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Effect of curing regime and maturation time on photopolymerisation and *in vitro* behavior of a polymeric light-cured calcium phosphate cement

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ABSTRACT

In this research, the release and degradation behavior of hydroxyethyl methacrylate (HEMA) and apatite formation of a polymeric light-cured calcium phosphate cement (PLC-CPC, we named Calcium Phosphate-ionomer) have been studied. Generally, this new kind of composites consists of solid and liquid phase; here, the solid phase was tetracalcuim phosphate (TTCP) and the liquid phase was the resin of commercial resin modified glass-ionomer cement (RMGIC) named Fuji II LC (GC Corporation). To decrease HEMA release, resin-modified glass-ionomers manufactures' strongly recommend using light-cured cements for at least the manufacturers' recommended time (MRT) at specific thickness. Three types of samples either have been exposed to radiation for the minimum manufacturers' recommended time (20 s) and named MRT, or were over-cured named 2MRT (40 s), or dark-cured named 0MRT. Specimens were kept in the mould for various maturation times (10 min, 60 min or 24 h) at 37°C until set or prior to immersion in deionized water at 37°C for 4 h.

KEYWORDS: *Cement, regenerative medicine, dental, orthopedic.*

1. INTRODUCTION

Glass ionomer cements (GICs) have been commonly used in dentistry since Wilson *et al.* introduced to dentistry and White to orthodontics in the 1970s [1, 2]. Using the glass ionomer cements is usual for orthopaedics applications, as they could overcome some of the limitations of 'conventional bone cement', such as shrinkage during polymerization, exothermic setting reaction, weak bond strength and none-degradation. While poly(methyl methacrylate) (PMMA) cements have not adhesive chemical bond to bone and mechanical stability due to mechanical interlocking only [3], glass ionomer cements chemically bond to hydroxyapatite [4, 5], which could allow for better bone ingrowth. Conventional Glass ionomer cements set by an acid-base reaction between a degradable glass and a polymeric acid such as poly (acrylic acid), PAA) [6].

In the late 1980s, The resin-modified glass ionomer cements (RMGICs) was introduced to supply a material with enhanced mechanical properties and a controlled cure competence at the same time as retaining the advantages of the earliest glass-ionomer [7, 8].

The RMGICs possess not only the components of a polyacid, acid-degradable glass and water, but also a water-compatible monomer frequently 2-hydroxyethyl methacrylate (HEMA) jointly with suitable setting mechanisms, the acid–base reaction of the original glass-ionomer cement and a free-radical addition polymerisation of the monomer. Somewhat, RMGICs have an extra polymerisation reaction concerning unsaturated side-chains on the modified polyacid will also happen. The set RMGIC contain of residual glass particles implanted in a mixture of polysalt and polymerised monomer matrix. Advantages of RMGICs consist of a reduced setting time, decreased early moisture sensitivity, extended working time and greater strength properties compared to conventional glass-ionomer cements [9-11]. The superior biocompatibility of the conventional glass-ionomer is well recognized and has been qualified to the minimal setting exotherm, fast acid neutralization and gradual release of useful ions [12]. Little or no organic species are leached out of conventional GIC [13]. In spite of this, the *in vitro* biocompatibility of some RMGICs has been reported insignificant, caused by leachable resin components, such as HEMA, which has frequently been added to this type of cements [9].

Under direct restoration conditions, the vinyl polymerisation reaction in composite resin cements doesn't complete [14]. Instead, in two paste composite systems, only about 50–70% of the methacrylate monomers reacts, whereas for the light-cured materials between 60–75% of the monomers reacts [15]. Under these conditions a significant proportion of the incorporated monomer has not reacted and may be degraded of the set cement. The degree of conversion of monomer to polymer of this type of cement has not been well recognized.

Some researchers examined the release of organic materials from a range of RMGICs and compomer cement. Their *in vitro* study looked at release from a cement sample directly into its storage medium and also release via diffusion through the dentine of a restored tooth. In both experiments all the materials tested were found to release measurable levels of HEMA. No other species were found in the storage solution [16].

This study investigates effect of the curing regimes on the measured HEMA leach from a new resin-modified Calcium

phosphate-ionomer cement before and after soaking in Phosphate Buffer Saline (PBS). After that, degradation behavior of the cements examined by measuring mechanical properties and released ions from cement to PBS. SEM and XRD analysis used for characterizing the cement during the soaking time.

2. EXPERIMENTAL SECTION

2.1. Samples preparation.

Calcium Phosphate ionomer (CP-ionomer) was used in this study, had the composition of tetracalcuim phosphate (TTCP) and Liquid component of a commercial light-cured resin modified glass-ionomer restorative named Fuji II LC (GC Corporation).

