Title: Sterilization effects on the handling and degradation properties of calcium phosphate cements containing poly ($_{D,L}$-lactic-co-glycolic acid) porogens and carboxymethyl cellulose

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Abstract

Injectable, self-setting calcium phosphate cements (CPCs) are synthetic bone substitutes considered favorable for the repair and regeneration of bone due to their osteocompatibility and unique handling properties. However, their clinical applicability can be compromised due to insufficient cohesion upon injection into the body coupled with poor degradation rates that restricts new bone formation. Consequently, carboxymethyl cellulose (CMC) was incorporated into CPC formulations to improve their cohesion and injectability while poly ($_{D,L}$-lactic-co-glycolic acid) (PLGA) porogens was added to introduce macroporosity and improve their biodegradation rate. Like most biomaterials, CPCs are gamma irradiated before clinical use to ensure sufficient sterilization. However, it is well known that gamma irradiation also reduces the molecular weight (Mw) of CMC and PLGA via chain scission which affects their material properties. Therefore, the aim of this study is to measure the effect that gamma irradiation has on the Mw of CMC at varying doses of 15, 40 or 80 kGy and investigate how this affects the handling (i.e. injectability, cohesion, washout, setting times) and *in vitro* degradation behavior of CPC formulations. Results reveal that the Mw of CMC decreases with increasing gamma irradiation dose, thereby reducing the viscosifying capabilities of CMC which causes CPCs to deteriorate more readily. Further, the addition of CMC seems to inhibit the degree of phase transformation during setting of the cement while the subsequent reduction in Mw of PLGA after gamma irradiation improves the *in vitro* degradation rate of CPCs due to the faster degradation rate of low Mw PLGA.

Keywords: Calcium phosphate cement, Gamma irradiation, carboxymethyl cellulose, PLGA, injectability, cohesion

Statement of Significance

CPCs are a class of bioceramics characterized as an injectable, self-setting bone substitute material. Through the addition of polymeric additives like CMC and PLGA, the osteocompatibility and clinical handling properties of CPCs can be tailored according to its application, which makes CPCs favorable in treating and repairing a wide array of bone defects. However, like many biomaterials, CPCs are sterilized using gamma irradiation and little is known about how this affects the handling and degradation behavior of CPCs. Here we investigate the effect that varying doses of gamma irradiation has on the Mw of CMC and its subsequent influence on the handling and degradation behavior of CPCs. By doing so, CPCs can be engineered to have a predictable, desired outcome for future clinical applications.

1. Introduction

Self-setting, injectable calcium phosphate cements (CPCs) are favorable bone substitute materials primarily due to their excellent osteocompatibility and handling properties [1-5]. Apatite-forming CPCs are produced by mixing calcium orthophosphate precursor powders with an aqueous solution to form a paste that, once mixed, undergoes a dissolution-precipitation reaction that leads to the setting and formation of a fully hardened calcium-deficient hydroxyapatite (CDHA) end product [5-10]. CDHA is the main inorganic constituent present in bone which is why CPCs are biocompatible and even osteoconductive [11-14]. As CPCs first form into a paste, they can be molded into arbitrarily-shaped bone defect sites and set under physiological conditions, which enables them to be utilized in minimally invasive surgical procedures [15-20]. These characteristic handling and biological properties have prompted CPCs to garner much attention in the dental and orthopedic field as an attractive alternative to other conventional bone substitute materials [16, 21, 22].

Moreover, CPCs do not exhibit many of the complications associated with auto- and allografts such as donor site morbidity and chronic pain, surgical harvesting, limited availability, and risk of disease transfer and/or infection [2, 3, 5, 16, 22, 23]. Nevertheless, the clinical applicability of CPCs has remained limited due to two factors: i) poor degradability due to a lack of interconnected macroporosity to allow for vascularization and bone tissue ingrowth and ii) poor cohesion and washout resistance, which is especially important when considering their indications for skeletal sites where perfusion is high, such as the spine [14, 23-26]. For the latter, extraosseous CPC leakage can lead to serious complications, as the release and subsequent washout of CPC particles into the blood stream can stimulate blood clotting and the formation of a pulmonary embolism that can lead to the death of the patient [25, 27]. Therefore, improvement of CPCs cohesive properties is of upmost importance while maintaining an appropriate degradation rate to promote bone tissue regeneration.

To this end, sodium carboxymethyl cellulose (CMC) was incorporated into CPCs to act as a binder to improve the cohesion and injectability [15, 20, 22, 24, 28]. CMC is an anionic derivative of cellulose, where carboxymethyl groups are bound to hydroxyl groups present along the anhydroglucose unit backbone [29-31]. Due to its unique structure, it is capable of forming viscous solutions at relatively low concentrations, depending upon its degree of polymerization (i.e. molecular weight), conformation and degree of substitution (i.e. replacement of hydrogen with carboxymethyl groups). Further, CMC is nontoxic, biocompatible, water-soluble, biodegradable, and cheap which makes it an important thickener and stabilizing agent in the industrial and medical sectors with common applications in food, cosmetics, paper and pharmaceutical products [28, 30-35]. It has also been shown that negatively charged carboxyl and hydroxyl functional groups present in the backbone of CMC can form ionic bonds with the positively charged calcium ions present in CPCs, thereby improving the cohesion and injectability of the CPC composite [15, 20, 28, 36] without

impeding the resorption of CPC or its osteoconductive properties [22, 31, 35, 37]. In fact, CMC solutions have been used as a carrier for bone grafts [35, 37] and have shown to improve the clinical properties when used as a binder for calcium sulfate bone grafts [37] and β-tricalcium phosphate particles [3]. Additionally, CMC has been reported to be well-tolerated biologically and even possess osteoconductive properties [3, 37, 38]. Consequently, CMC is an ideal binding agent and thickener to improve the clinical handling properties of CPCs.

CPCs are capable of degrading by either active or passive resorption. Active resorption is mediated by the cellular activity of osteoclasts during bone remodeling, while passive resorption is dictated by the chemical solubility of the CPC matrix [2, 21, 22]. However, the rate of resorption of apatite-forming CPCs is slower than the rate of new bone formation, which limits the potential for new bone to regenerate due to the lack of porosity to allow for bone ingrowth and vascularization [2, 17, 39-41]. This can be combated by introducing interconnected macroporosity to the CPC matrix to enhance the resorption pathways [41-45]. The presence of macroporosity promotes fluid diffusion as well as cell migration which are beneficial to CPC resorption and new bone formation [3, 17, 22, 46]. To achieve this, CPCs were incorporated with poly ($_{D,L}$-lactic-co-glycolic acid) (PLGA) microparticles to act as porogens [21, 24, 47]. PLGA is widely used in the biomedical sector as it is safe and biocompatible [41, 47-50]. Another advantage of PLGA is that it degrades hydrolytically via cleavage of the ester groups into acidic lactic and glycolic monomer units which are subsequently safely excreted from the body through the Krebs cycle as carbon dioxide and water [14, 21, 47-49, 51]. Consequently, the acidity of these lactic and glycolic by-products reduces the local pH, which further accelerates the passive resorption of CPC [17, 24, 46, 52, 53]. In fact, previous studies have shown that PLGA is an effective porogen capable of

degrading CPC up to 70% after 12 weeks of implantation in the femoral condyle of rabbits [14, 21, 22, 26].

