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## Effects of physical and chemical treatments on the molecular weight and degradation of alginate-hydroxyapatite composites

Daniel Alves Cardoso, Ann–Sissel Ulset, Johan Bender, John A. Jansen, Bjørn E. Christensen, Sander C. G. Leeuwenburgh*

Prof. J. Jansen, Dr. S. Leeuwenburgh, D. Alves Cardoso
Department of Biomaterials, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB Nijmegen, The Netherlands
E-mail: S. Leeuwenburgh@dent.umcn.nl

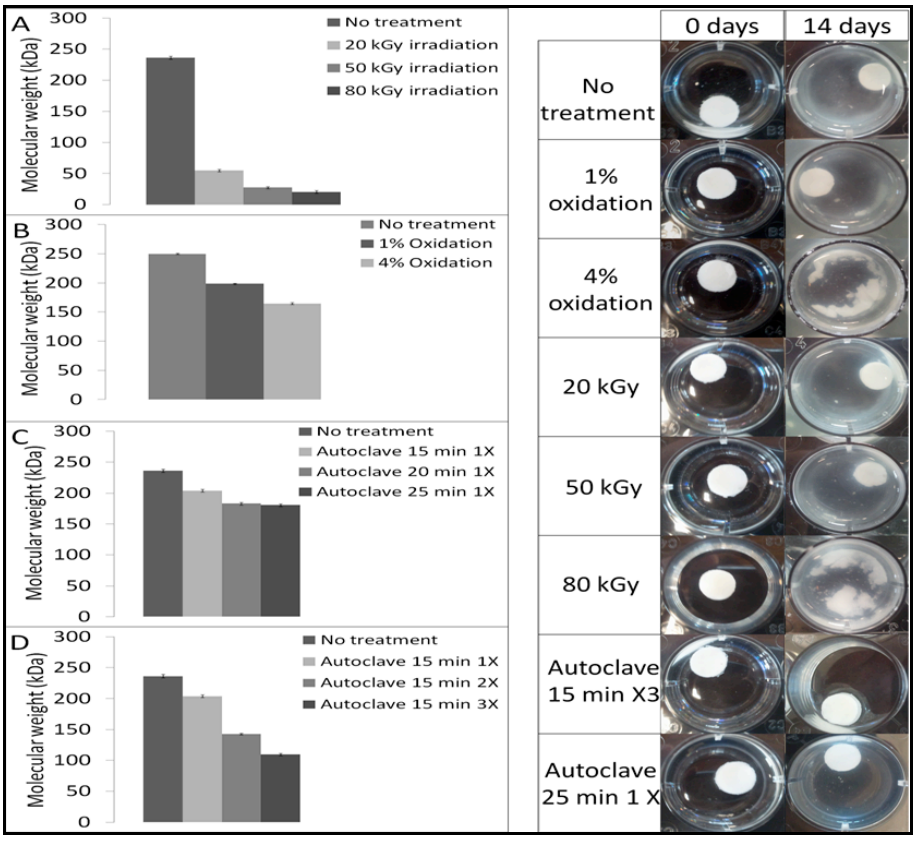
D. Alves Cardoso
EMCM B.V., Middenkampweg 17, 6545 CH Nijmegen, The Netherlands
E-mail: D.AlvesCardoso@emcm.com

Prof. B. Christensen, A-S. Ulset
NOBIPOL, Department of Biotechnology, NTNU Norwegian University of Science and Technology, Sem Saelands vei 6/8, NO-7491 Trondheim, Norway
E-mail: b.christensen@biotech.ntnu.no

J. Bender
Bender Analytical Holding B.V., Beukstraat 73, 3581 XE, Utrecht, The Netherlands
E-mail: Johan@bah-bv.nl

## Abstract

Degradation of alginate remains a critical issue to allow for predictable biological performance upon implantation of alginate-based materials. Therefore, the objective of the current study was to compare the effects of γ-irradiation (dry state, 20-80 kGy), partial (1 and 4%) periodate oxidation (aqueous solution) and autoclaving (dry state) on the molecular weight of alginate as well as the degradation behavior of alginate-based composites. The results show that γ-irradiation is by far the most destructive technique characterized by strongly reduced molecular weights and rapid loss of composite integrity upon soaking in Simulated Body Fluid (SBF). Partial periodate oxidation was less destructive as characterized by more moderate decreases in molecular weight, but the production of hydrolytically labile bonds compromised the integrity of the resulting composites. Autoclaving was shown to be a powerful tool to reduce the molecular weight of alginate in a controllable and mild manner without compromising the integrity of the resulting alginate-hydroxyapatite composites, simply by increasing the number of repetitive autoclaving cycles.

# 1. Introduction

In view of the increasing life expectancy and shortcomings of currently available synthetic bone substitutes, novel and effective bone regeneration strategies are urgently needed for an increasing number of elder patients. In that perspective, minimally invasive bone regenerative surgery has gained increasing interest in order to minimize patient trauma, accelerate postoperative surgical recovery and reduce costs. [1, 2] More specifically, the use of flowable or even self-setting formulations offers significant advantages compared to conventional, pre-shaped bone substitutes since flowable materials can be injected directly into irregularly shaped bone defects, where conformal filling offers enhanced contact to the host tissue. [3]

During the past decade, hydrogels have been extensively studied in view of their injectability, high water content and corresponding biocompatibility as well as their tunable degradability. [4-6] Nevertheless, most synthetic hydrogels are mechanically weak and lack biological cues that stimulate regeneration of tissues such as bone. The most straightforward method to functionalize hydrogels for application in bone regeneration involves biomimetic incorporation of calcium phosphate (CaP) nanoparticles into hydrogel matrices [5,7] since bone tissue itself can be describes as a nanocomposites consisting of a hydrated collagen matrix reinforced with finely dispersed, apatitic platelets. [8]

A plethora of both synthetic and natural hydrogels has been explored over the past decade for application in bone regeneration. [4,5,9] In that respect, alginate hydrogels are particularly interesting for applications in the biomedical field in view of their efficient and mild ionic crosslinking mechanism. [2,10] Alginates are a family of natural linear, unbranched polymers derived from brown algae. They are composed of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, which are linked together in different sequences. The content of G residues and their distribution along the chains are governed by several processive epimerases, and differ for different brown algae. [11] The G-blocks (.GGG… sequences) are responsible for ionic crosslinking by electrostatic interactions with divalent cations such as $Ca^{2+}$. [10]

