfibrils, with or without modified polysaccharides (such as curdlan phosphate), in order to obtain an injectable composite with proper features, that can be easily manipulated to fill the pulp chamber and dental cavity. The *hard component* of the system implied the obtaining of maleic copolymers functionalized with photopolymerizable groups, capable to ensure the sealer of the injectable composite and, in the same time, to resist at the masticatory forces. This system must resist few weeks until the clinician will decide to remove partially the occlusive system and to finish the tooth restoration.

The objectives were fulfilled through the following *activities*:

1. *Elaboration of the concepts and strategies for the synthesis protocols* for both components was based onto the scientific information and discussions with the specialists, and also was based on the mentor experience.

2. The *synthesis of the injectable substitute (soft component)* based on atelocollagen and hydroxyapatite was carried out in several steps as follows:

2.1. *Synthesis of the hydroxyapatite – atelocollagen composite*:

- premineralization of the atelocollagen using calcium chloride and small amounts of diammonium hydrogen phosphate;
- synthesis of nanoparticulate hydroxyapatite in the aqueous suspension of premineralized atelocollagen through a sol-gel method;
- alternative maturation, sonication and purification steps of the resulted hydroxyapatite-atelocollagen (HAP-AC) composite;
- final maturation step to ensure the transformation of the instable calcium orthophosphates into thermodynamically stable hydroxyapatite;

2.2. *Synthesis of phosphorylated curdlan*:

- chemical reaction between curdlan and phosphoric acid using urea as solvent;
- solvation of the phosphorylated curdlan (PCurd) in a 1M NaOH solution;
- precipitation of the PCurd in methanol;
- normal drying and purification of the PCurd;
- lyophilisation of the PCurd;

3. Characterization of the soft component (injectable substitute)

3.1. Characterization of the hydroxyapatite – atellocollagen composite:

The HAP-AC composite was characterized by FT-IR spectroscopy and X-ray diffraction (Fig. 1), and the results showed that a composite based on hydroxyapatite and collagen was obtained.

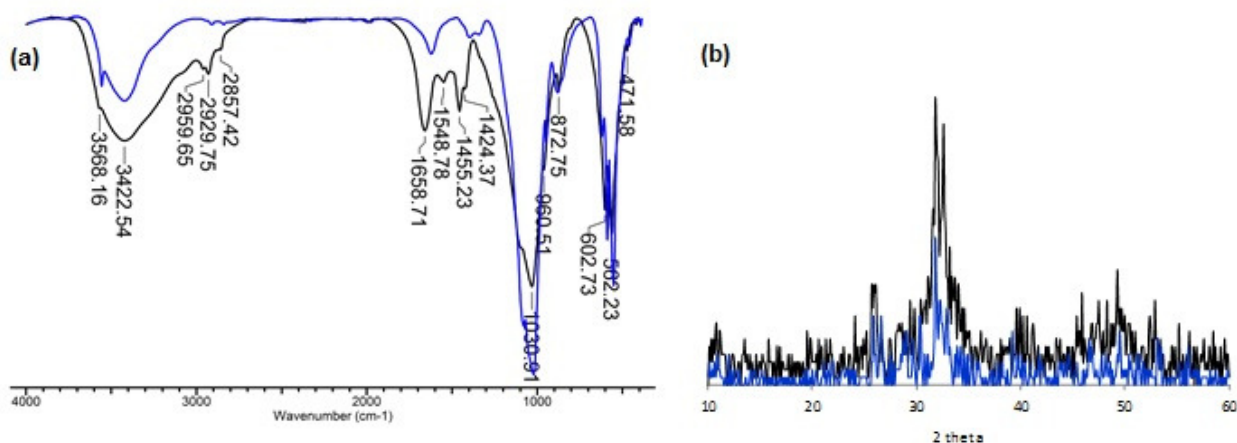


Fig. 1. FT-IR spectra (a) and XRD diffractogram (b) for the HAP-AC composite (black) and commercial hydroxyapatite (blue)

From the FT-IR spectra (Fig. 1a), the characteristic absorption band for amide groups of AC at ~ 1658 cm^{-1} (amide I) and ~ 1548 cm^{-1} (amide II), and the absorption band corresponding to PO_4^{3-} group of HAP at ~ 1030 and ~ 960 cm^{-1} , ~ 602 , 562 and 471 cm^{-1} , and also for HO^- group of HAP at ~ 3568 cm^{-1} and less evident at ~ 630 cm^{-1} , confirmed the structure of the HAP-AC composite. From XRD diffractogram (Fig. 1b) the specific diffraction angle at 26° , 32° , 40° si 50° indicate the obtaining of hydroxyapatite with low cristallinity.

3.2. Characterization of the phosphorylated curdlan:

The chemical structure of the PCurd was confirmed from FT-IR spectrum (Fig. 2a) where the absorption bands of phosphate group are observed at 2418 cm^{-1} for P–H, 1216 cm^{-1} for P=O, 1054 cm^{-1} for P–OH and at 828 cm^{-1} for P–O–C. The peak of about 2936 cm^{-1} is attributed for CH_2 group, while those at around 980 cm^{-1} is assigned to C–O–C from the glucoside units. Also, the signals from ^1H NMR spectrum, attributed to P–H bond at 6.17 ppm and 7.84 ppm confirmed the obtaining of PCurd (Fig.2b). The signal from 7.65 ppm and 5.99 ppm suggested that the group $-\text{H}_2\text{PO}_4$ can also be bind at C-2 at C-4 from glucoside units. The degree of substitution of PCurd was of about 0.8 determined from potentiometric titration.