Gamma irradiation is the most commonly used sterilization method for allograft materials used for clinical applications, with doses typically ranging between 10 to 35 kGy [30, 31, 54]. However, it is known that polysaccharides, such as CMC, degrade through the cleavage of glycosidic bonds present along the backbone of the polymer chain once exposed to ionic irradiation, thereby reducing the molecular weight (Mw) [30, 31, 34, 55-58]. This phenomenon, coupled with the formation of free radicals during the irradiation process, is known to accelerate the degradation kinetics of CMC and PLGA as well as the viscosifying properties of CMC [31, 48, 56, 58]. However, the degree at which this reduction in Mw affects the cohesion and degradation kinetics of CPCs has yet to be thoroughly investigated. In fact, two previous animal studies conducted by our group reported different degradation rates of CPCs that contained the same amount of both PLGA and CMC [22, 24]. However, the powder materials were gamma irradiated at two different doses, 20 kGy [24] versus 31.5 kGy [22]. We hypothesize that the different *in vivo* degradation rates can be attributed to this variation in gamma irradiation dose, where CPCs irradiated at 31.5 kGy experienced a two-fold increase in degradation that contributed to a five-fold increase in new bone formation when compared to CPCs irradiated at 20 kGy, as shown in **Table 1**.

Table 1. The degradation rate and new bone formation of CPCs containing PLGA and CMC after 26 weeks of implantation into the femoral condyle of rabbits.

Irradiation Dose	CPC Remnants	New Bone Formation	Study
20 kGy	59.1 ± 5.6%	6.5 ± 1.8%	An et al. [24]
31.5 kGy	20.8 ± 8.4%	32.3 ± 6.2%	Grosfeld et al. [22]

Therefore, the aim of this study is to measure the effect of varying doses of gamma irradiation on the Mw of CMC and, consequently, the handling and degradation behavior of CPCs. To

this end, premixed CPC powder compositions containing either CMC or a combination of both CMC and PLGA were gamma irradiated at doses of 15, 40 or 80 kGy after which the change in Mw of CMC was measured. Further, the handling properties of CPC compositions were evaluated with respect to their injectability, cohesion, washout and setting times. Lastly, an *in vitro* degradation study involving CPC/CMC and CPC/CMC/PLGA groups irradiated at either 15 or 40 kGy was conducted to investigate any differences in degradation kinetics due to irradiation dose.

2. Materials and Methods (1,294):

2.1. Materials and Cement Synthesis

α-tricalcium phosphate (α-TCP; CAM Bioceramics B.V., Leiden, The Netherlands) microparticles with a mean diameter of ~3.0 μm were used as the calcium phosphate solid phase precursor, while the liquid phase consisted of a 4 wt% $NaH_2PO_4 \bullet 2H_20$ (Merck, Darmstadt, Germany) aqueous solution. Poly ($_{D,L}$-lactic-co-glycolic acid) (PLGA) microparticles with a mean particle size of ~50 μm was supplied and marketed as Purasorb® PDLG 5002A (Mw = 17 kDa, L:G = 50:50, acid-terminated) (Corbion Purac, Gorinchem, The Netherlands) to act as porogens in the CPC formulations. Lastly, pharmaceutical grade sodium carboxymethyl cellulose (CMC) marketed as Blanose 9H4XF-PH (Barentz Raw Materials, Hoofddorp, The Netherlands) was sieved to remove any particles greater than 106 μm and used to modify the rheological properties of the cement paste.

To fabricate CPC specimens, all the solid components were first premixed in accordance with the specifications listed in **Table 2**. Premixed CPC compositions were then gamma irradiated (Synergy Health, Chusclan, France) at varying doses of 0, 15, 40 and 80 kGy before being added to the liquid phase at a liquid-to-powder ratio of 1:2. Once combined, the phases were

thoroughly mixed together with the aid of a spatula for ~30 s in order to fully wet the particles and obtain a cement paste.

Table 2. Solid phase specifications of CPC compositions.

CPC Composition	α-TCP (wt%)	CMC (wt%)	PLGA (wt%)
CPC	100.0	0.0	0.0
CPC/CMC	98.5	1.5	0.0
CPC/CMC/PLGA	59.1	1.5	39.4

2.2 Molecular Weight Analysis

Size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS; NTNU, Trondheim, Norway) was employed to measure the effect of gamma irradiation on the molecular weight distribution, including number and weight average molecular weights (M_n and M_w) using a protocol previously developed for γ-irradiated alginates (A.-S.T. Ulset, H. Mori, M.Ø. Dalheim, M. Hara, B.E. Christensen, The influence of amino acids, buffers and pH on the γ-irradiation induced degradation of alginates, Biomacromolecules, 15 (2014) 4590-4597) of CMC. However, before SEC-MALLS analysis, the premixed solid components needed to first be separated. For CPC/CMC compositions, the CMC was retrieved by first immersing the composition in Milli-Q water (Merck, Darmstadt, Germany) heated to 37°C under constant mixing to fully dissolve the CMC. Next, the solution was centrifuged at 5000 rpm for 30 min and the supernatant was retrieved and freeze dried to harvest the CMC. CPC/CMC/PLGA compositions underwent an additional step to ensure that the PLGA was completely removed from the system. After freeze drying, the harvested material was then immersed in acetone under constant mixing for 1 hr to dissolve any remaining PLGA. Again, the solution was centrifuged under the same conditions except in this case the supernatant, which contained the PLGA, was discarded and the precipitate was then freeze dried to retrieve the CMC to be utilized for SEC-MALLS analysis. Bulk CMC, which was not mixed with any other components, was used as a control.

2.3 Evaluation of Handling Properties

In order to investigate the influence of gamma irradiation on the handling properties of CPC/CMC and CPC/CMC/PLGA specimens, tests to measure the injectability, cohesion, washout resistance and setting times were conducted and performed in triplicate (n=3). The injectability of CPCs is defined as the ability of the paste to stay homogenous during manual extrusion from a syringe, independent of a defined injection force [15, 24]. For this study, CPC pastes of different compositions were extruded from a 2.5 ml syringe with a nozzle orifice 2 mm in diameter (Terumo Europe N.V., Leuven, Belgium) and the injectability was calculated as the mass percentage of CPC paste that was fully extruded [**Eq. (1)**] [50]. The time of extrusion was kept consistent at 1:30 min (i.e., from the time of mixing the solid and liquid phases together to the time of extrusion) in order to reduce the effect that the setting of the CPC may have on the overall outcome.

$$\%\ \text{injectability} = \frac{M_i - M_n}{M_i} \times 100\% \quad (1)$$

where M_n is the mass of CPC paste remaining in the syringe after extrusion and M_i is the mass of CPC paste before extrusion from the syringe.

The cohesion of CPCs is defined as the ability of a cement paste to retain its geometrical integrity and mass upon injection in to an aqueous environment [15, 24]. In this instance, CPC pastes were injected with the aid of a syringe into phosphate-buffered saline (PBS; Gibco®, Thermo Scientific, Waltham, MA, USA) solution heated to 37°C. Upon immersion, the cohesion was qualitatively analyzed and graded based on its degree of particulate cloud formation and fragmentation as specified in **Table A.1** of the Supplementary Data. The cohesion score was then calculated using **Eq. (2)**.

$$\text{Cohesion Score} = \frac{PC + F}{2} \quad (2)$$

Where *PC* is the particulate cloud formation grade and *F* is the fragmentation grade.

The washout resistance of CPC pastes refers to the mass loss percentage attributed to α-TCP particles fragmenting off the specimen and washing away, and is considered a quantitative method to measure the cohesion of the cements. In this instance, CPC pastes were injected in to tissue specimen bags with a porosity of 170 μm (Thermo Scientific, Waltham, MA, USA) and then placed in a 15 ml Falcon tube filled with PBS solution preheated to 37°C. Upon immersion of the CPC paste, the specimens were placed inside a 37°C incubator on shaker table running at 120 rpm. After 4 hrs, the tissue specimen bags containing CPC and the solution from the Falcon tubes were both freeze dried and the material remaining was weighed. The washout % was then measured according to **Eq. (3)** [17].

$$\text{Washout } \% = \frac{W_t}{W_t + W_b} \tag{3}$$

where W_t is the weight of CPC particles washed out in the tube and W_b is the weight of CPC remaining in the tissue specimen bag.