TTCP was obtained by solid-state reaction of equimolar amount of DCPA and calcium carbonate ($CaCO_3$, Merck, 2069, Germany) and the mixture was heated in an electric furnace (CARBOLITE BLF, 16/3, England) at 1500 °C for 6 h, then quenched to room temperature [17], crushed in an agate mortar and then grounded in a planetary ball mill. The size range of TTCP as determined by Laser Particle Size Analyser (Fritsch Analysate 22) was 5 μm. Dulbecco's Phosphate Buffer Solution (DPBS) was purchased from Sigma-Aldrich Company (56064C-10L).

All the calcium phosphates used in the experiment were weighed (±0.0001 g) and prepared at best consistency (Powder/Liquid ratio of 2.5 g/g) after that the composite powder was mixed with commercial resin of Fuji II LC to form paste according to the manufacturers' instruction. Briefly, mixture of powder and liquid was mixed with a stainless steel mixing spatula at room temperature of 24°C. Then, the paste was packed into glass-tubing template and cylindrical-shaped specimen formed with the height is twice the diameter (6×12 mm) by hand pressure on a glass surface to remove excess material [18].

After few seconds, specimen were taken out and light-polymerized by irradiating sequentially each face of specimen assembly according to minimum manufacturer's recommended time for 20 s (by code MRT) or alternatively, over-cured (irradiated for 40s, 2MRT) by light source (Farazmehr Isfahan) or dark-cured specimens (0MRT).

2.2. HEMA Release.

After the appropriate light-curing regime, the specimens were allowed to mature in their moulds in an incubator at 37°C. At the end of one of three maturation times (10 min, 60 min or 24 h), the specimens were removed from their moulds and any flash was removed. These maturation times were designed to represent different degrees of protection applied to the materials after placement.

After removal from the moulds, the specimens were weighed prior to immersion in a sample tube containing 20 ml of distilled water. The sealed tubes were placed in an incubator at 37°C and were gently agitated hourly. The HEMA released from the cement when stored in solution was determined by measuring the concentration of HEMA in the storage solution after 4 h of incubation.

Palmer *et al.* investigated the tolerance in HEMA concentration with time of few commercial GICs incubated for over 24 h after various cure methods and maturation times, confirmed that no supplementary HEMA was released from RMGIC experienced after three hours of soaking in pure water. They recommended an incubation time of 4 h as being proper for entire HEMA release from Vitrebond cement [19].

The HEMA concentration in the storage solutions was appointed using High Performance Liquid Chromatography (HPLC, KNAUER, Germany). The mobile phase was 15% methanol and 85% water at a flow rate of 1.7 cm/s. The injection volume was 20 ml, the stationary phase was a C18 column (PerfectSil Target ODS-3 5μm 250×4.6 mm) and the UV detector was set to 214 nm. The chromatograph and detector were calibrated using HEMA solutions of known concentration (1, 10 and 100 ppm). There was a linear dependence between detector response and HEMA concentration. The HPLC instrument was calibrated preceding to utilize.

To determine the quantity of HEMA present in the cement formulations, 4 mg of the liquid component was dissolved in 10 ml water. The concentration of HEMA in this solution was then determined using HPLC. From the data obtained the concentration of HEMA in cement liquid 256±3 ppm and then from the knowledge of the Powder:Liquid ratio (2.5 g/g) used to mix the cement, the quantity of HEMA in 1 g of each of the cements was found 60.910±0.002 [mg of HEMA/gram of cement].

2.3. *In vitro* Study.

According to Dulbecco methods to prepare the Phosphate-Buffered Saline (PBS), in this study Dulbecco's PBS was purchased from Sigma-Aldrich Company (56064C-10L). The *in vitro* degradation studies of specimens were performed by immersing the cylindrical samples in this PBS solution at 37 °C (solid to liquid loading of 1 g specimen per 100 mL water). After soaking for different preset time intervals (1, 7, 14 and 21 days), the samples were taken out from the PBS solution, rinsed gently with de-ionized pure water and dried in air condition for further characterization. It should be noted that during evaluation, the PBS solution was renewed every 24 h.

The compressive strength of samples with 60 min maturation time and soaked in PBS solution for various times was measured according to ASTM standard F451-08 using universal testing machine (Zwick/ Roell-HRC 25/400) with a crosshead speed of 1 $mm.min^{-1}$.

