Bioactive Glass Nanoparticles for Tissue Regeneration

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ABSTRACT: Sol–gel-derived bioactive glass nanoparticles have attracted special interest due to their potential as novel therapeutic and regenerative agents. Significant challenges are yet to be addressed. The fabrication of sol–gel-derived nanoparticles in binary and ternary systems with an actual composition that meets the nominal has to be achieved. This work addresses this challenge and delivers nanoparticles in a ternary system with tailored composition and particle size. It also studies how specific steps in the fabrication process can affect the incorporation of the metallic ions, nanoparticle size, and mesoporosity. Sol–gel-derived bioactive glass nanoparticles in the 62 SiO2–34.5 CaO–3.5 P2O5 (mol %) system have been fabricated and characterized for their structural, morphological, and elemental characteristics using Fourier transform infrared spectroscopy, X-ray diffraction analysis, scanning electron microscopy associated with elemental analysis, transmission electron microscopy, and solid-state nuclear magnetic resonance. The fabricated nanoparticles were additionally observed to form the apatite phase when immersed in simulated body fluid. This work highlights the effect of the different processing variables, such as the nature of the solvent, the order in which reagents are added, stirring time, and the concentrations in the catalytic solution on the controlled incorporation of specific ions (e.g., P and Ca) in the nanoparticle network and particle size.

1. INTRODUCTION

Bioactive glasses (BGs) are promising materials for tissue engineering due to their controlled degradability and capability to stimulate new tissue formation.1–5 BGs are especially attractive for orthopedic applications as they form a strong bond with the bone.6–9 Additionally, their degradation promotes osteogenesis by releasing ionic products that stimulate osteoinductivity.10–14 Depending upon the type of ion released and its concentration, specific properties can be achieved.15–17 Thus, there is great interest to gain control over the incorporation of each ion into the glass structure to achieve the desired performance. In particular, BG osteogenic properties are mainly attributed to the release of Si4+ and Ca2+ ions, which act as triggers for the upregulation of osteogenic gene expression as well as a spur for osteoblast metabolism and bone homeostasis.10,18–21 Although these properties have been achieved in several BG compositions, those containing CaO above 25 mol %, such as 45S5 Bioglass, S53P4, or S8S, are probably the most commercially exploited for bone grafts since a higher calcium content along with P provokes stronger cell mineralization.6,7

Tissue engineering and nanomaterials science have been merged to improve the material–cell interaction, presenting materials that mimic host tissue nanofeatures.22,23 Bioactive glass nanoparticles (BGNs) can be synthesized, tailoring their characteristics for the appropriate host response.24 Their small size favors cell uptake, granting an intracellular and localized release of therapeutic ions.15,25 The higher surface reactivity of BGNs compared to their micrometer counterparts causes faster network degradation, thereby advancing the bioactive properties and accelerating the regenerative process.26,27 Degradability, surface reactivity, and biological response depend on the network connectivity and thus can be tailored by adjusting the concentration of both network formers and network modifier ions in the ultimate composition of the glass structure.28,29 While addressing the desired composition in BG microparticles is a well-standardized process, the incorporation of metallic ions in BGNs is rarely achieved, challenging the ability to deliver the desired set of properties for tissue regeneration.30 Several techniques have been reported for the fabrication of BGNs such as microemulsion, flame spray, laser spinning, or post-modification.31 However, this work focuses on sol–gel-like approaches, utilizing polymer-free one-step basic catalysis methods. Thus, acid-catalyzed, two-step catalysis methods and polymeric surfactant methods will not be discussed. Silicate-based BGNs can be considered as silica nanoparticles in which various network modifier ions are introduced within the structure. The so-called Stöber
routine method for controlled silica nanoparticle synthesis, is the most adapted basic catalysis methodology for BGN fabrication. In this method, alterations in the pH, temperature, and reagent concentration can lead to silica particles with submicrometer (100−1000 nm) or nano (<100 nm) sizes, with the simultaneous formation of aggregates for the latter. The Stoëber method has been previously adapted to attempt the synthesis of BGNs but with limited success. The addition of network modifying metallic ion precursors in the synthesis process may impair the control over particle size, shape, and dispersity even at low concentrations. However, the main unsolved issue is the persisting discrepancy between the nominal composition and the actual one obtained after the fabrication process. Specifically, the concentrations of P and Ca ions in BGNs, which are both key elements for osteoconductivity and bone bonding, are consistently lower than that aimed.