The initial and final setting times of the CPC compositions were measured using a standardized Gillmore needle method (adapted from ASTM C266-89). Briefly, CPC pastes were inserted in to a bronze block mold 6 mm in diameter and 12 mm in height that was placed in a water bath heated to 37°C. With the aid of two needles of different dimensions the initial and final setting times were recorded, where the cement was considered set once the needle did not leave a full indentation when carefully placed on the surface of the cement.

2.4 In Vitro Degradation Test

Degradation studies were carried out on the following groups: CPC (control), CPC/CMC and CPC/CMC/PLGA groups irradiated at 15 and 40 kGy. Specimens were first fabricated into cylindrical specimens (6 mm in diameter; 12 mm in height) by being placed in a Teflon mold and left to set for 24 hrs. Next, the specimens were freeze dried and weighed to determine their initial dry mass. The specimens were then incubated in 10 ml of PBS (pH = 7.4) at 37°C

under static conditions for 1, 2, 3 and 4 weeks. After each time point, the pH of the PBS was measured using a pH electrode (Orion, Sigma Aldrich, St, Louis, MO, USA) before being replaced with new PBS. Further, inductively coupled plasma-optical emission spectrometry (ICP-OES; iCAP 6000, Thermo Fischer Scientific, Bremen, Germany) was utilized to perform elemental analysis on the PBS to measure the calcium release. To measure the mass change of the specimens, an additional set of CPC specimens was fabricated and incubated under the same conditions as mentioned previous, except in this instance after each time point the CPC specimens were removed from the PBS and freeze dried to measure their final dry mass and compared to their initial dry mass to determine the mass change.

2.5 Raman Mapping

Phase transformation profiles of CPC compositions used for the *in vitro* degradation studies were obtained by performing Raman mapping (DXRxi, Thermo Fischer Scientific, Madison, WI, USA) after each time point. 60 scans with a Raman shift of 200-3100 cm^{-1} were performed for each specimen using a 532 nm laser with a power of 10 mW. The Raman imaging spectrometer was set to the following specifications: a 50 μm slit aperture, a frequency of 200 Hz, a 50x lwd objective, and a pixel size of 50 μm. To determine the degree of phase transformation of the apatite-forming cements, the spectra of the specimens were compared to reference spectra of a fully-set, pure CPC using OMNICxi software (Thermo Fischer Scientific, Madison, WI, USA).

2.8 Statistical Analysis

All data from this study were presented as a mean ± standard deviation. Statistical analysis was performed using GraphPad Prism® software (version 5.03, GraphPad Software Inc., San Diego, USA) with a level of significance of $p<0.05$. A one-way analysis of variance

(ANOVA) with a post-hoc Tukey multiple comparisons test was used to compare data of CPC formulations between varying gamma irradiation doses for the injectability, cohesion, washout and setting times experiments whereas CPC formulations were compared between one another at each gamma irradiation dose for CMC Mw measurements. For the *in vitro* degradation study, CPC/CMC and CMC/CMC/PLGA formulations were compared amongst themselves at each time point using an unpaired t-test.

3. Results

3.1. Molecular Weight Analysis

From **Figure 1** it can be observed that the weight average molecular weight (M_w) of CMC decreased with increasing gamma irradiation dose, where M_w reduced considerably from 1814 kDa for non-irradiated bulk CMC to 66 kDa when irradiated at 80 kGy, a nearly 28-fold reduction. Interestingly, the reduction in the M_w of CMC was accelerated whenever CMC was irradiated with CPC. For this case, the CPC/CMC groups saw a reduction to 82, 80 and 64% when compared to the original Mw of bulk CMC irradiated at 15, 40, and 80 kGy, respectively. This phenomenon was even more pronounced when PLGA was also added to the composition, where the M_w of CMC for CPC/CMC/PLGA formulations reduced to 71, 50 and 35% of the original M_w for bulk CMC irradiated at 15, 40, and 80 kGy, respectively.

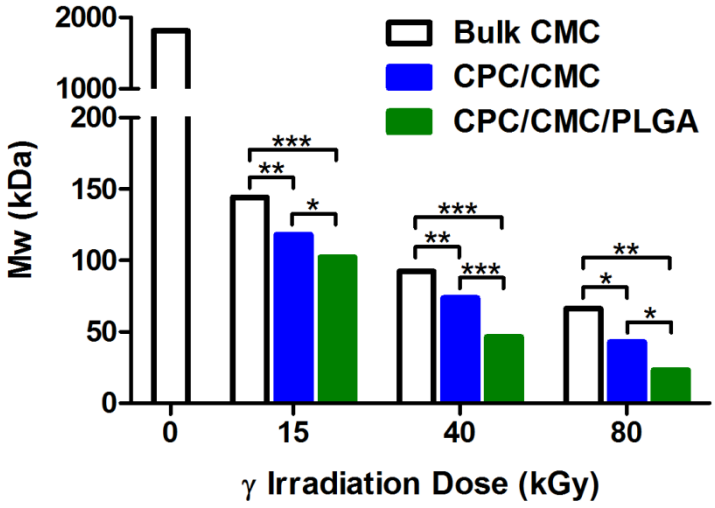

Figure 1. M_w (weight average molecular weight) of CMC as a function of gamma irradiation dose when premixed in powder form to compositions of CPC/CMC or CPC/CMC/PLGA. Bulk CMC, which was not mixed with any other components, was used as a control. Significant differences are indicated by *p<0.05, **p<0.01 and ***p<0.001.

3.2 Evaluation of CPC Handling Properties

Figure 2 reveals that the injectability of the control CPC, which contains no polymeric additives, was 70.5 ± 1.9% due to the occurrence of filter pressing. Filter pressing occurs when, during extrusion, the liquid phase flows at a faster rate than the solid CPC particles, thus leaving behind a portion of the solid phase in the syringe that cannot be extruded [1, 16, 20, 24, 59]. Filter pressing was avoided for CPC/CMC/PLGA groups, where the injectability remained stable at ~90% irrespective of the irradiation dose. For CPC/CMC groups, the injectability also remained constant at ~90% until it was subjected to an irradiation dose of 80 kGy, where the injectability reduced slightly to 83.3 ± 1.8%. It is important to note that for this test the injectability can only reach a maximum threshold of ~90% due to the design of the syringe. The plunger is unable to reach the tip of the nozzle, resulting in a portion of the cement paste to remain in the nozzle of the syringe.

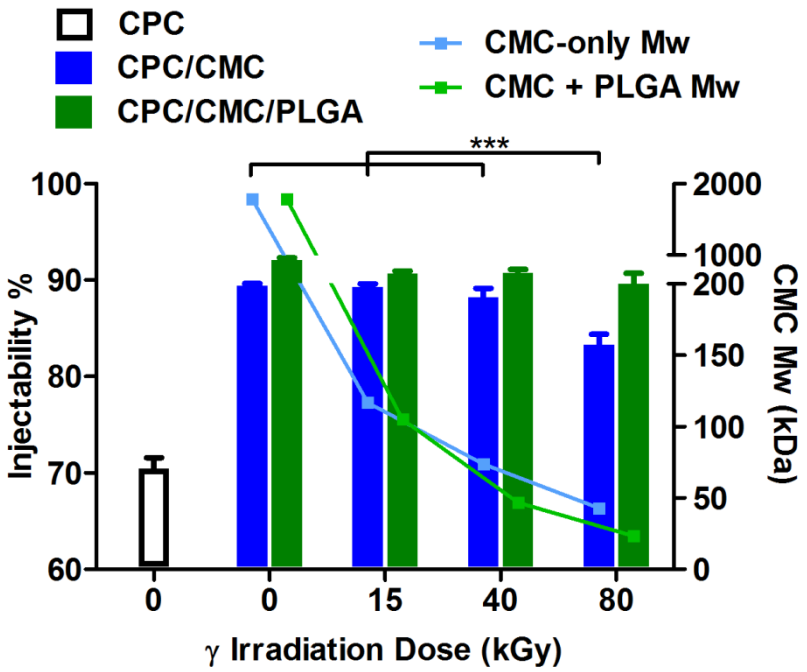

Figure 2. The injectability % of CPC compositions as a function of gamma irradiation dose. For comparative purposes, M_w of CMC is displayed along the right y-axis. Significant differences are indicated by ***p<0.001.