Surface microstructure of that soaked samples were analyzed by using a scanning electron microscope (SEM, Stereoscan S 360 Cambridge) that operated at an accelerating voltage of 20 kV and coupled with energy dispersive X-ray analysis (EDXA, Oxford, US). Due to the poor electrical conductivity of the samples, their surfaces were coated with a thin layer of gold before testing.

Phase composition and structural groups of the cement was characterized before and after soaking using X-ray diffractometry,

XRD (Philips PW3710) with Cu Ka radiation and Fourier transforming infrared (FTIR) spectroscopy, respectively.

For FTIR spectroscopy, two milligrams of the ground specimen was mixed with 800 mg of ground spectroscopic grade KBr and pressed to make a transparent KBr pellets. The Infrared spectra between 400 and 4,000 cm^{-1} was measured at a resolution of 2 cm^{-1} using BRUKER VECTOR 33 device. All specimens were grounded to powder and then analyzed.

3. RESULTS SECTION

3.1. Effect of maturation time/cure regime.

Altering the maturation time for dark-cured specimen seemed to have little effect on the measured HEMA release from specimens. In contrast, the HEMA release from 1MRT and 2MRT sample after 60 min maturation was significantly less than that after 10 min and 24 h maturation time ($p< 0.05$), but in the case of 1MRT and 2MRT, the 24 h matured specimen released the higher levels of HEMA than 10 and 60 min maturation time although the differences between 1MRT and 2MRT were not significant ($p< 0.05$). So, it seems 60 min maturation time is suitable for conventional process prior to immersion in storage solution.

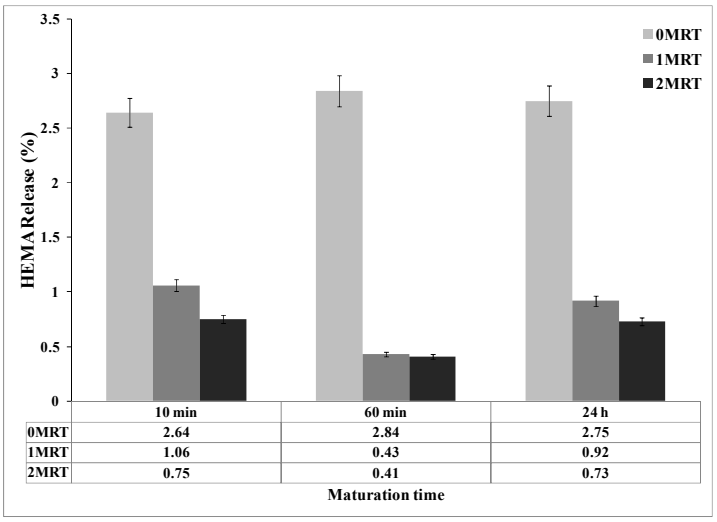


Figure 1. HEMA Release vs. maturation time. Results are expressed as the mean value with the error bar as one standard deviation n=6.

3.2. Effect of curing regime on *in vitro* behavior.

Figure 2 shows FTIR patterns of raw materials and samples after setting and maturation time of 60 min, as seen in section 3.1. The spectrum of cement liquid phase is also shown for comparison. These FTIR patterns help us to understand the mechanism of cement hardening and chemical characteristics of the crystals formed on the surface of sample in PBS. As it shows, important chemical groups with their related absorption wavenumber in these spectra has been noted. From the position and assignment of the peaks, the following features can be pointed out: For resin, the bands appeared at 813 and 1,650 cm^{-1} are respectively assigned to $C=CH_2$ and C=C groups in HEMA monomer, which are vanished in the pattern of cured cement. It confirms curing process of the cement through polymerization reaction. The bands around 1,330–1,450 and 1,550–1,650 cm^{-1} are specified to the symmetric and asymmetric stretches of the carboxyl group of calcium carboxylate salt [20].

Figure 3 shows Diffraction pattern of sample 1MRT and 2MRT and powder phase of the cement is also presented for comparison. The set cement specimen has similar XRD data to the cement powder after setting and before soaking in PBS solution, except that noise-like fluctuations found in this pattern, which are probably due to the amorphous nature of polymerized resin and setting reaction products.