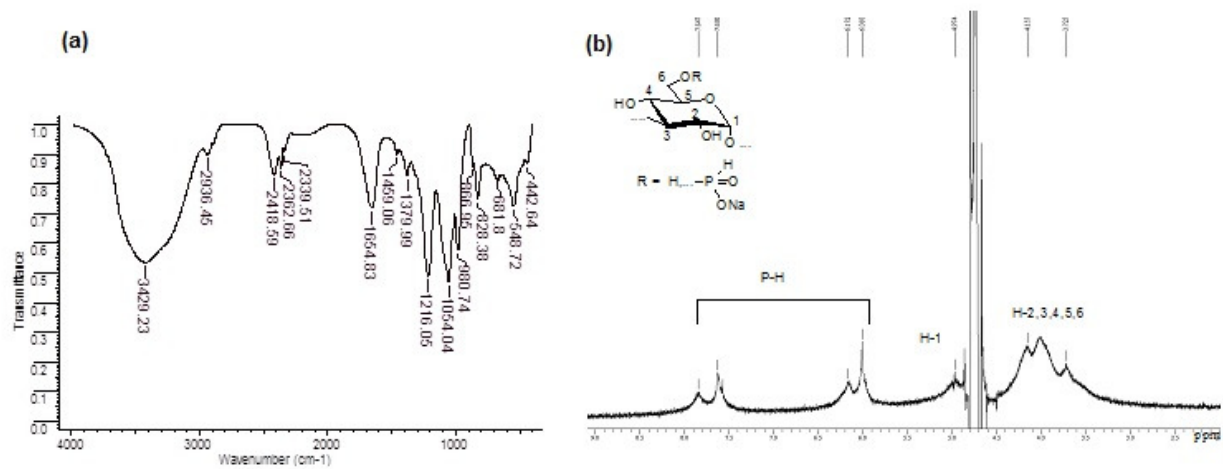


Fig. 2. FT-IR spectrum (a) and ^1H NMR spectrum (b) of PCurd

3.3. Characterization of the injectable substitute based on HAP-AC composite and PCurd:

In order to study the influence of the PCurd added into HAP-AC composite on its properties, few samples were prepared in which the amount of polysaccharide was varied.

FT-IR analysis

The chemical structure of some pastes was confirmed by FT-IR spectra (Fig. 3), and the characteristic peaks for HAP, AC and PCurd (mentioned above) was observed.

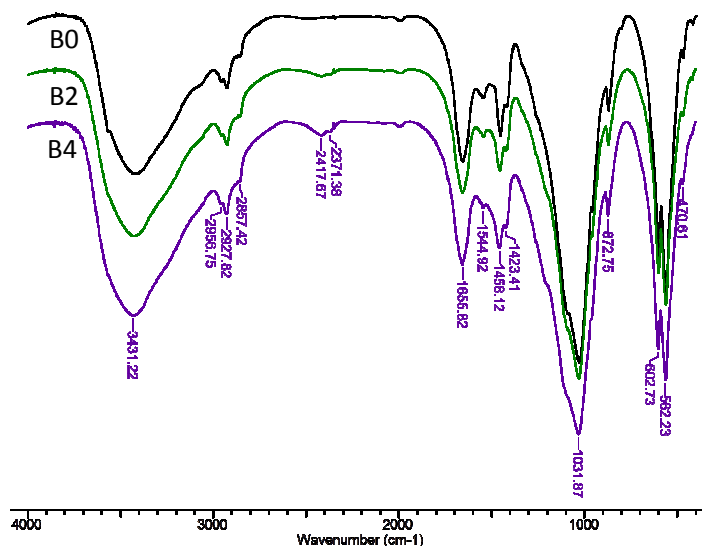


Fig. 3. FT-IR spectra of pastes: B0: HAP-AC, B2: (HAP-AC):PCurd = 1: 0.5, B4: (HAP-AC):PCurd = 1: 1

Rheological test

To characterize the rheological properties of pasted resulted from the mixture of a solid phase – S (resulted from freeze drying) with a liquid phase – L (ultrapure water), in a S/L ratio of 0,33 g/ml, complex viscosity as well as the storage (G') and loss (G'') moduli were measured as a function of angular frequency or temperature.

For C0, C1 and C2 systems, G'' is always lower than G' , the viscoelastic behavior of the investigated systems being controlled by the elasticity of the network with only a minor contribution from the viscous response (Fig. 4).

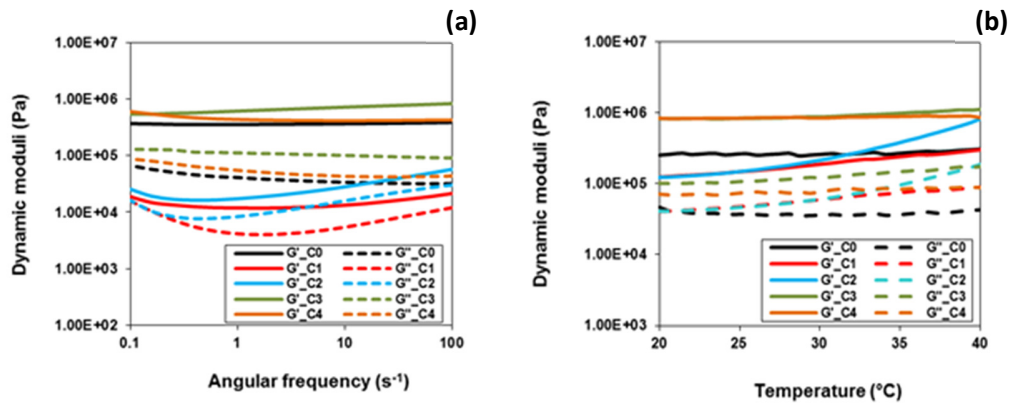


Fig. 4. The variation of the dynamic moduli of the pastes with angular frequency (a) and temperature (b): C0= HAP-AC; C1= (HAP-AC) : PCurd = 1:0,5; C2= (HAP-AC) : PCurd = 1:1; C3= HAP-PCurd; C4=HAP-(AC : PCurd, 1 : 1)

The measurements of the dynamic modulo as a function of increasing temperature has indicated the same behaviour, with $G'' < G'$, suggesting a strong gel structure. With increasing of temperature, the samples which have incorporated PCurd, a slow increase of the moduli can be observed after 30 °C, probably due to the macromolecules rearrangements.