Since the 1970s, alginate hydrogels have been combined with calcium phosphates (CaPs), but the vast majority of these formulations were crosslinked prior to implantation and did not gel in situ. [12-14] Recently,

an injectable, in situ setting alginate-CaP composite was developed at our laboratory that can be extruded from a two-component dual syringe which can combine the bone healing properties of calcium phosphate with the gentle gelling of alginate polymers [15] This formulation was crosslinked by calcium ions derived from CaP powder precursors in the presence of slowly acidifying glucono-delta-lactone (GDL). Although the biocompatibility of these formulations was confirmed in vivo, the degradability of the alginate-based hydrogels was not addressed and these properties are important if they are to be further considered for application in human patients. Generally, disintegration of alginate hydrogels and alginate-based composites proceeds by exchange of divalent calcium ions with monovalent sodium ions. It should be realized, however, that the human body lacks enzymes that can degrade alginate macromolecules at the molecular level. As a consequence, several approaches have been explored to control degradation of the alginates, since it was reported that molecular weight of alginates should be below 50 kDa in order to allow for clearance by the kidneys. [16] Partial oxidation [17-19] or γ-irradiation [6,20,21] of alginate have been studied quite extensively and together with thermal treatment [22] these treatments are known to decrease the molecular weight of alginates. Nevertheless, a direct comparison between the effect of these treatments (or combinations thereof) on the molecular weight and corresponding viscoelastic properties of alginate-based composites has never been performed before. Therefore, the objective of the current study was to compare the effects of γ-irradiation (dry state, 20-80 kGy), partial (1 and 4%) periodate oxidation (aqueous solution) and autoclaving (dry state) on the molecular weight of alginate as well as the degradation behavior of alginate-hydroxyapatite composites for bone regeneration. The molecular weight of alginate was measured using Size-exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) as a function of irradiation dose, extent of oxidation and duration as well as number of repetitive cycles of autoclaving. In addition, the viscoelastic and physiochemical properties of resulting alginate-hydroxyapatite composites were characterized by measuring storage moduli (using rheometry), swelling ratios and calcification behavior as a function of time of soaking in Simulated Body Fluid.

## 2. Experimental Section

### 2.1 Chemical Reagents

Ultrapure sodium alginate was provided by EMCM (Nijmegen, the Netherlands) obtained according to the ASTM F 2064 – 00 (Reapproved 2006) standard resulting into an endotoxin level of less than 50 EU/g (Table 1). [23] The alginate had a pH of 6.8 when dissolved in water at a 3.5% (wt%) concentration. For preparation of oxidized alginate, sodium alginate was obtained from FMC BioPolymer (Manugel DMB) since alginate from EMCM was temporarily unavailable (**Table 1**). For preparation of alginate-calcium phosphate composites, hydroxyapatite was obtained from AAP Biomaterials (Ostim® powder, VPP132). Glycerol was purchased from Sigma-Aldrich (G5516) while glucono-delta-lactone (GDL) was purchased from Merck (HS code 2932 20 90).

### 2.2 Chemical and Physical Treatments of Alginate

All alginate samples were subjected to three types of physical or chemical treatment.

1) Ultrapure sodium alginate powders were subjected to γ-irradiation (Cobalt-60) at 20 (10s exposure), 50 (15s exposure) or 80 (30s exposure), kGy (Isotron B.V., The Netherlands).

2) Ultrapure sodium alginate powders were subjected to sterilization by autoclaving by either one, two or three cycles of 15 min at 120 ºC, or one cycle of either 15, 20 or 25 min, also at 120 ºC.

3) (Partial) oxidation of alginate was performed as described previously [17]. In brief, 250 ml of n-Propanol was added to 2.25 liter of aqueous alginate solutions (4.44 g/L) followed by cooling to 4 °C. Subsequently, 0.1080 g or 0.4231 g of sodium periodate was added to the solutions to obtain 1% and 4% partially oxidized alginate monomers, respectively. The solutions were put in darkness for 48 hours for total consumption of

sodium periodate, followed by addition of NaCl (3g/L) to obtain sodium alginate. The alginate was precipitated with ethanol by progressively washing with 70, 90 and 95% ethanol. The precipitated alginate was dissolved in water and lyophilized for 24 hours to obtain a dried powder. All the reactions were done without high temperature and under constant stirring. [17]

### 2.3 Determination of Molecular Weight of Alginate

Size-exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) was used to analyze the molecular weight average (Mw) of the alginate samples before and after irradiation, oxidation and autoclaving, as previously described [17,24]. In brief, measurements were carried out at ambient temperature on an HPLC system consisting of a solvent reservoir, on-line degasser, HPLA isocratic pump, autoinjector, precolumn, and three columns (serially connected) of TSK G-6000PWXL, 5000 PWXL, and 4000 PWXL. The column outlet was connected to a Dawn DSP multiangle laser light scattering photometer (Wyatt, U.S.A.) ($\lambda_0$ = 633 nm) followed by Optilab DSP differential refractometer (P-10 cell) with a flow rate of 0.5 mL/min. The injection volume was 100−250 μL, and the sample concentration was adjusted to obtain the best possible light scattering signal without influencing the RI profile (overloading). Samples were filtered (pore size 0.22 or 0.45 μm) prior to injection. Data from the light scattering and the differential refractometers were collected and processed using Astra software (Wyatt, U.S.A.), using a refractive index increment $(dn/dc)_\mu$ of 0.150 ml/g.

### 2.4 NMR spectroscopy

NMR spectroscopy was carried out as described earlier [24].

### 2.5 Preparation of Alginate/Hydroxyapatite Composites and Soaking Studies in SBF

Alginate/hydroxyapatite composites were prepared after subjecting alginate to one of the above-mentioned physical or chemical pre-treatments using dual syringes by mixing an aqueous alginate phase with a mineral phase consisting of hydroxyapatite powder dispersed in glycerol in the presence of an acidifier (GDL). The alginate phase was obtained by dissolving alginate powder in Milli-Q water (pH 6.8) under constant agitation to obtain a polymer concentration of 3.5 w/v%. The mineral-containing phase was obtained by mixing hydroxyapatite, glycerol and the acidifier GDL at 25, 73, and 2 wt%, respectively, yielding a final hydroxyapatite content of 12.5 wt%. The alginate phase and mineral phase were loaded into the separate chambers of a dual syringe system for mixing and injection (MEDMIX®, L-system, 2.5 mm chamber, mixing tip 25 mm) leading to extrusion of an homogeneous composite (**Figure 1**). The composites were injected in cylindrical molds (8 mm in diameter and 5 mm in height) for 24 hours at 37 °C. Subsequently, the prepared composite disks (n=3) were immersed in Simulated Body Fluid (SBF) for up to 28 days. Rheological and swelling characteristics were tested for up to 14 days while calcium uptake was monitored until 28 days of soaking.

The in vitro experiments were carried out in conventional SBF with an ionic composition almost equal to human plasma. [25] Ionic concentrations of this SBF were 142.0 mM $Na^+$, 5.0 mM $K^+$, 1.5 mM $Mg^{2+}$, 2.5 mM $Ca^{2+}$, 103,0 mM $Cl^-$, 4.2 mM $HCO_3^{2-}$, 1.0 mM $HPO_4^{2-}$ and 0.5 mM $SO_4^{2-}$. Tris-HCl served as buffer to maintain a constant pH value of 7.4.

## 2.5 Rheological Characterization

The viscoelastic properties of the composite gels were analyzed before and after immersion in SBF (1, 3, 5, 7 and 14 days of immersion in SBF) using a rheometer (TA Instrument, AR2000ex) equipped with a flat steel-plate geometry (20 mm diameter). As described previously the composites had been injected in cylindrical molds (8 mm in diameter and 5 mm in height) for 24 hours at 37 °C to obtain round samples for testing. Storage moduli (G´) were determined in oscillatory time sweep tests for 5 min at a variable gap distance and fixed normal force of 0.1 N by subjecting the samples (n=3) at an oscillatory stress of 0.1 Pa and a frequency of 1 Hz.