The cohesion of CPC pastes significantly improved from 2.7 ± 0.4 for the CPC control group to 1.0 ± 0.0 for non-irradiated CPC/CMC and CPC/CMC/PLGA groups (**Figure 3A**). However, upon irradiation, the cohesiveness of the CPC compositions began to deteriorate. This trend was more pronounced for the CPC/CMC group, where the cohesion score reached as high as 3.2 ± 0.3 when irradiated at 80 kGy. When subjected to the maximum irradiation dose of 80 kGy, the M_w of CMC substantially reduces to <70 kDa which negatively affects its viscosifying properties and, consequently, the cohesion of the CPC paste. This is further evidenced in **Figure 3B**, where representative digital images of CPC/CMC and CPC/CMC/PLGA groups clearly demonstrate how, as the irradiation dose increases, the cement paste begins to fragment into more pieces and CPC particles break off and wash away from the paste, forming particulate clouds.

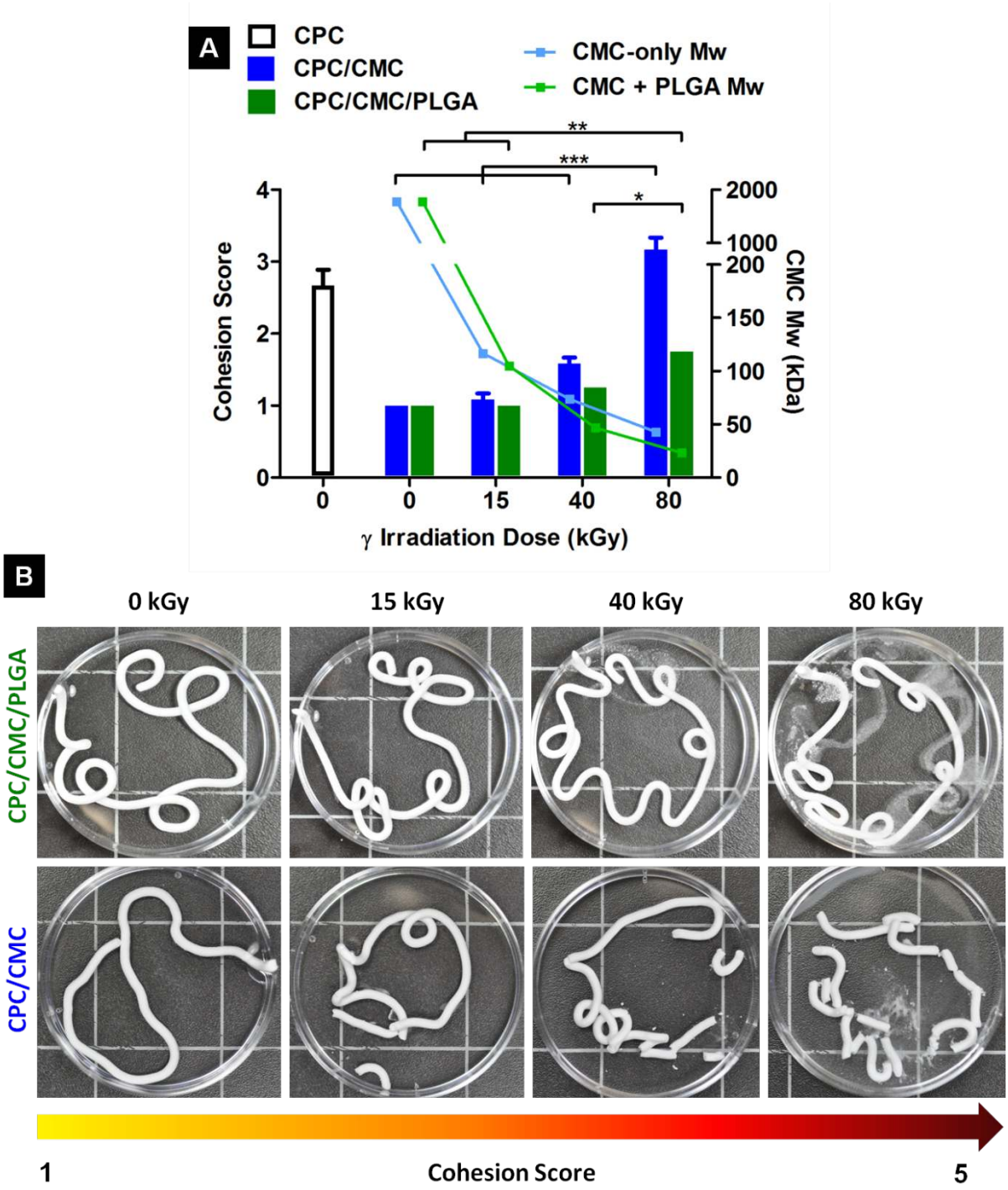

Figure 3. The cohesion score (A) of CPC compositions as a function of gamma irradiation dose. For comparative purposes, M_w of CMC is displayed along the right y-axis. Representative digital images of CPC/CMC and CPC/CMC/PLGA groups injected into PBS heated to 37°C after being subjected to varying doses of gamma irradiation (B). Significant differences are indicated by *p<0.05, **p<0.01 and ***p<0.001.

The cohesion of CPCs was also evaluated quantitatively by performing washout tests to measure the amount of CPC particles that washed away when the cement paste was placed under dynamic conditions in PBS heated to 37°C (**Figure 4**). Similar to the results of the cohesion test, an improvement in the washout resistance was observed with the addition of non-irradiated CMC and PLGA. However, upon irradiation, the washout resistance began to decline back to values comparable to the CPC control, irrespective of the dose amount.

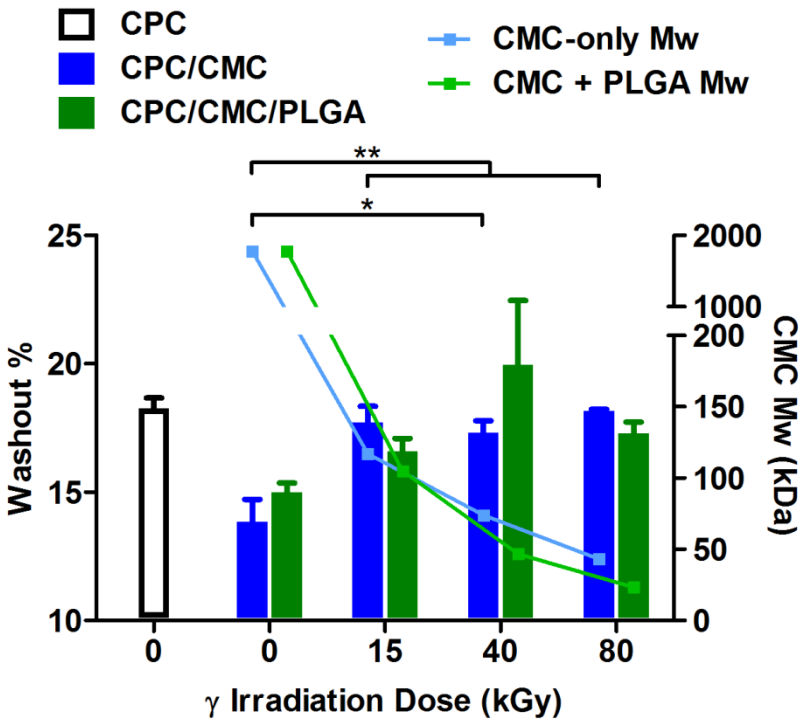

Figure 4. The washout % of CPC compositions as a function of gamma irradiation dose. For comparative purposes, M_w of CMC is displayed along the right y-axis. Significant differences are indicated by *p<0.05 and **p<0.01.