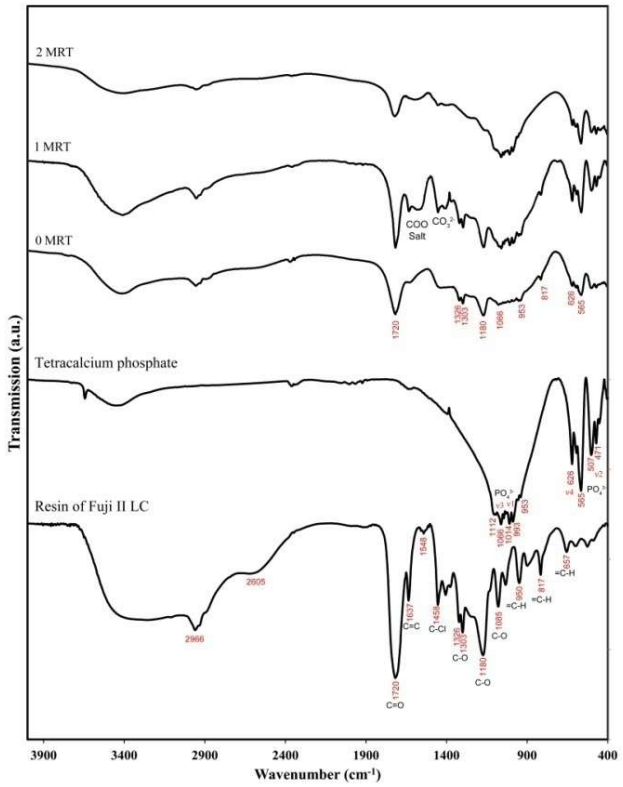


Figure 2. FTIR Patterns of Resin and Tetracalcuim phosphate as the raw materials and samples (0MRT,1MRT, 2MRT) after setting.

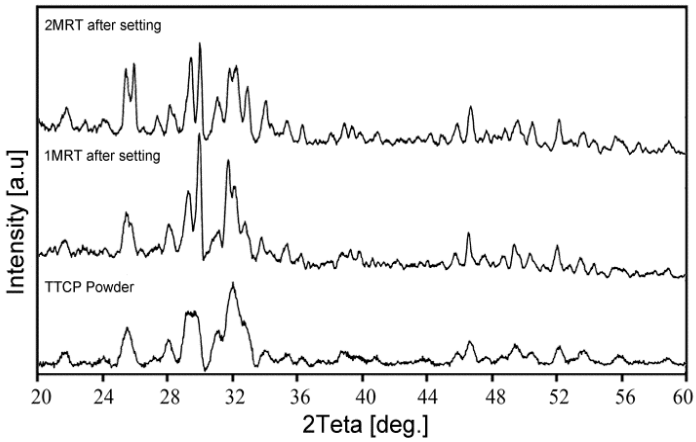


Figure 3. XRD pattern of TTCP powder as the raw material and samples (1MRT, 2MRT) after setting.

3.3. Effect of soaking time.

Figure 4 shows FTIR patterns of samples after 1 and 21 day(s) soaking in PBS solution. The spectrum of cement for the

soaked light-cured specimens reveals the formation of phosphate and carbonate groups that are individually found in apatite lattice. The bands observed at 562 and 603 cm^{-1} and 1,060 relate to stretching mode of PO_4^{3-} in apatite crystal and the band at 865 cm^{-1} is assigned to vibrational mode of CO_3^{2-} substituted for PO_4^{3-} group [21]. The bands observed at 1,416 and 1,457 cm-1 can also confirm the substitution of CO_3^{2-} groups for PO_4^{3-} in apatite lattice [21]. Appearance of the absorption band at around 630 cm^{-1} indicates the formation of hydroxylated apatite phase during the soaking process, even though the resulted phase may not be stoichiometric in chemistry, i.e. $Ca_{10}(PO_4)_6(OH)_2$.

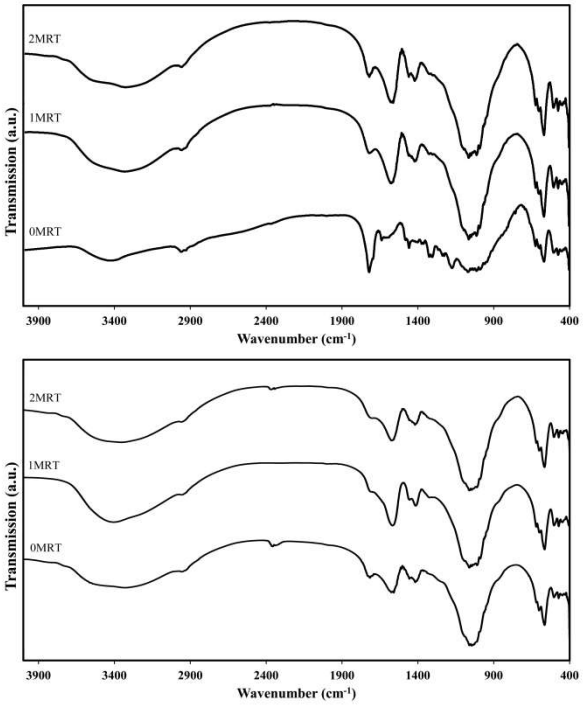


Figure 4. FTIR patterns of samples after 1 and 21 day(s) soaking in PBS.