## 2.6 Swelling Behavior

Swelling of the composites was measured by immersing the samples in SBF for 14 days as a measure for the mechanical stability of alginate-hydroxyapatite composites. The mass of the samples was measured before immersion and after 1, 3, 5, 7 and 14 days of immersion in SBF. After each time point, the samples were removed from the solution and the adsorbed liquid removed using tissue paper. The calculated swelling was obtained using the following formula: Swelling % = $(W_t - W_0)/W_0$ X 100 where $W_t$ is the weight of the sample after immersion in SBF and $W_0$ is the weight of the composite before immersion in the solution. Representative image of the gels were recorded using photography at days 3, 7 and 14 to visualize the process of composite disintegration.

## 2.7 Calcium Deposition

The calcium (Ca) content in the supernatant solution was quantified at various time points using the orthocresolphtalein complexone (OCPC) assay (Sigma) [25]. Samples were immersed in SBF (at a temperature of 37 °C under agitation) for 28 days and the solution was replaced with freshly prepared SBF at days 3, 7, 10, 14, 21, 24 and 28. To this end, the supernatant solutions were incubated overnight in 1 ml 0.5 N acetic acid on a shaker table. For analysis, 300 µl working reagent was added to 10 µl sample or standard in a 96-wells plate. Subsequently, the plate was incubated for 10 min at room temperature. The absorbance of each well was measured on a microplate spectrophotometer at 570 nm. The standards (range: 0-100 µg/ml) were prepared using a $CaCl_2$ stock solution. Data were obtained from triplicate samples and measured in duplo. The depletion of Ca in the supernatant was plotted cumulatively by measuring the difference between the Ca concentration in the sample-free SBF control solutions and the SBF solution in the presence of alginate/HA composites.

## 2.8. Statistical analysis

Data are presented as mean ± standard deviation. Statistical analyses were performed using SPSS, version 20.0 (SPSS Inc., Chicago, IL, USA). Significant differences were determined using a one-way analysis of variance (ANOVA) with a Tukey multiple comparison post-test. Differences were considered significant at p-values $< 0.05$ (* symbol was used to indicate significant difference).

# 3. Results

## 3.1 Alginate Molecular Weight Before and After Treatment

**Table 2** shows the weight average molecular weights ($M_w$) of alginates subjected to γ-irradiation, partial oxidation or autoclaving. Physical treatment by γ-irradiation reduced the molecular weight of the alginates considerably to 27% of the original molecular weight for treatment at 20 kGy, 17% for 50 kGy and 12 % for 80 kGy. Partial oxidation, on the other hand, resulted into moderately decreased molecular weights of 79% and 66% of the original molecular weight for 1% and 4% oxidation, respectively. Single cycles of autoclaving of variable duration (between 15 and 25 minutes) decreased the molecular weight of the original alginate to a minimum of 76% (25 min cycle). Repeated cycles of autoclaving at 15 min strongly reduced the molecular weight from an initial value of 86% (1 cycle), to 60% (2 cycles) and finally 40% (3 cycles) of the original molecular weight of alginate. NMR analysis revealed that the G/M ratio between guluronic and mannuronic acid was unaffected for all samples by the various physical or chemical treatments (data not shown). A graph of the $1/M_w$ versus each alginate treatment dose was plotted to confirm that the degradation occurred randomly and at a constant rate (**Figure 2**)

## 3.2 Composite degradation in SBF

### 3.2.1 Rheological Characterization

Rheological characterization revealed that storage moduli of alginate-hydroxyapatite composites decreased with increasing intensity of γ-irradiation (**Figure 3**A) or oxidation (Figure 3B), while autoclaving did not affect the elasticity of the resulting composites (Figure 3C and 3D). Upon soaking in SBF, storage moduli of all samples decreased in time. This effect was most pronounced for composites composed of γ-irradiated alginate, which revealed hundredfold smaller storage moduli after 14 days of soaking (~0.1 kPa) than composites consisting of untreated alginate (~10 kPa). After 3 days of soaking in SBF, composites

prepared from alginate pre-treated at 80kGy became too fragile for further testing (**Figure 4**). Samples prepared from oxidized alginate presented a similar behavior since storage moduli of alginate-hydroxyapatite composites decreased with increasing extent of oxidation and soaking time in SBF (Figure 2B). Composites composed of 4% oxidized alginate already disintegrated after 1 day of soaking in SBF (Figure 3), thereby impeding further rheological characterization at later time points. Composites made of 1% oxidized alginate, on the other hand, retained their integrity up to 14 days of soaking but their storage modulus decreased to about 1 kPa after 7 days of soaking. The samples containing alginate pre-treated by autoclaving were not affected by the physical treatments since storage moduli hardly decreased with increasing soaking time, autoclaving duration (Figure 3C) or number of repetitive autoclaving cycles (Figure 3D). All the composites had a tanδ lower than one, which indicates that they maintained gel-like characteristics throughout the experiment

### 3.2.2 Swelling behavior and physical degradation

In order to study the effect of the different treatments on physical degradation of the alginate/HA composites, the swelling of the gel was calculated from the mass changes observed upon soaking in SBF. The composite prepared from non-treated alginate showed an initial swelling phase lasting for 7 days up to a maximum fold swelling of 14%. The composites obtained from γ-irradiated alginate did not swell but revealed negative swelling ratios characteristic for mass loss induced by sample disintegration as observed most clearly for samples consisting of alginate pre-treated at 80 kGy which fell apart completely between days 3 and 5 (**Figure 5**A). Regarding the influence of oxidation it was shown that a low degree of partial oxidation (1%) did not affect the swelling of the composites, whereas a high degree of 4% oxidation resulted into mass loss and complete disintegration by day three (Figure 5B). Composites containing alginate pre-treated by autoclaving revealed moderate degrees of swelling up to 7 days followed by mass loss - as evidenced by negative swelling ratios - after 14 days of soaking in SBF. A single cycle of autoclaving did not reduce the swelling of the composites, whereas two or three repetitive autoclaving cycles resulted into significantly increased mass loss of the composites after 14 days of soaking (Figure 5C and Figure 5D).

### 3.3 Calcium deposition

Calcium uptake of the composite materials was measured quantitatively by assessing the calcium concentration in the SBF supernatant solution as a function of soaking time (**Figure 6**). Composites prepared with alginate γ-irradiated at 80 kGy as well as 4% oxidized alginate are not presented in Figure 6 due to their rapid degradation which impaired accurate measurements of calcium in SBF. The calcium concentrations in the SBF solution decreased for all tested alginate/hydroxyapatite formulations from day 0 until day 9, corresponding to Ca uptake by the hydrogels from metastable SBF solutions. The amount of calcium uptake increased with increasing extent of γ-irradiation, indicating that calcium uptake was more pronounced with decreasing molecular weight of the original alginate (Figure 6A). Due to the smaller difference in Mw (Table 2), only a smaller increase in calcium uptake was observed by increasing the number of cycles of autoclaving (Figure 6B). The Ca uptake was similar regardless of duration of autoclaving time (Figure 6C). Between days 9 and 28, all composites displayed a decrease in calcium depletion corresponding to release of calcium into SBF rather than consumption of calcium from SBF.