Results of the initial and final setting times are illustrated in **Figure 5A and 5B**, respectively. The addition of only CMC into the cementitious formulations caused minimal differences in the initial setting time, irrespective of the irradiation dose. Conversely, the final setting time for CPC/CMC specimens experienced an initial reduction for non-irradiated specimens from 11:3 ± 0:1 to 7:3 ±0:3 min when compared to the CPC control, followed by a substantial

increase of ~2x when subjected to irradiation, reaching up to 16:2 ± 0:4 min for the final setting time. For CPC/CMC/PLGA specimens, the addition of PLGA caused a considerable increase in both the initial and final setting times, which progressively increased once exposed to irradiation. In this case, the initial setting time reached as high as 9:2 ± 0:2 min and the final setting time reached a maximum of 25:4 ± 1:1 min after exposure to irradiation doses of 15 kGy and 80 kGy, respectively.

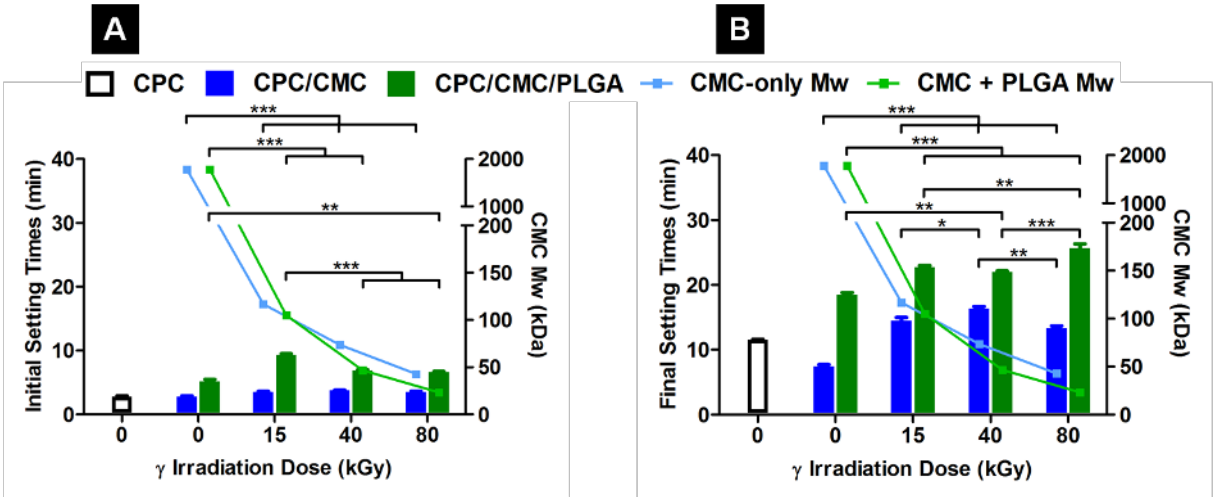

Figure 5. The initial (A) and final setting times (B) of CPC compositions as a function of irradiation dose. For comparative purposes, M_w of CMC is displayed along the right y-axis. Significant differences are indicated by *p<0.05, **p<0.01 and ***p<0.001.

Table 3. Handling properties of CPC composites with respect to their setting times, cohesion, washout and injectability.

CPC Composition	Irradiation Dose (kGy)	Initial Setting Time (min)	Final Setting Time (min)	Cohesion Score	Washout (%)	Injectability (%)
CPC	0	2:5 ± 0:1	11:3 ± 0:1	2.7 ± 0.4	18.3 ± 0.7	70.5 ± 1.9
CPC/CMC	0	2:5 ± 0:1	7:3 ± 0:3	1.0 ± 0.0	13.9 ± 1.5	89.4 ± 0.4
	15	3:3 ± 0:1	14:3 ± 0:5	1.1 ± 0.1	17.7 ± 1.1	89.3 ± 0.5
	40	3:5 ± 0:1	16:2 ± 0:4	1.6 ± 0.1	17.3 ± 0.8	88.3 ± 1.5
	80	3:3 ± 0:1	13:2 ± 0:3	3.2 ± 0.3	18.2 ± 0.1	83.3 ± 1.8
CPC/CMC/ PLGA	0	5:1 ± 0:3	18:3 ± 0:4	1.0 ± 0.0	15.0 ± 0.6	92.1 ± 0.4
	15	9:2 ± 0:2	22:5 ± 0:3	1.0 ± 0.0	16.6 ± 0.9	90.7 ± 0.4
	40	6:6 ± 0:2	22:0 ± 0:2	1.3 ± 0.0	20.0 ± 4.3	90.8 ± 0.6
	80	6:4 ± 0:1	25:4 ± 1:1	1.8 ± 0.0	17.3 ± 0.7	89.6 ± 1.9

3.3 Evaluation of CPC Degradation Properties

The *in vitro* degradation of CPC/CMC and CPC/CMC/PLGA composites exposed to gamma irradiation doses of 15 or 40 kGy was assessed by measuring the changes in mass and pH as well as calcium release after immersion in PBS at 37°C for 1, 2, 3 and 4 weeks. As shown in **Figure 6A**, all CPC and CPC/CMC specimens experienced a slight weight gain of 2-3 wt% after 4 weeks, possibly due to the apatite crystal growth or precipitation of salts from the PBS solution after freeze drying. Conversely, all CMC/PLGA-containing CPCs exhibited a linear weight loss from 3.6-3.7 wt% to 43.5-43.7 wt% from week 1 to 4, irrespective of irradiation dose, due to the accelerated degradation behavior of PLGA [17, 24, 46, 52, 53]. These results are in accordance with the calcium release results in **Figure 6B**, where CMC/PLGA-containing CPCs saw a considerable increase in the release of calcium from 156 to 1708 μg/ml for weeks 1 thru 4 when irradiated at 40 kGy, whereas the CPC and CPC/CMC groups experienced virtually no calcium release, irrespective of the irradiation dose. Interestingly, the CMC/PLGA-containing CPCs irradiated at 15 kGy exhibited a similar trend as their counterparts irradiated at 40 kGy, although the extent of calcium release was lessened to 1486 ± 147 μg/ml, a reduction of nearly 15%. The pH measurements in **Figure 6C** show a substantial drop in the pH of the incubation media of CMC/PLGA-containing CPCs from 7.4 (pH of PBS) to 4.2-4.3 after week 3, followed by an increase to 5.2-5.6 after week 4. This is to be expected due to the accelerated hydrolytic degradation of PLGA in to lactic and glycolic acid by-products which reduce the pH of the surrounding environment [17, 24, 46, 52, 53]. Contrary to this, the incubation media for CPC and CPC/CMC groups only saw a slight drop in pH to ~6.4 after week 1 followed by a gradual increase to ~6.8 after week 4. This initial drop is attributed to the occurrence of the dissolution-precipitation reaction that takes place during the setting of the cement, where a supersaturation of Ca^{2+} and PO_4^{3-} ions lead to a pH

drop in the incubation medium [9, 10, 60], but does not decrease further due to the absence of PLGA.