The decrease of complex viscosity with increasing of PCurd (Fig. 5) could be due to the lubricating effect of modified polysaccharide. In this respect the injectability of the paste is increased and the filling of dental cavity can be improved, and even more, the filling of dental tubules could be assured. When temperature increase, for the samples containing PCurd, complex viscosity has a constant trend with the other samples (Fig. 5b) till 27°C, followed by a gradual increased of complex viscosity, probably due to ionic interactions between phosphat groups of PCurd and Ca ions from HAP.

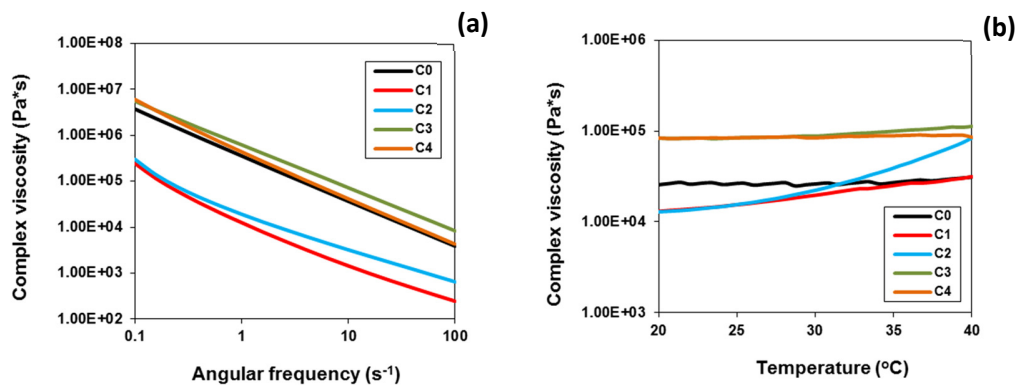


Fig. 5. The variaton of complex viscosity of the pastes with angular frequency (a) and temperature (b): C0= HAP-AC; C1= (HAP-AC) : PCurd = 1:0,5; C2= (HAP-AC) : PCurd = 1:1; C3= HAP-PCurd; C4=HAP-(AC : PCurd, 1 : 1)

SEM investigation

The morphology of the pastes deposited onto dentin slices were investigated by SEM and are displayed in Fig. 6 at two magnifications.

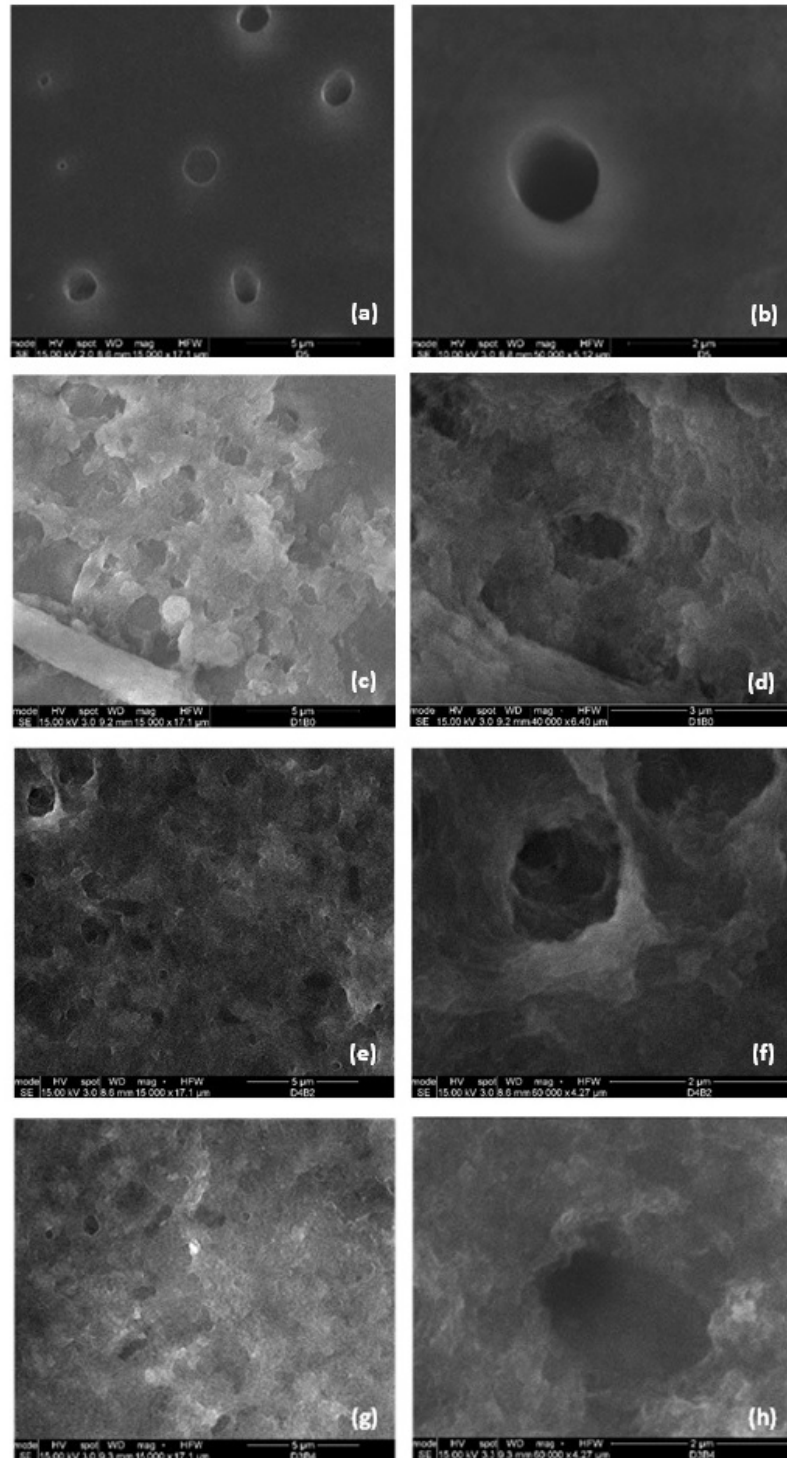


Fig. 6. SEM images of two magnifications of bare dentin (a, b), B0 = HAP-AC (c,d), B2 = (HAP-AC):PCurd = 1: 0.5 (e, f), B4 = (HAP-AC):PCurd = 1: 1 (g, h)

4. The synthesis of a photopolymerizable viscous fluid (hard component) based on modified maleic copolymer bearing methacrylate groups

A maleic anhydride - methyl methacrylate copolymer (COP) was synthesized and functionalized with a urethane derivative bearing methacrylate groups (COP-UMA), and by its mixing with commercial dental monomers, such as Bis-GMA, TEGDMA, and HEMA, two light-curing formulations were prepared and investigated.