## 4. Discussion

The objective of the current study was to compare the effects of γ-irradiation, oxidation and autoclaving on the molecular weight of sodium alginate as well as the degradation of alginate-based composites. To this end, alginate was subjected to i) γ-irradiation at a dose range between 20 and 80 kGy, ii) periodate oxidation at low (1%) and high (4%) extent, and iii) autoclaving at variable duration (15, 20 and 25 min) and number of repetitive cycles (1x, 2x, 3x). The molecular weight of the treated alginates was determined using SEC-MALLS while the degradation behavior of the composites made of treated alginate and hydroxyapatite was characterized as a function of soaking time in SBF by measuring storage moduli G'

(using rheometry), swelling ratios (based on quantification of mass loss) and calcification behavior (by quantifying Ca depletion in SBF).

Characterization of molecular weight by SEC-MALLS revealed that γ-irradiation of dry sodium alginate (up to 80 kGy) was by far the most effective treatment to reduce the molecular weight of alginate compared to the other methods. The ionizing γ-irradiation degrades alginates by formation of free radicals which break glycosidic bonds, [6,21] thereby causing a rapid decrease in molecular weight upon irradiation at 20 kGy. This decrease in molecular weight leveled off at higher irradiation doses, which is logical given the fact that reduction in molecular weight upon chain fission is evidently most effective for polymers of high molecular weight. This is corroborated by essentially linear plots of $1/M_w$ versus dose (Figure 2), which corresponds to a random degradation at a constant rate. The relative reduction in molecular weight as observed in the current study are close to the values obtained by Lee et al. [27], who reported reductions in molecular weight to ~26% of the original molecular weight of alginate at 20 kGy and 13% at 50 kGy (vs. 27% and 17% as observed in our study at 20 and 50 kGy, respectively). This results were similar even given the fact that alginate was irradiated as dry powder in the current study, since irradiation of aqueous solutions - as performed by Lee et al - was suggested to be more effective by Nagasawa.[21] Alsberg [6] performed γ-irradiation of alginate powders in air and observed slightly higher molecular weights specially after irradiation at 20 kGy (~42% of original molecular weight) with more similar values for 50 kGy (~21%), and 80 kGy (~15%). We speculate that differences the γ-irradiation equipment may have contributed to the lower efficacy of γ-irradiation as observed by Alsberg. [6]

Bouhadir [18] introduced partial periodate oxidation as a means to indirectly reduce the molecular weight of alginate, and improve the degradability of alginate-based gels. According to this method, carbon-carbon bonds of the cis-diol group in the uronate residues (C2-C3 bond) are cleaved, thereby forming the corresponding dialdehydes, which are highly susceptible to alkaline β-elimination even at pH 7.4. [17] This is the basis for enhanced biodegradability. The decrease in molecular weight observed during periodate oxidation may to a certain extent be due to β-elimination, but it is generally known that the main process is actually a free-radical induced degradation occurring as a side reaction. It can to some extent be reduced by adding free radical scavenges such as n-propanol. [26,28] The results presented herein confirm that oxidation

can be used to reduce the molecular weight of alginate to ~65% of the molecular weight of the original alginate. Compared to γ-irradiation, however, the chain fission efficacy of partial periodate oxidation was much lower, which stresses that sterilization by γ-irradiation is much more destructive than partial periodate oxidation. Regarding the effect of autoclaving, it was observed that the duration of autoclaving hardly reduced the molecular weight of the sterilized alginate in the range between 15-25 min, but increasing the number of repetitive autoclaving cycles reduced the molecular weight of alginate to values that were even lower than those obtained for partial periodate oxidation (i.e. 53% after 3 cycles of 15 min). These results confirm that repetitive heating and cooling render the autoclaving process more destructive. Autoclaving is itself not a degradation tool, but accelerates other degradation mechanisms due to high temperatures. In alginates the underlying mechanisms are: acid hydrolysis, alkaline beta-elimination and free radicals (oxidative-reductive depolymerisation). [22] Compared to the highly destructive γ-irradiation, the reduction in molecular weight of alginate could be controlled more precisely by varying the number of repetitive autoclaving cycles. In addition, alginates with lower pH when dissolved will degrade faster. By controlling the Na+/H+ ratio (alginate/alginic acid) before autoclaving would permit a higher degree of control. In view of the simplicity of the autoclaving process, it can be concluded that sterilization by repetitive autoclaving is a useful tool to sterilize and tailor the molecular weight of alginate-based formulations in a simultaneous step.

Composites composed of treated alginate and hydroxyapatite were soaked in SBF followed by monitoring of storage modulus, swelling ratio and calcification behavior. Without treatment, alginate-hydroxyapatite composites displayed only moderately decreased storage moduli and swelling ratios and moderately increased calcium uptake from SBF solutions. Composites containing irradiated alginate, however, lost their integrity as evidenced by continuously decreasing storage moduli and swelling ratios as well as more pronounced uptake of calcium from SBF. The rate of composite disintegration increased with irradiation dose and exposure time, which indicated that composite disintegration directly depended on the molecular weight of alginate after γ-irradiation. Apparently, the amount of calcium as released from hydroxyapatite was not sufficient in the current study to compensate for exchange of crosslinking, divalent

calcium ions for non-crosslinking monovalent sodium ions. As a consequence, calcium uptake from SBF by alginate-hydroxyapatite composites increased with decreasing molecular weight of alginate.

Although oxidation of alginate resulted in less reduction of molecular weight than γ-irradiation, composites containing highly oxidized (4%) alginate also disintegrated within less than 3 days of soaking in SBF. Composites made of 1% oxidized alginate revealed slightly reduced storage moduli but similar swelling ratios and calcium uptake compared to composites made of untreated alginate. Apparently, high degrees of oxidation produced labile bonds which gradually broke (by β-elimination) upon soaking in aqueous SBF solutions. As before, the amount of calcium released from hydroxyapatite induced by gradual acidification of GDL was not sufficient to counteract the disruptive process of alginate degradation. It has previously been shown [29] that periodate oxidation directly influences the gelation of alginates with calcium ions, producing weaker gels under otherwise equal conditions.

Interestingly, the storage moduli of alginate-hydroxyapatite composites were hardly affected by both the duration and number of repetitive cycles of autoclaving, since storage moduli only decreased after one day of soaking and hardly decreased afterwards irrespective of the duration and number of repetitive cycles of autoclaving. Evidently, autoclaving did not impair the stability of the composites on long term even though the molecular weights were reduced upon autoclaving to values comparable to or even lower than periodate oxidation. Apparently, the hydrolytical stability of the alginate macromers was not affected by the autoclaving treatment as opposed to the more destructive γ-irradiation and partial periodate oxidation treatments which caused long-term damage and disintegration of the hydrogel matrix. This can also be seen in the swelling ratios of the different autoclaved composites which remained almost constant, although a slight decrease was observed after 7 days of soaking for composites which were autoclaved more than once or for more than 15 min. Furthermore even though there was a decrease in molecular weight this did not impact the cross-linking density of the alginate polymer which therefore lead to composites with similar storage modulus, but which afterwards lead to more mechanically unstable materials.

The amount of calcium uptake from SBF moderately increased with increasing number of repetitive cycles, whereas increasing autoclaving duration did not affect calcium uptake by alginate-hydroxyapatite composites. We suggest that the lower decrease in Mw by increasing autoclaving duration process lead to

more stable composites which lead to lower amounts of calcium binding to the alginate phase of the composite. These results indicate that repeated autoclaving of alginate resulted into reduction of molecular weight without producing excessive amounts of hydrolytically cleavable moieties that compromise the integrity of alginate in aqueous environments. As a result, sterilization of alginate powder by repeated cycles of autoclaving appears to be a powerful and mild technique to control the molecular weight of alginate without compromising the integrity of resulting alginate-hydroxyapatite composites.