Another considerable point in FTIR spectrum of the soaked light-cured specimens, compared to unsoaked samples, is reduction of its carbonyl and CH band intensities, which confirms typical degradation of polymeric phase. In other words, omission of these bands indicates that the polymeric phase of the LCCPC is degraded which confirms the results of SEM images. In other words, using the FTIR spectra, it can be stated that the pores observed in the SEM images of 21-soaked samples is obtained due to the leaching out of the polymerized resin and poly salts into the PBS solution during soaking period.

Figure 5 XRD pattern of samples after 1,21 day(s) soaking in PBS solution. Signs of apatite phase are observed in XRD pattern of the samples after immersion in PBS for 21 days; however TTCP phase still exists in cement composition even after 21 days. Regarding to the peak intensity of apatite and TTCP, equal amounts of these phase are suggested in 1MRT specimen and more apatite in 2MRT after soaking in PBS for 21 days.

Figure 6 shows SEM images of the surfaces of different specimens before and after soaking in PBS. In all samples, before soaking, fine particles of reactants (calcium phosphate) have been surrounded by a monolithic polymer phase to yield a compacted microstructure. The micropores observed in the composite microstructures are due to the air bubble trapping during mixing powder and liquid phases. After 21 days of soaking, large pores (5-20 μm) with good interconnectivity are observed in the microstructures of 0MRT and 1MRT samples. The size, number and interconnectivity of the pores increase with decreasing irradiation time of cements (2MRT).

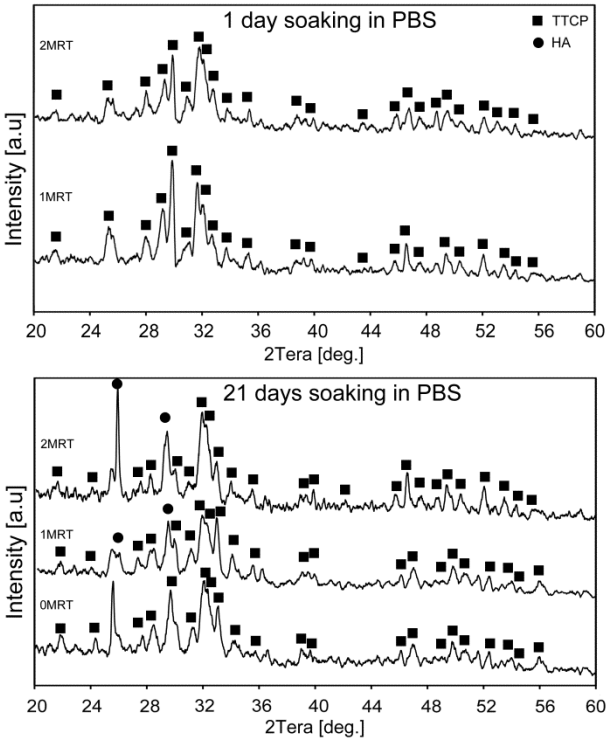


Figure 5. Pattern of samples after 1,21 day(s) soaking in PBS.

SEM pictures with higher magnifications reveal deep important difference in morphologies of different samples after soaking for 21 days. Confluent nanosized spherical crystals with thickness of 10–20 and length of 50–100 nm are observed in the microstructures of 1MRT and 2MRT. These crystals have been produced on samples during soaking them in PBS solution and are quite similar to the morphology of apatite layer formed on bioactive glasses and glass–ceramics [19]. No sign of these crystals is observed in the microstructure of 0MRT specimen.

Generally, the resin modified glass ionomer cements demonstrate an improvement of mechanical strength after soaking in water. Nonetheless, due to the existence of OH groups that is hydrophilic, these GICs absorb water, which operates as a plasticizer agent. therefore, a decline of the compressive and flexural strengths was expected [22].

Therefore, the compressive strengths of specimens before and after soaking in PBS solution show in Figure 7 for various soaking times. The 0MRT specimen reveals an ultimate compressive strength value of about 30 MPa after incubation at 37 °C for 4h (as-cured). The value of compressive strength first increase but then reduces with increasing soaking time.