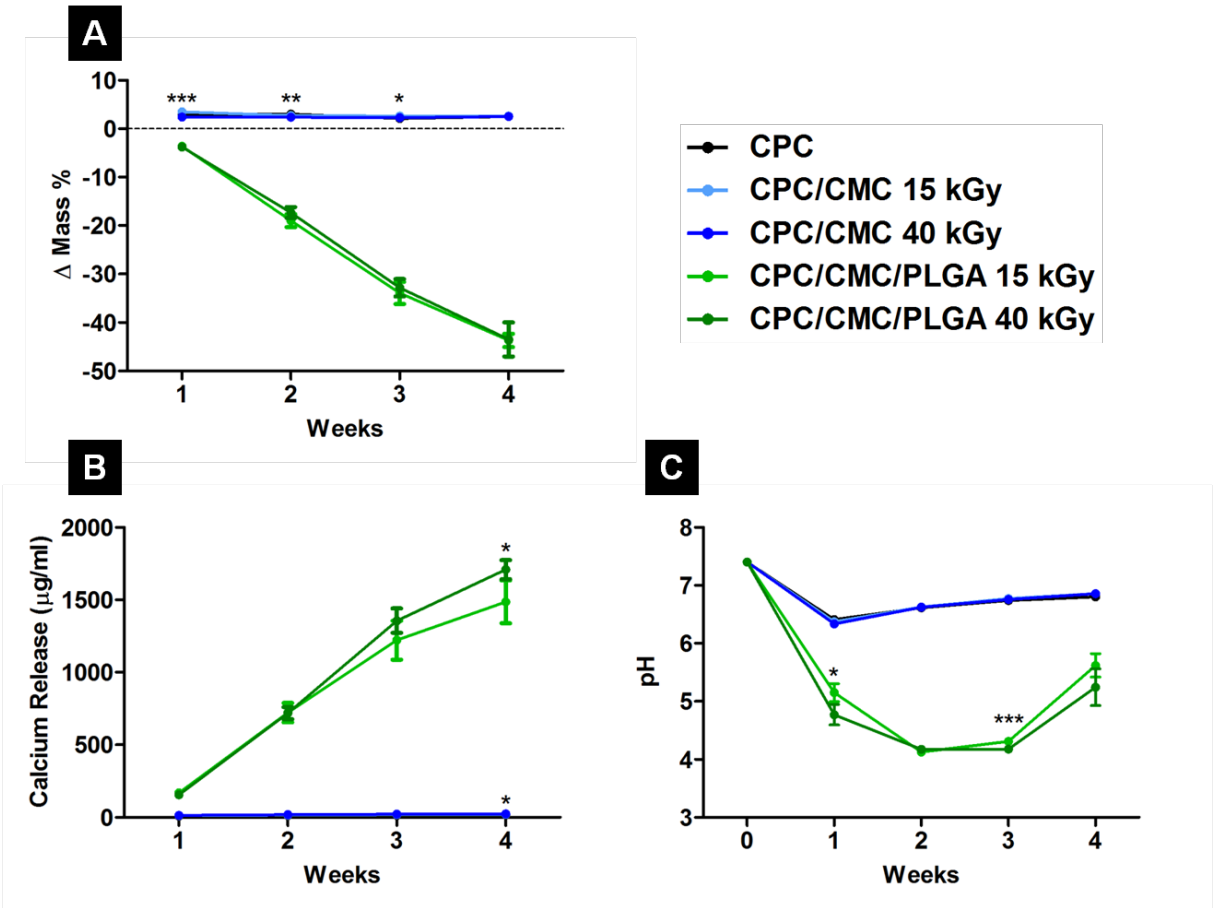

Figure 6. Degradation results with respect to the Δ mass % (A), cumulative calcium release (B) and Δ pH (C) of CPC/CMC and CPC/CMC/PLGA composites gamma irradiated at doses of 15 or 40 kGy. Significant differences are indicated by *p<0.05, **p<0.01 and ***p<0.001.

Phase transformation profiles were also performed by applying Raman mapping to the cross-sections of each specimen. The goal of this measurement was twofold: (i) to obtain information on the location and migration of CMC in the cement over time and (ii) decipher if the presence of CMC has any influence on the phase transformation of α-TCP particles to apatite. Consequently, only the results of CPC/CMC composites are presented in **Figure 7**. Further, the presence of PLGA has already been reported to have no inhibitory effect on the phase transformation [50]. When comparing the Raman mapping profiles (**Figure 7**) of

CPC/CMC groups irradiated at 15 (top) or 40 kGy (bottom), it is clear that CPCs irradiated at a higher dose exhibit a spectral profile that is less similar to that of a fully-set, pure CPC reference. This indicates that higher doses of gamma irradiation inhibit the phase transformation. Lastly, it can be stated that CMC was evenly distributed within the cement matrix and there was no indication of any migration after 4 weeks of incubation.

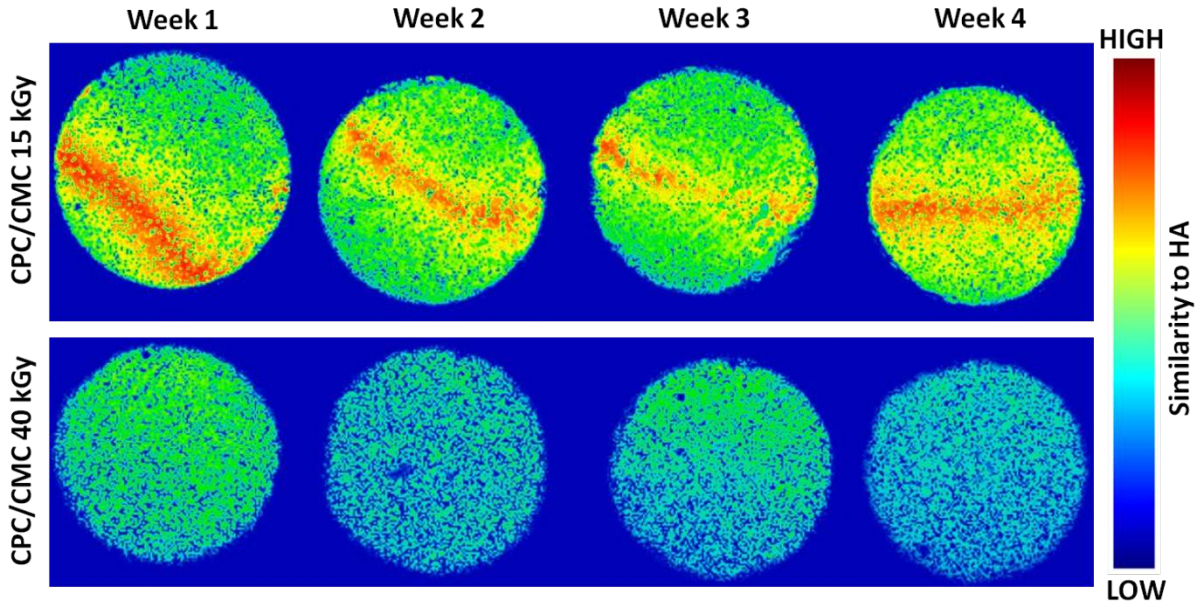

Figure 7. Raman mapping outlining the phase transformation profile of CPC/CMC specimens irradiated at 15 (top) or 40 kGy (bottom) after degradation time points of 1, 2, 3 and 4 weeks. The color scale represents the degree of similarity between the spectra of each specimen when compared to the spectra of a fully-set, pure CPC control specimen soaked in PBS for 4 weeks.

4. Discussion

To this end, sodium carboxymethyl cellulose (CMC) was incorporated into CPCs to act as a binder to improve the cohesion and injectability [15, 20, 22, 24, 28]. CMC is an anionic derivative of cellulose, where carboxymethyl groups are bound to hydroxyl groups present along the anhydroglucose unit backbone [29-31]. Due to its unique structure, it is capable of forming viscous solutions at relatively low concentrations, depending upon its degree of

polymerization (i.e. molecular weight), conformation (chain extension) and degree of substitution (i.e. replacement of hydrogen with carboxymethyl groups). Further, CMC is nontoxic, biocompatible, water-soluble, biodegradable, and cheap which makes it an important thickener and stabilizing agent in the industrial and medical sectors with common applications in food, cosmetics, paper and pharmaceutical products [28, 30-35]. It has also been shown that negatively charged carboxyl and hydroxyl functional groups present in the backbone of CMC can form ionic bonds with the positively charged calcium ions present in CPCs, thereby improving the cohesion and injectability of the CPC composite [15, 20, 28, 36] without impeding the resorption of CPC or its osteoconductive properties [22, 31, 35, 37]. In fact, CMC solutions have been used as a carrier for bone grafts [35, 37] and have shown to improve the clinical properties when used as a binder for calcium sulfate bone grafts [37] and β-tricalcium phosphate particles [3]. Additionally, CMC has been reported to be well-tolerated biologically and even possess osteoconductive properties [3, 37, 38]. Consequently, CMC is an ideal binding agent and thickener to improve the clinical handling properties of CPCs.