## 5. Conclusion

In the current study, the effects of γ-irradiation (dry state), partial periodate oxidation (in solution), as well as duration and number of repetitive cycles of autoclaving (dry state) on the molecular weight of sodium alginate and degradation of alginate-hydroxyapatite composites was investigated systematically. The herein presented results show that among the methods tested γ-irradiation is by far the most destructive technique characterized by strongly reduced molecular weights and rapid loss of composite integrity upon soaking in Simulated Body Fluid (SBF). Partial periodate oxidation was less destructive than γ-irradiation as characterized by more moderate decreases in molecular weight, but the production of labile bonds compromised the integrity of the resulting composites. Autoclaving was shown to be a powerful tool to reduce the molecular weight of alginate in a controllable and mild manner without compromising the integrity of the resulting alginate-hydroxyapatite composites, simply by increasing the number of repetitive autoclaving cycles.

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*Table 1.* Molecular weight and G-content of original alginate powders

| | Mw [kDa] | % G residues |
|---|---|---|
| Alginate (EMCM) | 237 | 65-75 |
| Alginate (FMC BioPolymer) | 250 | 65-75 |

*Table 2.* Molecular weight of alginate samples after treatment.

| | | Molecular Weight (kDa) |
|---|---|---|
| Irradiation | 0 kGy | 237 (100%) |
| | 20 kGy | 64 (27%) |
| | 50 kGy | 41 (17%) |
| | 80 kGy | 38 (12%) |
| Oxidation | 0% | 250 (100%) |
| | 1% | 199 (79%) |
| | 4% | 165 (66%) |
| Autoclaving | Non-treated | 237 (100%) |
| | 15 min 1X | 204 (86%) |
| | 15 min 2X | 143 (60%) |
| | 15 min 3X | 110 (46%) |
| | 20 min 1X | 183 (77%) |
| | 25 min 1X | 181 (76%) |

*Figure 1.* Extrusion of alginate/HA composite

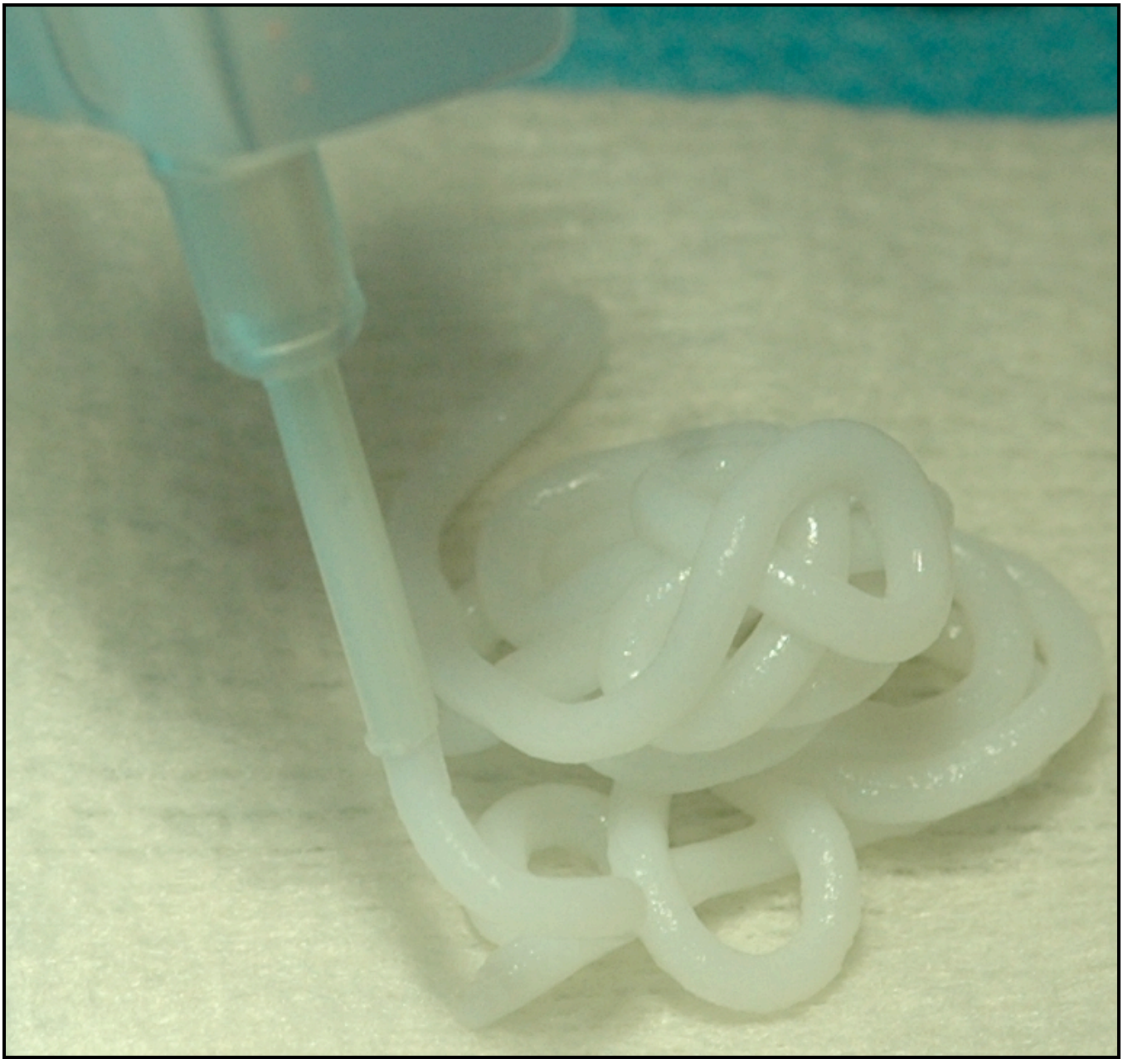


*Figure 2.* Effect of (A) γ-irradiation, (B) periodate oxidation, (C) duration of autoclaving, and (D) number of repetitive autoclaving cycles on absolute Mw of alginate