Besides, all specimens tend to weaken by immersion in PBS solution in a time-dependent approach. Typically, about 54 % decrease in compressive strength of 0MRT specimen is observed during 1 day to 21 days, which reaches to 20.8 MPa after 21 days soaking in PBS. Compressive strength of 38.8 and 40.7 MPa have been observed for 1MRT and 2MRT after 4 h of

incubation and reach to 35.4 and 38.8 MPa after 21 days of immersion, respectively. Figure 8 shows the changes in the concentrations of Ca and P released ion in PBS after soaking.

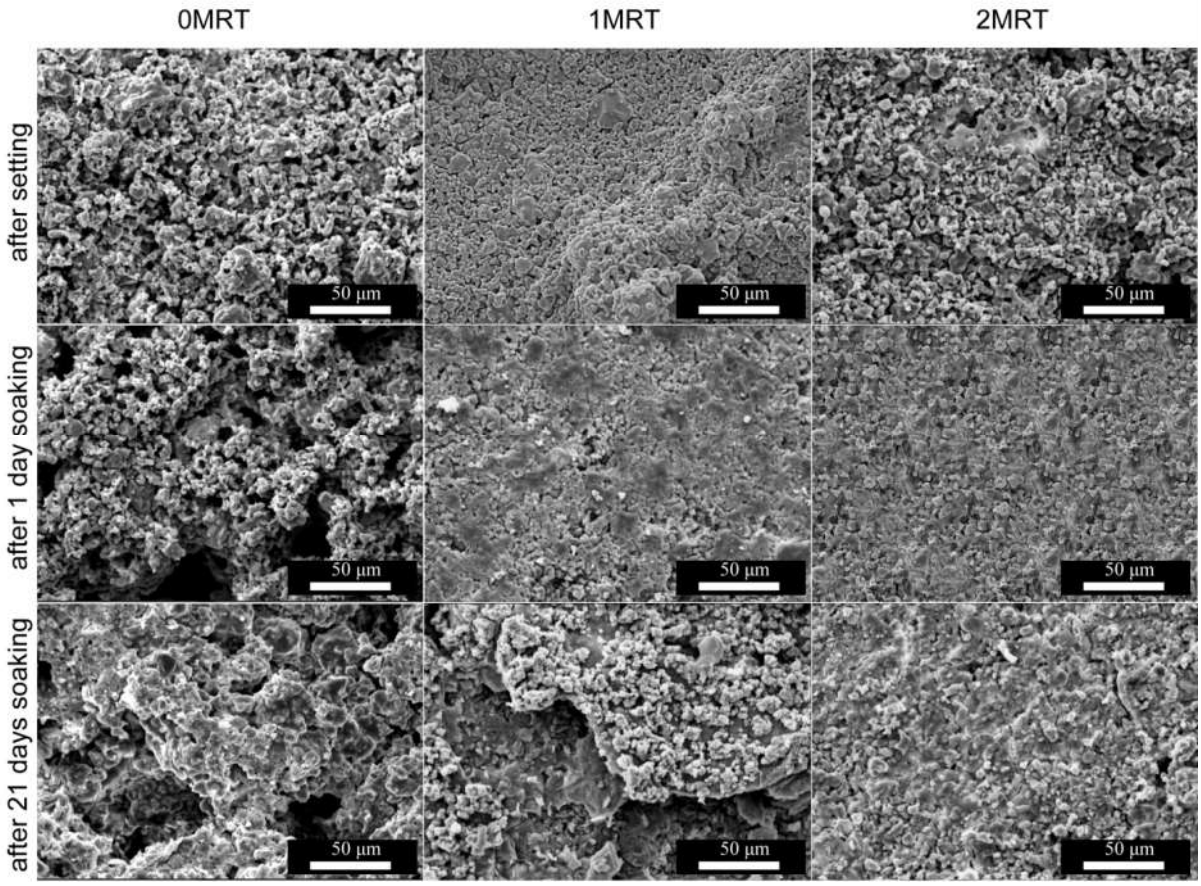
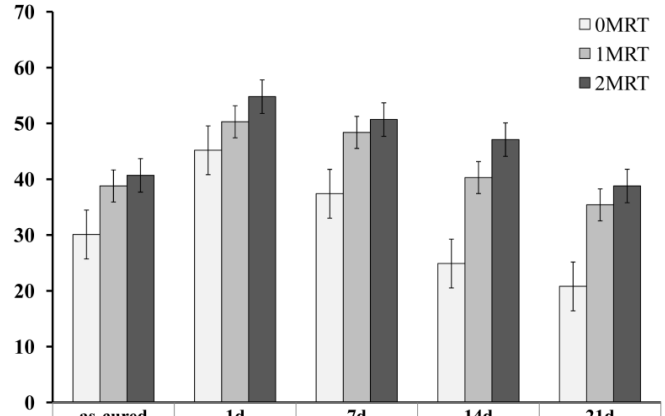


Figure 6. SEM Images of samples after and before soaking in PBS.



	as-cured	1d	7d	14d	21d
0MRT	30.1	45.2	37.4	24.9	20.8
1MRT	38.8	50.3	48.4	40.3	35.4
2MRT	40.7	54.8	50.7	47.1	38.8

Figure 7. Mechanical strength of different samples before and after soaking in PBS.