CMC has been frequently incorporated into CPC formulations to act as a binder and viscosifying agent to improve their cohesion and injectability [15, 20, 22, 24, 28]. The results from this study further confirm that CPC formulations containing non-irradiated CMC improve the handling properties of the cements. However, upon gamma irradiation, it was observed that the handling and degradation properties of the CPC composites become altered. In fact, two *in vivo* animal studies were recently conducted by our research group that involved the implantation of CPC/CMC and CPC/CMC/PLGA cements in the femoral condyle of rabbits for up to a period of 26 weeks [22, 24]. Although the experimental setup was identical, the two studies reported a different degradation rate of the CPC composites and percentage of newly formed bone (**Table 1**). Upon further investigation, it was revealed that

the powder phase of these cements were gamma irradiated at different doses of 20 kGy [24] and 31.5 kGy [22]. It is hypothesized that this difference in CPC degradation and new bone formation is attributed to the effect that gamma irradiation has on the molecular weight of the polymeric additives, specifically on CMC. However, the degree at which gamma irradiation affects the molecular weight of CMC premixed with CPC and/or PLGA particles has yet to be investigated. Therefore, the aim of this study is to measure what effect gamma irradiation, at varying doses, has on the molecular weight of CMC and, consequently, how this influences the handling and degradation behavior of CPCs.

4.1 CMC Molecular Weight Analysis

Irradiation of CMC induces two reactions that affect the overall properties of the polymer: i) cleavage of glycosidic bonds present along the backbone of the CMC chain leading to degradation and reduction in the weight average molecular weight (M_w) [30, 31, 34, 55-58, 61] and ii) crosslinking of CMC chains that result in the formation of a hydrogel network [30, 57, 58, 62-65]. The degree at which these reactions takes place is dependent upon several factors including the concentration and degree of substitution of CMC, irradiation dose, concentration of hydroxyl free radicals formed after irradiation, and whether CMC was irradiated in an aqueous or dry state [30, 57, 62-64]. When CMC with a high degree of substitution is irradiated at in an aqueous environment, crosslinking of the CMC chains is the dominant reaction at play. By increasing the concentration of CMC and the irradiation dose, the crosslinking effect is further enhanced [30]. The presence of water is especially important for crosslinking to occur as it contributes to two key factors. First, radiolysis of water forms highly reactive species of hydrogen atoms and hydroxyl free radicals which form a higher concentration of CMC macroradicals when they abstract hydrogen from the polymer main chain. Second, water promotes the mobility of the CMC molecules, allowing them to become

closer in proximity where they can interact with other macroradicals and, consequently, bind together [30, 56-58, 63, 66]. Conversely, when CMC is irradiated in a dry state, as is the case for our study, random cleavage of the glycosidic bonds attributed to the breakdown of inter- and intramolecular hydrogen bonds is the primary reaction that takes place [58]. Further, the absence of water means less macroradicals are formed and less crosslinking takes place.

In the present study, SEC-MALLS analysis indicates that the average chain length of CMC decreases upon exposure to increasing doses of gamma irradiation (**Figure 1**). This is to be expected as CMC was irradiated in a dry state and a reduction in the average chain length due to the random cleavage of the glycosidic bonds is the prominent reaction. Interestingly, the degradation rate of CMC was more pronounced when CMC was premixed with PLGA and CPC particles. This phenomenon can be attributed to the hygroscopic nature of PLGA and CPC particles. The addition of more water molecules present on the surface of these particles allows for the formation of hydroxyl free radicals through water radiolysis [56-58, 63]. The subsequent addition of hydroxyl free radicals results into abstraction of hydrogen atoms from the glycosidic bonds, thereby rendering them weaker, less rigid and easier to cleave [30, 58]. The addition of free radical-induced oxidative degradation causes a stronger reduction in the average chain length of CMC and the formation of smaller, more mobile anionic polymer chains that reduce the viscoelastic and gelation properties of CMC [30, 31, 57].

4.2 Evaluation of Handling Properties

When subjected to high doses of irradiation (i.e. ≥ 40 kGy), the M_w of CMC substantially reduces to < 70 kDa, a 28-fold reduction compared to non-irradiated CMC. This considerable reduction strongly reduces the viscosifying capabilities of CMC as the long anhydroglucose chains cleave in to smaller, more mobile anionic chains. The result is a reduction in the clinical handling properties of CMC-containing CPCs. Specifically, the injectability reduced

from a maximum threshold of ~90% to 83% for CPC/CMC pastes irradiated at 80 kGy since the smaller CMC chains were less effective as acting as a suspending agent for the CPC particles, resulting in a phase separation to occur, known as filter pressing. In this instance, the liquid phase flows at a faster rate out of the syringe than the powder phase, thereby leaving behind a portion of the powder phase in the syringe [1, 16, 20, 24, 59]. This alteration in the powder-to-liquid ratio of the extruded paste makes the physiochemical properties of the CPC less stable and unpredictable.

The addition of non-irradiated CMC was able to curtail the occurrence of filter pressing and improve the injectability of CPCs two ways: i) by acting as a viscosifying agent and ii) by improving the distribution of CPC particles by introducing electrostatic repulsion forces between the CPC particles. Due to their high surface area, CPC particles have a tendency to agglomerate due to strong Van der Waals attraction forces. However, CMC is able to reduce this agglomeration by binding to the surface of the CPC particles and creating a negatively charged gel-like coating. This negative charge produces an electrostatic repulsion force between the particles which aids in their dispersion and increases the interparticle space, thereby improving the homogenous distribution of particles and removing the flocculation effect [20, 59, 67]. Further, the gel coating reduces the frictional and shear flow stresses among the CPC particles, making it easier to inject the CPC paste. Lastly, high Mw polymeric additives, such as non-irradiated CMC, are better able to retain liquid and reduce any migration of CPC particles [20].

Similar to other studies [14, 41], the addition of PLGA improved the injectability which remained at the maximum threshold of ~90% irrespective of the irradiation dose. One explanation for this phenomenon can be correlated to the effect that the acidic byproducts of PLGA have on the CPC particles. Similar to CMC, radiation-induced degradation of PLGA occurs through random scission of the main chain, where long chains cleave into shorter

chains due to the excess energy from radiation exceeding the attractive forces between the atoms, causing bond scission and creation of tertiary macroalkyl and peroxyl free radicals. According to the principles of radiation chemistry, these highly reactive free radical species can initiate further chain splitting through hydrogen abstraction [48, 49]. Chain scission results in the formation of lactic and glycolic acid molecules which, once mixed with the liquid phase of CPC, causes local acidification of the setting cement that increases the positive charge of the CPC particles and precipitated CDHA nanoparticles. Consequently, the interparticle repulsion forces are increased which improves the injectability of the cement paste [14, 67].

Similar to the injectability, the cohesion and washout resistance of the cements began to deteriorate for all irradiated CPCs, especially for CPC/CMC groups. This reduction is attributed to the reduced viscosifying effect of CMC caused by radiation degradation, which makes it more difficult for CMC to bind the CPC particles together [30, 31, 57, 68]. Consequently, the cements are more susceptible to particulate fragmentation and leakage.