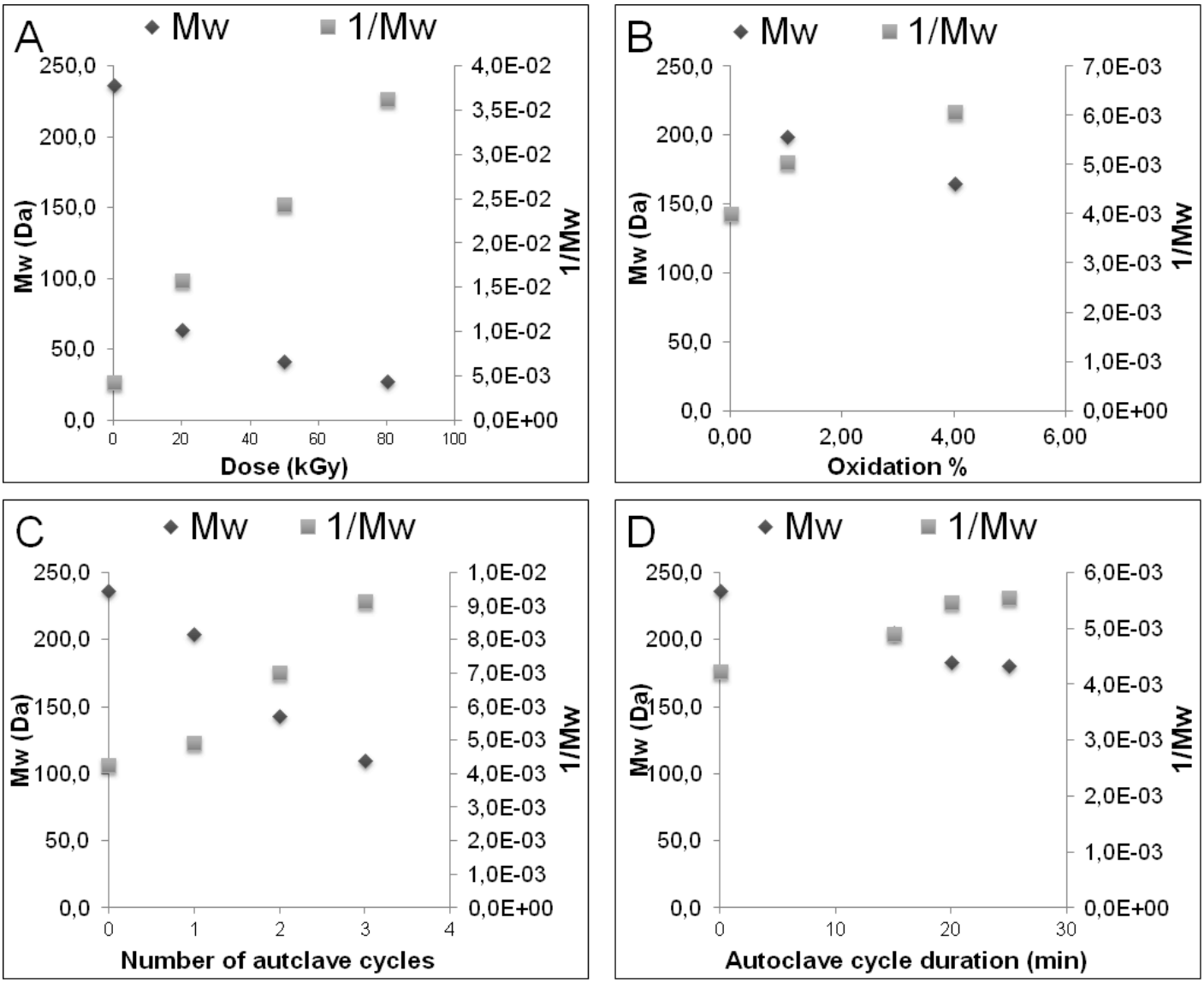


*Figure 3.* Storage moduli (G') of alginate/hydroxyapatite composites obtained from physically pre-treated alginate after soaking in SBF for up to 14 days; (A) effect of γ-irradiation, (B) effect of oxidation, (C), effect of autoclaving duration, and (D) effect of number of autoclaving cycles. * < 0.05 between each group indicated at each time point.

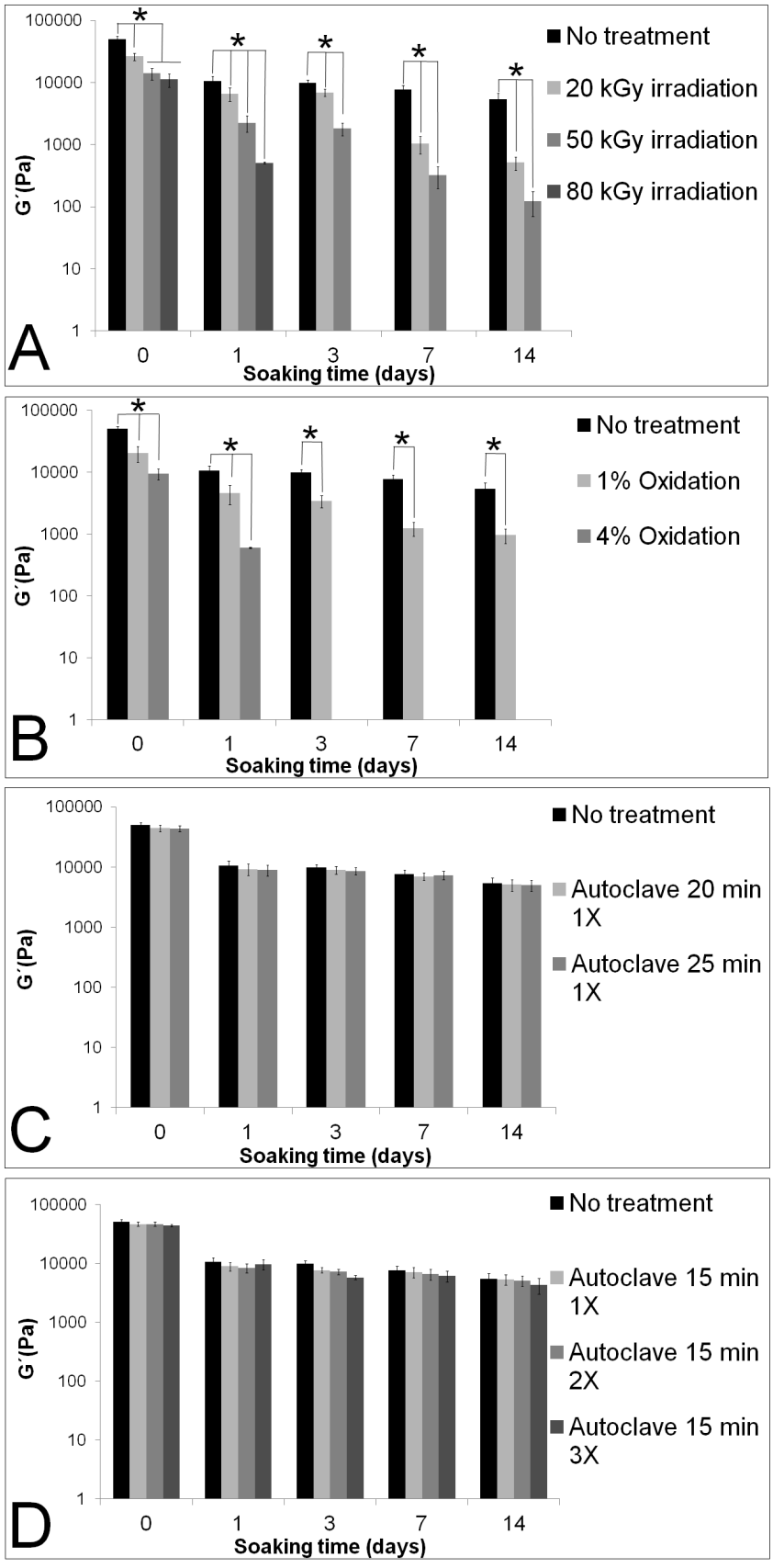



*Figure 4.* Photography of disintegration of alginate-hydroxyapatite composites obtained from γ-irradiated and periodate oxidized alginate after soaking in SBF for up to 14 days (representative images of autoclaved composites).