The concentration change show flocculated behavior related to dissolution-precipitation process of apatite formation [23].

Both the Calcium and the phosphorus concentrations decreased after 540 h (21 days) that can be involved to decreasing rate of apatite formation.

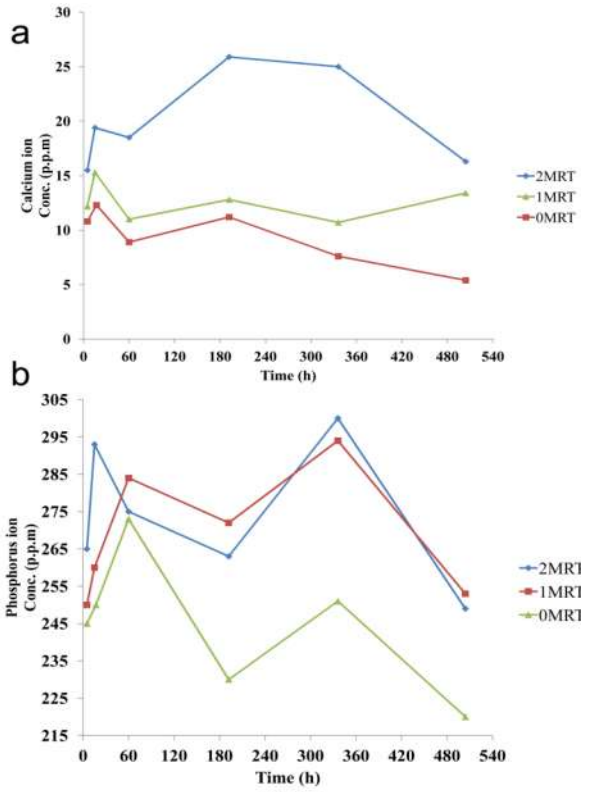


Figure 8. Calcium and Phophorus ion relaese from samples after soaking in PBS.

As other researchers reported [16, 19], this kind of specimens disregarding of cure regime or maturation prior to

immersion in water, released measurable quantities of HEMA into the storage solution. The scatter in the results indicates a significant variation in measured HEMA release from specimens prepared under the same experimental administration. The large variations are not associated with the chromatography of the storage solutions, because repeated injections of the same solution were highly reproducible and losses by evaporation were minimised by the use of vigilantly sealed storage containers. The scatter is more likely to be associated with curing variations within the specimens and variable rates of diffusion of the unpolymerised HEMA from the cement.

The preparation and curing of the specimens under a standardised method, all materials being weighed before mixing and then was covered with a glass slide to reduce oxygen inhibition and the end of the curing-lamp light guide was placed in contact with the slide.

Palmer *et al.* [19] reported no change in the concentration of monomer in solution could be detected after 3 h storage. Although, Kanchanavasita *et al.* [24] reported water sorption on the same materials by specify that specimens of this size do not become saturated with water until 2–3 weeks after immersion in water. It was noted that the water absorption in the first 6 h was high (at least 85% of the maximum value for Fuji Lining LC and Fuji II LC). The values obtained for HEMA release in these researches may therefore be lower than the total potential release of HEMA from these specimens.

As could be expected the liner/base grade GIC contained significantly more HEMA than the restorative cements. This reflects the thinner consistency (lower powder:liquid ratio) required for the liner/base materials. It was 15 min before the dark-cured specimens had set sufficiently to enable them to be removed from the mould. About 50% of the included HEMA was released into the storage solution. Without the presence of dark-cure initiators the cements had set by the acid–base reaction alone. Despite the lack of a polymerisation reaction, the percentage of HEMA released was unexpectedly low. This may be a reflection of the sensitivity of the material to ambient light. Although the specimens were matured in light-free conditions, the cements were mixed in a laboratory with similar ambient lighting conditions to those found in a dental operation. In this circumstances may be adequate brightness to initiate a partial polymerization. however, This is doubtful to consider for such a high percentage of material maintain within the cement.