The composition of COP was determined by conductometric titration with 0.1 N NaOH aqueous solution in acetone : water mixture (1 : 1 v/v) and the obtained result of 1 : 2 (MA : MMA) was in good agreement with NMR data. The average molecular weight determined in tetrahydrofuran solvent by gel permeation chromatography (GPC) analysis was of about 30000 g/mol. The COP and COP-UMA were characterized by nuclear magnetic resonance (^1H NMR) and Fourier transform-infrared (FT-IR) spectroscopy.

The ^1H NMR spectrum of the COP-UMA (Fig. 7) shows additional peaks at 3.5 ppm (methylene protons linked to the NH), 4.23 ppm (methylene protons of the ester function), 6.12 and 5.6 ppm (unsaturated protons in trans/cis configuration), all confirming the new structure of the modified copolymer. The functionalization degree of the maleic anhydride units of the maleic anhydride – methyl methacrylate copolymer was estimated by comparing the integral of the unsaturated protons with the integral of the methyl protons (3.61 ppm) of the MMA. The COP-UMA contains about 10 % urethane methacrylate groups attached to the polymer backbone which can cross-link with dimethacrylates through radical polymerization.

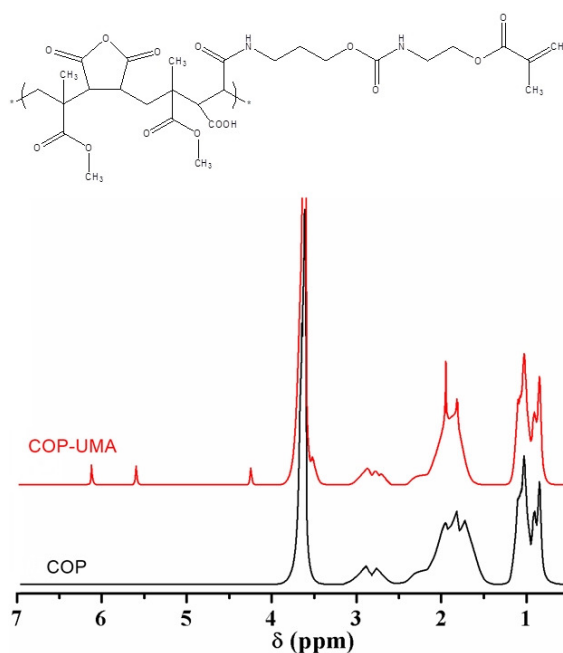


Fig. 7. Structure of poly(maleic anhydride - methyl methacrylate) copolymer (COP) functionalized with methacrylate groups (COP-UMA) and ^1H NMR spectra of COP and COP-UMA in CDCl_3

5. Characterization of the hard component (photopolymerizable products):

COP-UMA was dissolved in methylene chloride and then mixed with dental monomers: Bis-GMA, TEGDMA and HEMA, using 1 wt. % Irgacure 819 as photoinitiator. Two organic compositions incorporating

COP-UMA (Table 1) were prepared in thin film with around 1 mm thickness and then photopolymerized for one minute using a dental-curing unit. A control sample (F-0) was obtained in the same manner.

Table 1: Composition of dental formulations with and without modified copolymer

Samples	COP-UMA wt. %	Bis-GMA wt. %	TEGDMA wt. %	HEMA wt. %
F-0	0	50	30	20
F-1	30	30	20	20
F-2	50	22	14	14

The degree of double bond conversion

To evaluate the photopolymerization behavior of the experimental compositions, the formulations were irradiated, and the progress of the curing reaction was monitored by FT-IR spectroscopy (Fig. 8.). The degree of conversion (DC) of the double bonds attained after 120 s irradiation was of 62.14 % for F-1. A gradual decrease of the double bond absorption was also observed in the case of F-2 formulation, where the DC of the methacrylic function was of only 55.3 %. The DC determined for the control sample had a higher value (66.7%) than that for the formulations which contain the modified copolymer, thus confirming the reactivity of the dental monomers alone.

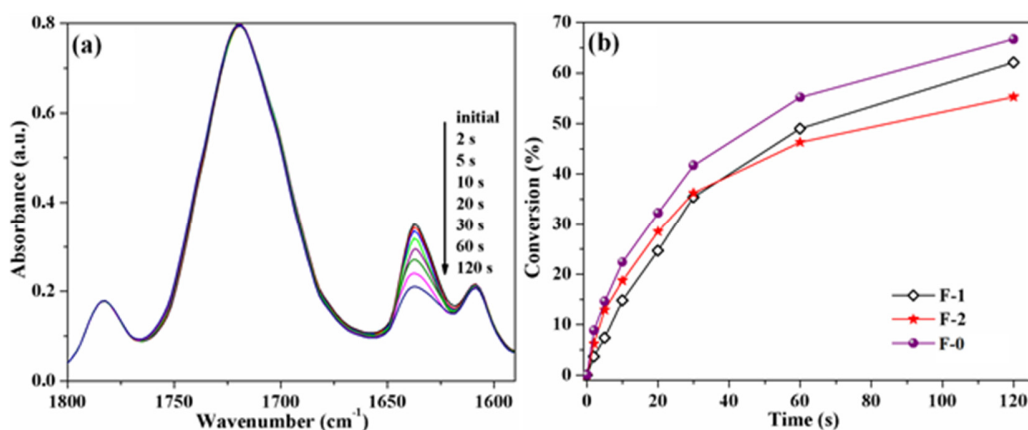


Fig. 8. Changes in the double bond absorption band from the FT-IR spectrum of F-1 formulation during photopolymerization (a) and degree of conversion of the double bonds attained after 120 s of irradiation in all formulations (b)

Surface analysis

The AFM height images revealed the homogenous structure of the films at nanometer scale, and the difference between the morphology of the polymeric films could be due to the COP-UMA amount incorporated in the organic compositions. It is assumed that the topography features and the presence of some holes on the film surface (observed by SEM) in the case of F-1 and F-2 could ensure a proper morphology of the polymer film which can be able to maintain the hydration state of the soft component localized beneath the hard component.