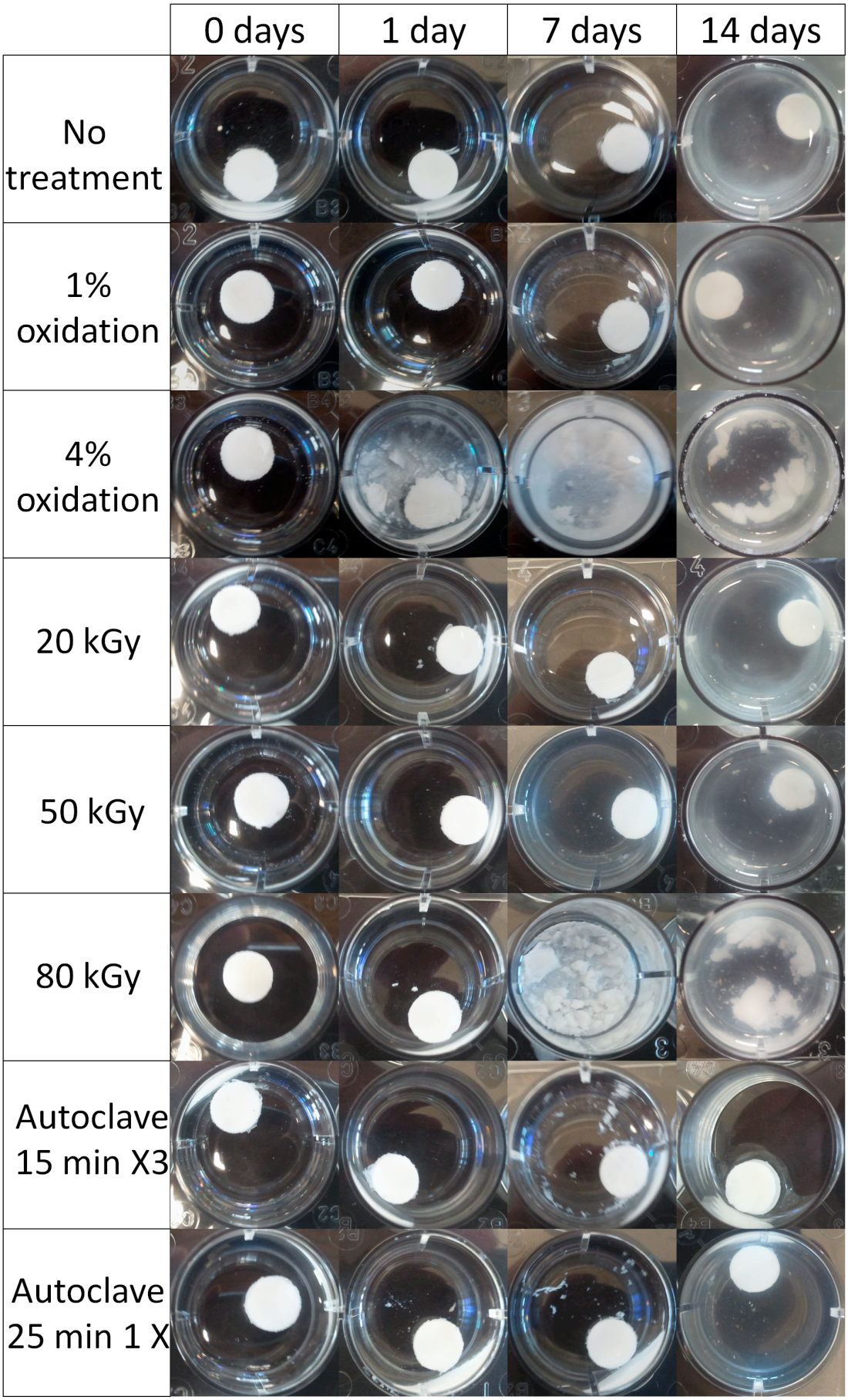



*Figure 5.* Swelling behavior of alginate/hydroxyapatite composites upon soaking in SBF; (A) effect of γ-irradiation, (B) effect of oxidation, (C) effect of autoclaving duration, and (D) effect of number of autoclaving cycles. * p-value <0.05 no treatment compared to irradiated composites, [a] p-value <0.05 no

treatment compared to autoclaved 20/25 min 1X, [b] p-value <0.05 no treatment compared to autoclaved 15 min2X/3X