As known in the cement, the presence of the organic species considerably decreases the rate of the ionic reaction [25]. Further, when methanol was present during an acid–base reaction, the expected ionic structure was completed by the formation of supplementary species, probably including methanol. Similar species containing HEMA could have been formed in this research which would cause the HEMA to be retained, in part, within the cement. This potential for the formation of supplementary species could also be the source of some of the observed variation in HEMA release. Given the low concentrations of HEMA measured, it is unlikely that saturation of the storage solution was inhibiting further diffusion.

Although the cement had set sufficiently to permit removal from the mould after 5 min, the dark-cure polymerization was still ongoing within the cement. This was confirmed by those specimens that were allowed to mature for at least 10 min as they released a lower level of HEMA. As expected from the data on degree of conversion in composite resin cements, the HEMA release from the dark-cured specimens was always higher than that from similar light-cured materials.

Direct comparisons between the dark and light cures are not possible because for example, in Vitremer the dark-cure initiators will always be activated, thus the ‘‘light-cured’’ results are in fact the product of both curing regimes.

These two reactions may be additive or competitive. The higher release from the dark-cured material could be the result of the inclusion of lower levels of dark-cure initiators in comparison to that of light-cure initiators. This would lead to a lower degree of conversion of monomer and hence a higher concentration of HEMA for release. The light-cured materials released very low levels of HEMA, the highest mean value was only 0.4% which is equivalent to the release of about 0.19 mg of HEMA from 1 g of cement.

Dark-cured specimen set in only 5 min. The instructions for use of Fuji II LC make no claim for the presence of a dark-cure reaction, but the dark-cure release level combined with the short setting time show that an effective dark-cure polymerisation reaction is occurred. The lower level of HEMA release from the overcured (2MRT) specimen demonstrated that this specimen profited from overcuring. Also, Kanchanavasita *et al.* [26] investigated temperature rise on curing during polymerisation and also reported that the polymerisation reaction of Fuji II LC would advantage from a longer cure-time than recommended administration by the manufacturer.

The manufacturer’s instruction for Fuji II LC, notify that it should not be used in thickness greater than 2 mm. The level of HEMA release from the dark-cured specimens (0MRT) was significantly higher than that from the specimens light-cured for the manufacturer’s recommended time. The distinctions were doubtful to be the result of the different degrees of conversion for the two different polymerization reactions. As an alternative, they suggest the content of a lower level of dark-cure initiators in the cement combination. This specifies the optimum conversion to be achieved, the specimens must be exposed to radiation for at least the manufacturer’s recommended time.

Varying the time of maturation of the specimen prior to immersion in the storage solution beyond 10 min had little effect on the measured HEMA release from any of the materials. Although the polymerization reactions were still continuing within the cements, the bulk of the polymerisation reaction had occurred. The higher level of HEMA released from Fuji II LC at their shortest possible maturations compared to that released after 10 min shows that there is a significant variation in the degree of monomer conversion between 5 min and 10 min.

The low biocompatibility of the GICs in cell culture is perhaps because of HEMA release. HEMA has been indicated to be cytotoxic in cell culture (for example a 35% solution) and is also recognized to cause postponed hypersensitivity reactions and contact dermatitis [19].

The polymerization reaction of the HEMA in the resin-modified glass-ionomer should therefore be suitable in order to

minimize the release of free monomer of HEMA. It should be noted that increasing the degree of monomer conversion in the polymerization reaction would cause an increase in polymerization shrinkage and polymerization exotherm.

4. CONCLUSIONS

A resin of Fuji II LC and Tetracalcuim phosphate were selected to investigate HEMA release and measurable extent of HEMA were released into their storage solutions. The acid–base reaction in the cement caused the formation of an ionic polysalt crosslinked matrix. Some research shows that some GICs set by the light-activated polymerisation and acid–base reactions alone such as Vitrebond resin modified glass ionomer [19]. These materials have a significant dark-cure polymerisation initiator(s). Also, in that GIC, the HEMA release from the dark-cured specimens was higher than that from the equivalent light-cured materials. For Vitremer this is due to the lower efficiency of the dark-cure reaction, but for Fuji II LC and also Fuji Lining LC the dissimilarity was more expected because of utilizing of low concentrations of dark-cure initiator [19].

The bulk of the polymerisation reactions occurred within 10 min because increasing the maturation time had little effect on the concentration of HEMA released. The Pilot study of the hydraulic cements had been experienced under conditions similar to this study [27]. These data claim that the CalciumPhosphate-ionomers are more strength than Hydraulic-CPCs about 5 to 8 times greater than Hydraulic-CPCs.

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