Adhesion force

Figure 9 depicts a typical experimental pull-off force curve with a single step of adhesion, and the histograms corresponding to the adhesion force (F_{adh}) recorded for the polymeric films. For all samples the histograms reveal three populations with median values of the F_{adh} around 0.05 nN, 0.75 nN, and 1.25 nN.

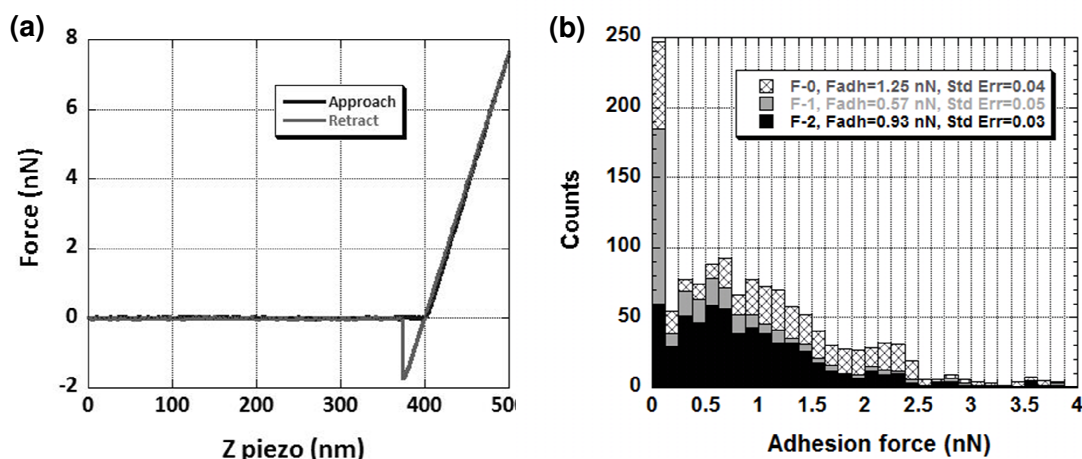


Fig. 9. Typical pull-off force curve (a) and the experimental histograms of the adhesion force measured in water (b) for all specimens

From the experimental histograms the first population with very low adhesion (~ 0.05 nN) indicates that irrelevant interactions between the AFM tip and the hydrophobic surface occur. In the case of the control sample, the mean value of the F_{adh} between the hydrophobic tip and the polymeric films was of about 1.25 nN suggesting a less hydrated surface, but more adhesive comparing with those having COP-UMA.

Diffusion coefficient

The fluorescence recovery after photobleaching (FRAP) method was chosen to measure the molecular diffusion speed. A slight increase of the diffusion coefficient is remarked in the case of the F-2 sample ($5.85 \mu\text{m}^2/\text{s}$) comparatively with F-1 sample ($3.58 \mu\text{m}^2/\text{s}$), and it is supposed that the value is dependent both on the content of the COP-UMA in film composition and on the roughness of the surface. For the control sample the diffusion coefficient had a low value ($0.38 \mu\text{m}^2/\text{s}$) and this could be caused by the lack of the copolymer in the denser cross-linked matrix.

Contact angle, water sorption and water solubility

The values of the water contact angle, water sorption and water solubility measured for the copolymer films and for the control sample are listed in Table 2.

Table 2: Static water contact angle, water sorption and solubility

Sample	Water contact angle ($^\circ$)	Water sorption (%)	Water solubility (%)
F-0	74 (± 2)	4.94 (± 0.12)	1.23 (± 0.03)
F-1	80 (± 4)	12.08 (± 0.04)	0.67 (± 0.02)
F-2	86 (± 5)	15.25 (± 0.05)	9.32 (± 0.06)

The small difference between the values of contact angle could be explained in terms of hydrophobicity of the polymer films created by the heterogeneous composition and the network structure formed by photopolymerization, without neglecting roughness. The control sample F-0 presented a small value of water sorption (4.94%) and water solubility (1.23%) due to the different structure and topography of the polymeric film. Concerning the water solubility, a higher value was observed in the case of the specimen with 50% COP-UMA and this value may be regarded as a drawback in dentistry as the final purpose is to better seal the dental cavity.

Dynamic vapor sorption

In Fig. 10., changes in the weight of moisture is not observed between 0 - 20 % RH in the case of the samples having COP-UMA, indicating that the film surface has already got a small amount of moisture compared with the control sample. This plateau is followed by an exponential rise in the water vapor uptake meaning that the materials become “moderately” hydrophobic (the maximum value for the moisture content is around 1.6 %).

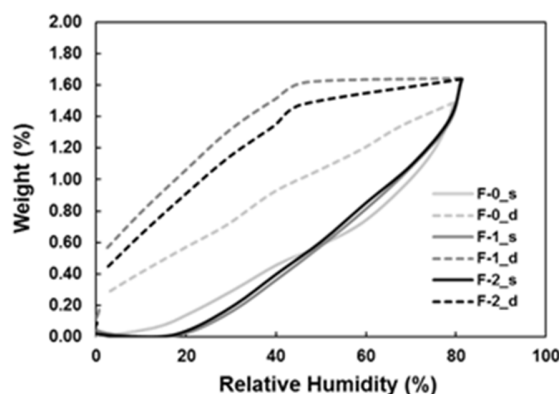


Fig. 10. Vapor sorption – desorption isotherms of all polymeric films

The isotherms present hysteresis (the rate of desorption is lower than the rate of sorption) and this is related to the chemical composition and the surface roughness of the cross-linked films. Taking into account that around 90% of the anhydride cycles from the copolymer structure are not modified, they can lead to carboxylic groups by a ring opening reaction with water molecules, and a small fraction of water could be chemically bonded, so that the samples will not reach the initial weight as it can be seen from the end part of the desorption curves.