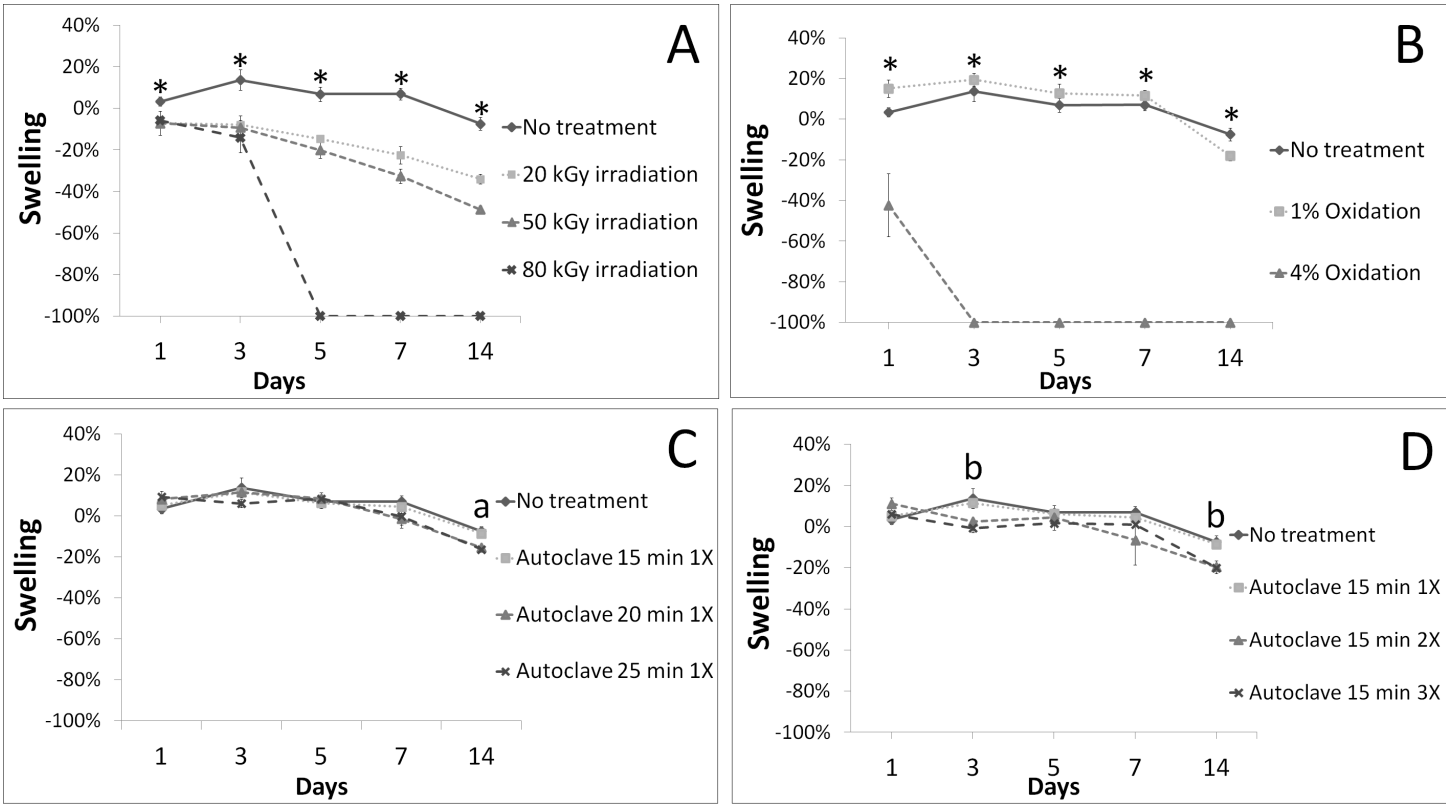
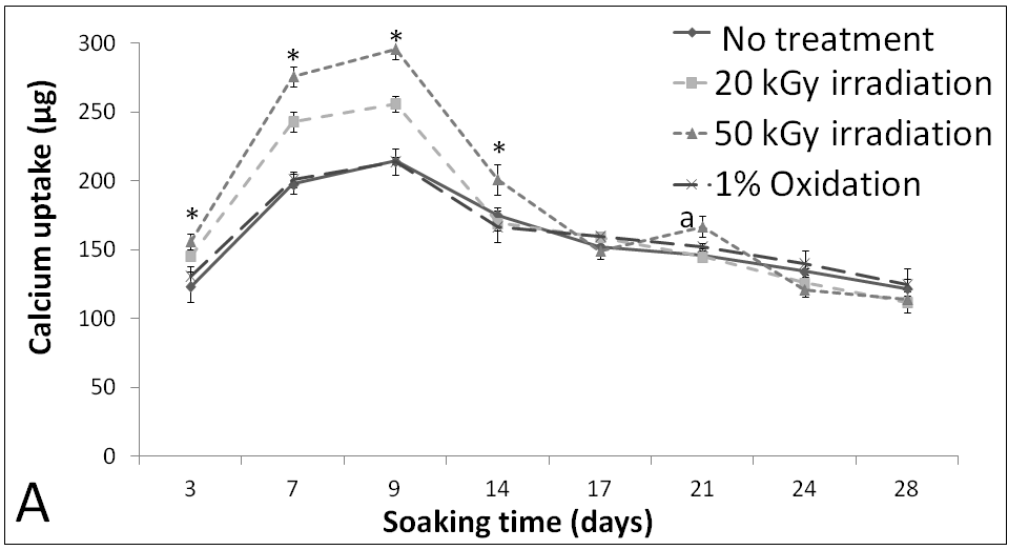
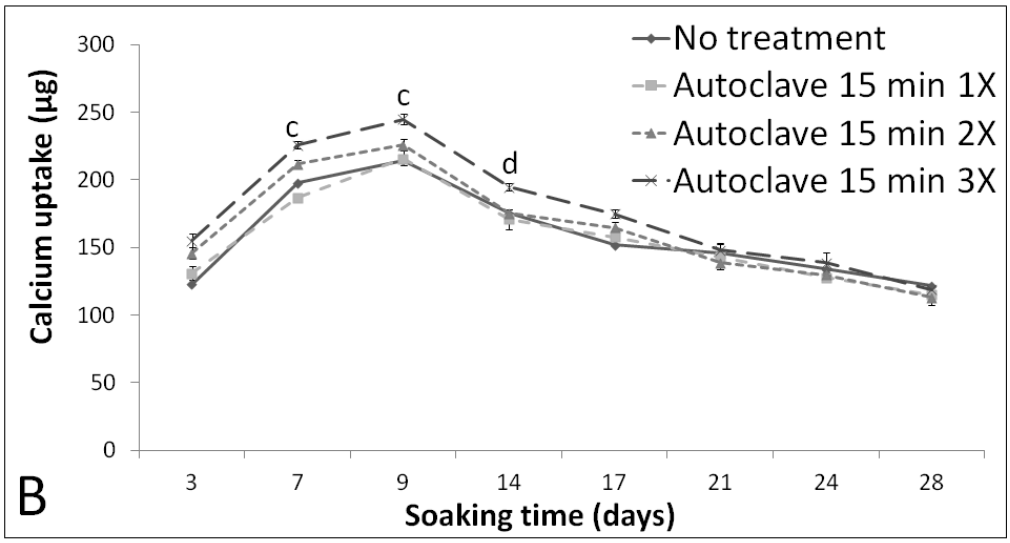



*Figure 6.* Cumulative calcium uptake by alginate-hydroxyapatite composites from SBF as a function of time; (A) effect of γ-irradiation and oxidation, (B) effect of autoclaving duration, and (C) effect of number of repetitive autoclaving cycles. * p-value <0.05 no treatment compared to 20/50 kGy irradiation, [a] * p-value

<0.05 no treatment compared to 50 kGy irradiation, [c] p-value <0.05 no treatment compared to autoclaved 15 min2X/3X, [d] p-value < 0.05 no treatment compared to autoclaved 15 min 3X



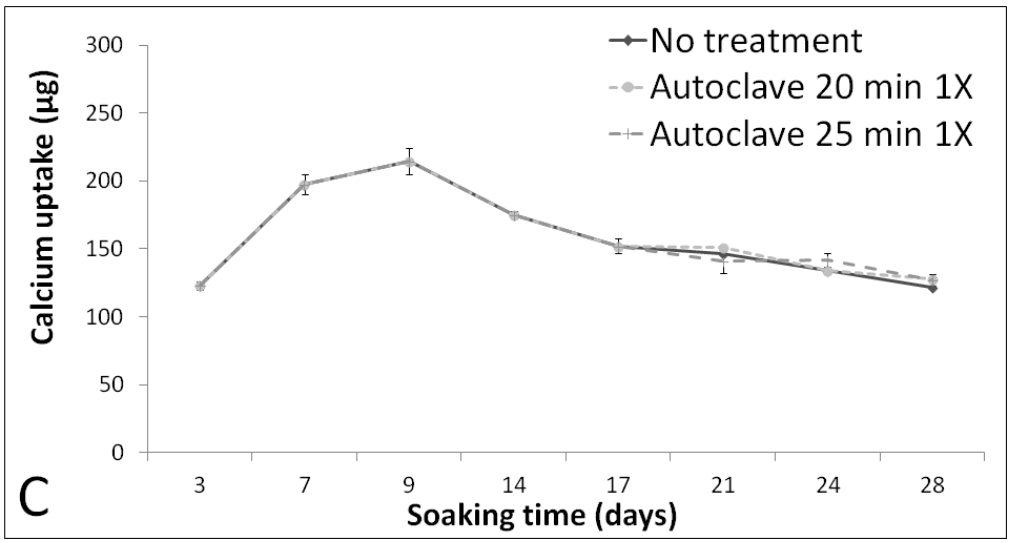




**Table of Contents**

**Alginate gels are interesting for applications in the biomedical field in view of their efficient ionic crosslinking mechanism but the human body lacks enzymes that can degrade the polymer.** Alginate treated by irradiation, autoclaving or partial oxidation can lead to a more refined control of polymer properties and obtention of more degradable injectable alginate/CaP gels for bone regeneration.