The addition of CMC and PLGA also prolonged the setting times of CPCs, which became more pronounced when subjected to gamma irradiation. A similar trend has also been reported in other studies [17, 24] where PLGA increased the initial and final setting times, although they still remained within the clinically acceptable range of 15-20 min [20, 24, 69]. However, our results indicate that the final setting times of all irradiated CMC/PLGA-containing CPCs were above this time limit which suggests that irradiation of PLGA had an even stronger effect on the setting times. This trend can be associated to several reasons, both physically and chemically. First, PLGA is a non-setting material, and since a large quantity (39.4 wt%) is added to the CPC composites it is to be expected that the setting time would increase. Second, it is believed that the acidic lactic and glycolic acid byproducts of

radiolyzed PLGA can inhibit the setting reaction. Similar to the effect of citric acid [67, 70, 71], the addition of lactic and glycolic acid decreases the local pH of the setting reaction through the addition of protons that interrupt the ionic equilibrium [46]. Although this increases the dissolution rate of α-TCP, it also reduces the supersaturation level and so more time is needed before a critical calcium-to-phosphorus ratio can be reached. As a result, the re-precipitation of Ca^{2+}, PO_4^{3-} and OH ions that form CDHA is delayed [10, 67].

It should also be noted that CMC is known to be a CDHA growth inhibitor [72] which explains why irradiated CMC prolongs the final setting times. Additionally, the irradiation of CMC forms smaller, more mobile anionic CMC chains that facilitates their ability to bind to α-TCP particles and form a gel-like coating [36]. As a consequence, the coating can act as a barrier and inhibit the dissolution-precipitation reaction and movement of the Ca^{2+}, PO_4^{3-} and OH ions which prolongs the setting reaction, as reported elsewhere [28, 59]. These results are in agreement with the Raman mapping profiles for CPC/CMC groups irradiated at 15 and 40 kGy. In this case, specimens irradiated at 40 kGy share a less similar spectral profile to that of a fully set CPC reference, as compared to specimens irradiated at 15 kGy. This result is attributed to the fact that, at higher irradiation doses, CMC is able to adhere more closely to the surface of α-TCP particles and form a gel-like interfacial layer. This layer not only inhibits the setting reaction but also influences the degree of phase transformation [36] and may interfere with the Raman spectral signal due to its low penetration depth.

The irradiation of CPC particles has also been shown to inhibit the degree of phase transformation for apatite-forming CPCs [19, 73], and can induce the formation of undesired free radicals on the particles surface [68]. Chen et al. investigated the effect of gamma irradiation on calcium phosphate-sulfate composite cements and determined that non-irradiated cements had a ~10% higher apatite conversion ratio compared to irradiated cements

in the range of 25-100 kGy [73]. This can also explain why the phase transformation appears to be diminished for CPCs irradiated at a higher dose in our study.

4.3 Evaluation of Degradation Properties

For the *in vitro* degradation study, a slight increase in mass was observed for the CPC and CMC-only groups which is due to the precipitation of salts from the PBS solution after freeze drying [17, 74], while the linear reduction in mass for CMC/PLGA-containing groups is attributed to the hydrolytic degradation of PLGA [17, 24, 41, 46, 52, 53, 74]. The acidic byproducts of PLGA degradation also caused a reduction in the pH which mimics the acidic environment produced by osteoclasts during bone remodeling [21, 46]. This, coupled the formation of a macroporous structure, contributed to an accelerated dissolution rate of the CPC matrix as evidenced by the calcium release profile [21, 24, 46, 47, 50]. In contrast, the CMC-only groups experienced only a slight reduction in pH in the first week followed by a gradual increase to near neutral pH conditions. This initial pH drop is associated with the dissolution of Ca^{2+} and PO_4^{3-} ions [9, 10, 60] followed by the hydration process of CPC particles that neutralizes the pH [20]. In this instance, virtually no calcium was released due to the poor resorption rate of the CPC and lack of macroporosity [2, 17, 39-41].

Contrary to the clinical handling properties, virtually no differences in the degradation properties were observed for CPC/CMC groups irradiated at different doses. The same can be said for the mass loss measurements of CPC/CMC/PLGA groups, although slight variations in the pH and, more evidently, the calcium release can be seen. In this instance, specimens irradiated at a higher dose experienced a generally lower pH when compared to specimens irradiated at lower dose. Moreover, the calcium release after weeks 3 and 4 was greater for specimens irradiated at 40 kGy versus specimens irradiated at 15 kGy, suggesting that higher irradiation may improve the degradation rate of the cement. This effect may be more

pronounced under *in vivo* conditions, as observed in the comparison of two animal studies conducted by our research group where CPC/CMC/PLGA specimens irradiated at 31.5 kGy [22] experienced a two-fold increase in degradation compared to specimens irradiated at 20 kGy [24]. The main reason behind these considerable differences is attributed the effect that gamma irradiation has on the multiple phases present in the composite cement. First, gamma irradiation has been shown to decrease the degree of phase transformation from α-TCP to CDHA due to i) the formation of free radicals on the surface of α-TCP particles and ii) the formation of a gel-like CMC interfacial coating that inhibits the diffusion of ions during the dissolution-precipitation setting reaction. This is an important factor to consider since the resorption of cements *in vivo* is dictated by their solubility [10] and α-TCP is more soluble than CDHA [11, 75]. Second, the Mw of CMC decreases with increasing irradiation dose which increases its degradability. An increase in degradability of CMC can help eliminate any remaining hydrogel coating which has been suggested to inhibit the CPC resorption process [24]. Lastly, irradiating PLGA also results in a greater reduction in its molecular weight, leading to PLGA particles that are able to degrade faster, thereby increasing the biological activity of the cements [26]. The effect that this reduction in PLGA molecular weight has on the degradation of composite cements is twofold: i) the acidic byproducts of radiolyzed PLGA creates an acidic environment that promotes further degradation of PLGA autocatalytically [21, 46] while at the same time accelerating the degradation of the CPC matrix under acidic conditions and ii) introducing a well-interconnected macroporous structure at an earlier stage that promotes the diffusion of fluids and cell migration which are key factors in the resorption of CPC and the formation of new bone [3, 17, 22, 41-46]. By increasing the resorption rate of the CPC matrix, more Ca^{2+} and PO_4^{3-} ions are released into the surrounding body fluid which has recently been postulated to influence the migration, proliferation and differentiation of osteoblast precursor cells [76, 77]. Based off these factors, it is suggested that variations in the

irradiation dose can have strong implications on the overall degradability and biological response of CPC composites.

5. Conclusion

Based off the present study, it can be concluded that the addition of non-irradiated CMC contributed to a significant improvement in the clinical handling properties of CPCs. However, gamma irradiation caused a hyperbolic reduction in the Mw of CMC that was even more pronounced when CMC was irradiated with CPC and PLGA particles. This reduction in average molecular weight negatively affected the viscosifying capabilities of CMC which caused the clinical handling properties of CPCs to deteriorate and reduced the degree of phase transformation. On the other hand, gamma irradiation seemed to improve the *in vitro* degradation and subsequent *in vivo* resorption of CPCs. This is attributed to faster degradation rates of lower M_w PLGA and CMC polymers that benefited the resorption of the CPC matrix. When developing CPCs for future clinical applications, one must consider which properties, handling or biodegradation, are more paramount for its intended use and tailor the CPC, with respect to its composition and irradiation dose, to meet those needs and obtain a desired outcome.

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Conflict of Interest

Ralf-Peter Herber is an employee of CAM Bioceramics BV. No benefit of any kind is received either directly or indirectly by the author(s).

Appendix A. Supplementary Data

Table A.1. Grading scheme to qualitatively assess the degree of particulate cloud formation and fragmentation after injection of a CPC slurry in to an aqueous environment.

Grade	Particulate Cloud Description
1	Virtually no particulate cloud formation
2	Minimal particulate cloud formation upon injection into aqueous environment
3	Visible particulate cloud formation present in the vicinity of the injected CPC slurry
4	Large particulate cloud formation present that spreads over the majority of the aqueous environment
5	Virtually no cohesion present with near total disintegration of CPC slurry

Grade	Fragmentation Description
1	A continuous slurry of CPC is extruded
2	CPC slurry remains almost continuous with an average slurry length >5 cm
3	CPC slurry fractures in several points with an average slurry length of 3 cm
4	Prevalence of fracture points in CPC slurry is frequent with the average tube length <1 cm
5	Virtually no cohesive properties present with total disintegration of CPC slurry

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