The amount of water that is adsorbed on specific sites can be estimated from the value of the monolayer. The BET and GAB models are accepted as the most useful to calculate the value of the monolayer and the surface area. The values of the monolayer and the surface area calculated using these models are presented in Table 3.

Table 3: The main parameters from water sorption - desorption isotherms

Sample	Coefficient of sorption (%)	BET		GAB	
		Monolayer (g/g)	Surface area (m ² /g)	Monolayer (g/g)	Surface area (m ² /g)
F-0	1.49	0.00648	22.727	0.00743	26.076
F-1	1.65	0.00976	34.269	0.01732	60.813
F-2	1.63	0.00859	30.156	0.01426	50.065

Apparent elastic modulus

From the rheometric measurements, the slope coefficient was used to estimate the apparent elastic modulus of the light cured polymeric films, and the calculated values are: 0.96 (\pm 0.005) MPa for F-1 and 0.56 (\pm 0.005) MPa for F-2 sample. In the case of F-0 the value of the modulus is higher, 22.56 (\pm 2) MPa, compared with F-1 and F-2, and this could be due to the higher concentration of the methacrylate groups from Bis-GMA and TEGDMA which are able to provide a rigid cross-linked film after photopolymerization.

Compressive strength, flexural strength and Vickers hardness

To compare the mechanical properties of the COP-UMA based materials with those of the control sample, some representative mechanical tests were performed and the results are listed in Table 4.

Table 4: Mechanical properties of the prepared materials

Sample	CS (MPa)	FS (MPa)	VH
F-0	51.84 (\pm 5.25)	27.09 (\pm 1.58)	29.08 (\pm 1.5)
F-1	45.80 (\pm 8.66)	20.40 (\pm 2.85)	14.72 (\pm 0.67)
F-2	47.77 (\pm 5.82)	7.86 (\pm 2.36)	15.04 (\pm 0.75)

From a clinical practice point of view, compressive (CS) and flexural (FS) strength are important because if the stresses exceed the strength of the material, this will break down. Also, the hardness of the material offers noticeable data about the material ability to resist at scratching or abrasion. Due to the fact that the dental formulations obtained in this experiments are intended as a hard component for a temporary occlusion system, their mechanical properties must be over those of the capping materials (with low values) and above glass ionomer cements or dental composites (with high values).

The measurements of the resistance of each specimen to indentation or penetration by a point under a specified load (Vickers hardness, VH) were also determined. At a load of 4.9 N (500 g) the surface of the specimens F-1 and F-2 showed no significant differences in the value of hardness. For F-0 sample a higher value of VH can be related to the composition effect. In literature it is known that the hardness of the dental materials depends on their composition, degree of conversion, but also on the nature of inorganic filler.

The presence of 30% COP-UMA in the dental formulation appeared to encompass the features necessary for a polymer sealer film which can be used as hard component in a temporary system designed for dental applications.

6. Interaction between pastes and dental tubules

CLSM visualization

To investigate the penetrability of the composite paste inside the dentine tubules through CLSM (Confocal Laser Scanning Microscopy) another series of pastes was prepared. The pastes having low quantity of PCurd were mixed with a fluorescent marker, Rhodamine 6G, and then the pastes were deposited onto dentin slices and conditioned for 24h. In order to compare the results with a commercial product which is currently used as capping material, Dycal paste was prepared in the same conditions. In Fig. 11 can be observed that the C7 paste better advance into dentine tubules, at a depth of penetration of about 10 μm , while for the C6 sample, the depth of penetration are between 5 and 10 μm , and the C5 paste display a lower penetration depth, around 5 μm , due to its higher viscosity. On the other hand, due to its rapid hardness, Dycal has displayed no penetration into tubules (Fig. 12).

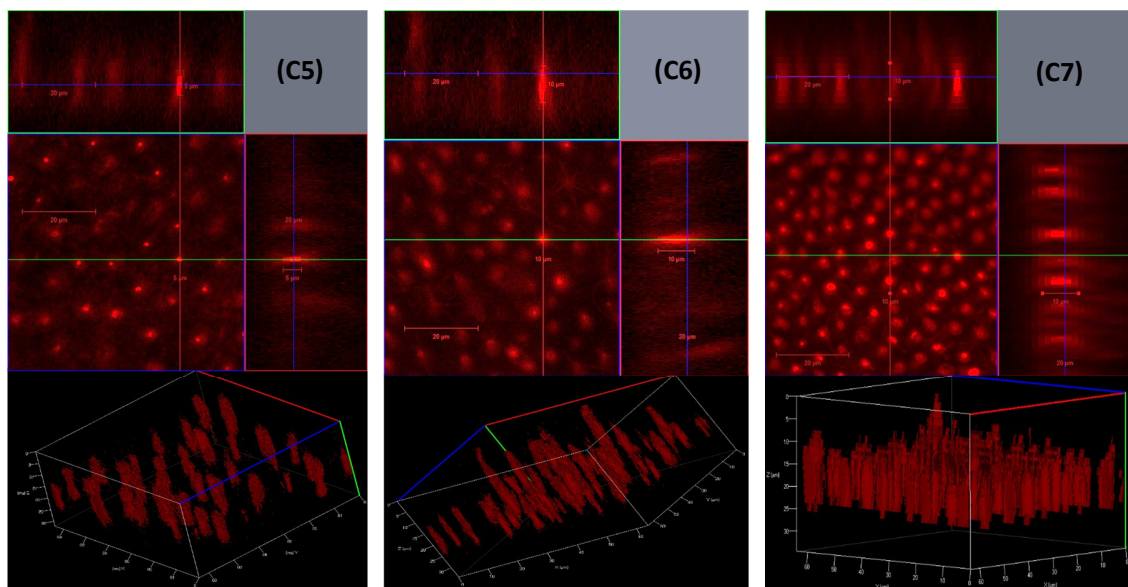


Fig. 11. CLSM images of three pastes: C5 = HAP-AC, C6 = (HAP-AC):PCurd = 1: 0.05; C7 = (HAP-AC):PCurd = 1: 0,1

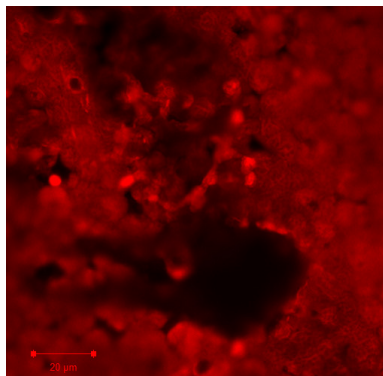


Fig. 12. CLSM image of Dycal

Adhesion investigation

The adhesion investigation at nanometric level consisted in the measure of the adhesion force (Fadh) between tip and surfaces of the samples deposited onto dentin slice using AFM in force spectroscopy mode. The Fadh is considered a pull-off force necessary to separate the tip from the surface and can give informations about the local properties of the material. The experimental histograms are presented in Fig. 13. The Fadh decreased with amount of PCurd added into HAP-AC composite suggesting a better adhesion between the paste and substrat in the case of paste with more PCurd.

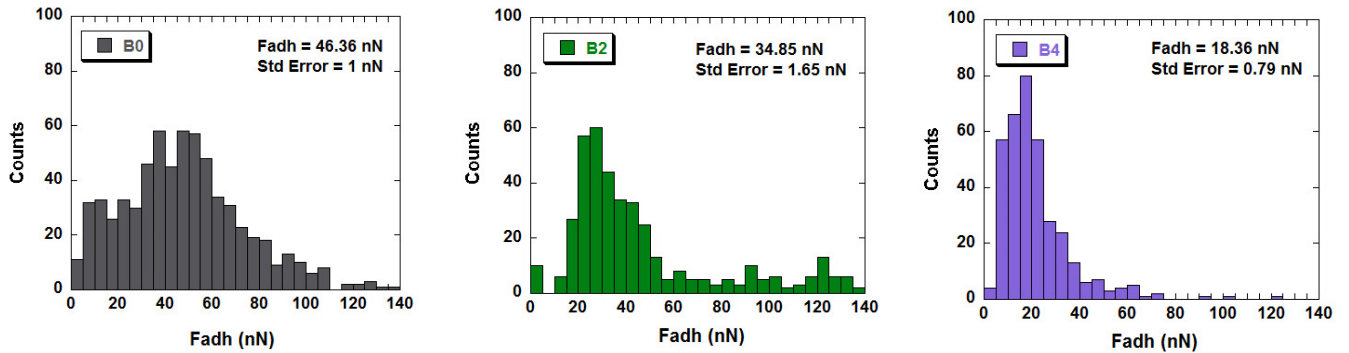


Fig. 13. Experimental histograms of the Fadh measured on all specimens:
B0 = HAP-AC, B2 = (HAP-AC):PCurd = 1: 0.5, B4 = (HAP-AC):PCurd = 1: 1

7. Biocompatibility test

The biocompatibility of the samples was evaluated from the cell culture using the pre-osteoblasts from the MC3T3-E1 cell line. The MTT test was performed with the extract from the C5, C7 samples and a commercial product, Dycal, using the indirect contact method. The cultures were tested at the incubation time at 24, 48 and 72 h. In Fig. 14 the results of the viability test are presented as a function of time. It can be observed that the cell viability for the tested samples is comparable with that of control, having the values higher than 90%. The extract from the Dycal sample become cytotoxic from a concentration of 2 mg/ml, the cytotoxicity increased with the increasing of time incubation.

The cytocompatibility of the pastes were evaluated through optic microscopy when the cells were in direct contact with samples. The Fig. 15 shows that the cells populate the material forming a dense agglomerations on the surface, but also there are at the edge of the paste.

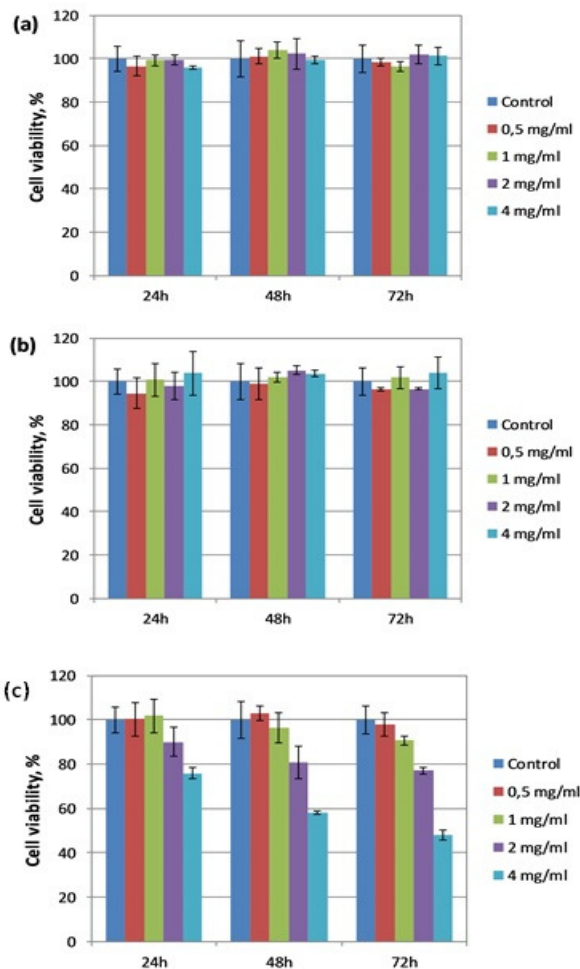


Fig. 14. The results of MTT test for the samples: C5 (a), C7 (b) and Dycal (c)

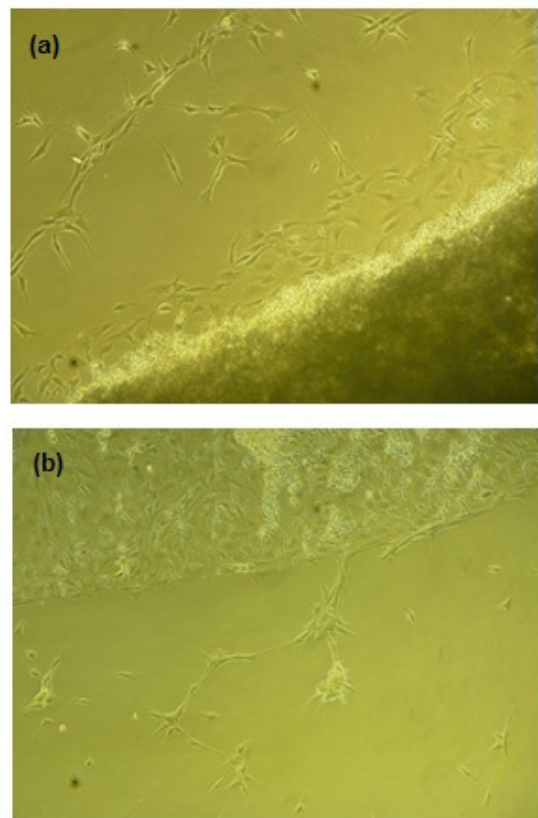


Fig. 15. Optical microscopy images of the cells proliferation and differentiation on the surface of sample C7

In conclusion, in this project, the experiments were focused on the: designing on biomimetic principles, synthesis and investigation of a *temporary occlusion system* based on two components: soft and hard. The *soft component* based on collagen, hydroxyapatite and a modified polysaccharide, was conceived to have the composition somewhat close to the dentin, to be easily for manipulation in order to fill the pulp chamber and dental cavity as to ensure the dentin remineralization or more complex dentin-pulp regeneration. The *hard component*, based on maleic copolymer modified with photopolymerizable groups, was designed to be easily applied in a thin film, to set rapidly, to cover the cervical margin of the dental cavity and the soft component, to be insoluble in oral or dental fluids, and to resist several weeks to masticatory forces. It is expected that the film will ensure protection and mechanical strength for a short period of time, until the clinician decides to remove the cover film and a portion of the soft component of the temporary system, and to finalize the restorative procedure of the tooth.

Dissemination of the results:

Articles:

1. *A novel light-cured dental material based on maleic copolymer functionalized with urethane derivative for dental applications*, authors: Irina M. Pelin, Tinca Buruiana, Violeta Melinte, Sylvie Descartes, Ana-Maria Trunfio-Sfarghiu, Pierre Farge, *Int. J. Polym. Mater. Polym. Biomater.* 64 (2015) 551 – 562.
2. *Cationic curdlan. Synthesis, characterization and application of quaternary ammonium salts of curdlan*, authors: Dana M. Suflet, Irina Popescu, Irina M. Pelin, Alina Nicolescu, Gabriela Hitruc, *Carbohydr. Polym.* 123 (2015) 396 – 405.
3. The influence of phosphorylated curdlan on the calcium orthophosphates formation at low temperature, Magda Aflori, Irina M. Pelin, Dana M. Suflet, for submission at *Powder Diffr. J.*
4. The effect of the phosphorylated polysaccharide on a composite paste as novel dental capping material, Irina M. Pelin, Ana-Maria Trunfio-Sfarghiu, Stelian S. Maier, Maria Butnaru, Raluca N. Darie, Dana M. Suflet, Tinca Buruiană, Pierre Farge, for submission at *Mat. Sci. Eng. C.*

Oral presentation:

1. *L'investigation des proprietes physico-chimiques des films a base de copolymere d'anhydride maleique modifie pour des applications dentaires*, authors: Irina M. Pelin, Tinca Buruiana, Violeta Melinte, Ana-Maria Trunfio-Sfarghiu, Pierre Farge, participation at XI^{eme} Colloque Franco-Roumain sur les Polymères, Pitești, Roumanie, 27–29 aout 2014.

Posters:

1. *Maleic copolymers modified with photopolymerizable groups as new hard component for dental applications*, authors: Irina Mihaela Pelin, Tinca Buruiana, Violeta Melinte, Emil C. Buruiana, Irina Popescu, Dana M. Suflet, participation at 12th International Conference Polymers for Advanced Technologies, Berlin, Germany, 29 september – 2 october 2013.
2. *The crystallization of calcium orthophosphates in the presence of maleic acid copolymers*, authors: Irina Mihaela Pelin, Tinca Buruiana, Irina Popescu, Dana M. Suflet, participation at 12th International Conference Polymers for Advanced Technologies, Berlin, Germany, 29 september – 2 october 2013.
3. *Quaternary ammonium salts of curdlan: synthesis, characterization and applications*, authors: Dana M. Suflet, Irina Popescu, Irina M. Pelin, participation at 6th International Conference “Biomaterials, Tissue Engineering and Medical Devices”, Constanta, Romania, 17-20 september 2014.
4. *The effect of the incorporation of phosphorylated curdlan into an injectable composite for dental applications*, authors: Irina M. Pelin, Ana-Maria Trunfio-Sfarghiu, Stelian S. Maier, Dana M. Suflet, Tinca Buruiana, Pierre Farge, participation at 6th International Conference “Biomaterials, Tissue Engineering and Medical Devices”, Constanta, Romania, 17-20 september 2014.
5. *The influence of a modified polysaccharide on the formation of hydroxyapatite-based composite*, authors: Irina M. Pelin, Vasilica Maier, Raluca N. Darie, Dana M. Suflet, participation at 8th Edition of the Symposium: New trends and strategies in the chemistry of advanced materials with relevance in biological systems, technique and environmental protection, Timișoara, Romania, 4 – 5 june 2015.