The most frequently used precursors for the incorporation of calcium and phosphorous ions during BG sol−gel synthesis are calcium nitrate and triethyl phosphate (TEP), respectively. The incorporation of P ions depends on the hydrolysis of TEP, usually performed in a solution already containing tetraethyl...
orthosilicate (TEOS) as the main reagent for SiO₂. The low amount of P₂O₅ (mol %) in the final BGN system has been attributed to the different hydrolysis rates between these two precursors, TEOS and TEP, under elevated pH, which causes the rapid condensation of SiO₂ nanoparticles lacking P ions.⁵⁶ In the case of Ca⁺² ion incorporation, the addition of calcium nitrate takes place after hydrolysis and condensation of nanoparticles. In this process, Ca⁺² ions cover the particles’ surface by bonding to hydroxyl species and get diffused during calcination above 400 °C, thus modifying the network.⁵⁹ This mechanism results in very low amounts of CaO (mol %) in the final BGN system, resulting in a composition significantly different in the nominal one. Different reasons explain this outcome, such as the lack of sufficient hydroxyl groups at the nanoparticles’ surface to bond with elevated concentrations of Ca⁺² ions in solution or the low strength of these bonds, which cannot withstand the washing steps before the calcination.⁴⁰ Additionally, Ca⁺² ions are likely to form other species such as carbonate groups or calcium-rich components, without being properly incorporated into the amorphous structure.³⁵,³⁷

Different approaches have been explored to overcome these challenges. For example, the addition of calcium nitrate during the early stages of particle condensation allowed higher detection of calcium by EDS but that resulted in a drop in the particle dispersity.⁶⁴ Additionally, it was unclear if the observed calcium was modifying the silica network of the BGNs as modifier ions or calcium was trapped as CaO molecules in BGNs or as calcium carbonate molecules. Another approach reported the increase in the actual concentration of calcium into the BGN network by increasing the Ca/Si ratio in the synthesis protocol beyond the expected ratio of the nominal composition.³⁵ The Ca⁺² ion supersaturated solution, along with the absence of the washes before calcination, resulted in the detection of the higher calcium content as well as the formation of calcium-rich areas in the delivered BGNs.³⁵,³⁷ However, once these calcium-rich areas were removed by applying washes after the heat treatment, the measured amount of the CaO in the BGNs was only around 10 mol %. Lately, Kesse et al. have shown the effect of different concentrations of the CaO content in BGN by adjusting their protocol to achieve 15.4 mol %, the maximum amount reached up to date in monodispersed submicrometer BG particles by a one-step base-catalyzed synthesis.⁴¹

In this work, the challenges of incorporating P and Ca in amounts equal to the nominal in BGNs were addressed. This work reports for the first time a novel approach to synthesize nanoparticles in the 62 SiO₂−₃₄.5 CaO−₃₂ P₂O₅ (in mol %) system where both nominal and actual compositions agree. Initially, submicrometer particles (e.g., 400 nm) were reproduced according to the protocol described by Zheng et al.,⁵⁰ and they were used as a reference for this study. Then, systematic modifications were applied to the synthesis protocol in terms of the utilized solvent, the stirring time, and the order in which reagents were added to incorporate the desired concentrations of P and Ca⁺² ions. The solvent promoting hydrolysis of TEP was used to allow the incorporation of P ions in the SiO₂ network before the nucleation of nanoparticles. The processing protocol was tailored so that the actual amount of CaO in the fabricated BGNs was measured above 30 mol % and the particle size was below 100 nm. This work also reports for the first time the impact that the stirring time before catalysis has on controlling the size of the fabricated nanoparticles. Moreover, it was showed that mesoporosity and particle distribution can be further customized by modifying the concentrations of the reagents in the catalyst (e.g., ammonium hydroxide and distilled water). Overall, the changes introduced in the synthesis process not only yielded BGNs with a composition that meets the nominal but also revealed possible means of controlling particle size, particle dispersity, and mesoporosity.

### 2. RESULTS AND DISCUSSION

#### 2.1. Morphology, Particle Size, and Distribution. The particle size, dispersity, and composition of sol–gel-derived BGNs were studied as a function of processing parameters. Particles were synthesized following two main protocols: method 1 (M1) and method 2 (M2). Figure 1 shows the size, morphology, and distribution using the SEM and TEM images and elemental analysis using the EDS spectrum of synthesized BGN. The average particle size for each fabrication protocol was calculated by analyzing the TEM images, and the mean values are reported in Table 1. Dense, spherical, and monodispersed particles with a diameter of around ~400 nm were achieved in the M1 methodology (Figure 1A–C-G).

The addition of CaNT before catalysis in M2 (Figure 1J-M, Q–S, U–W) led to a reduction in particle size below 100 nm and consequently a decrease in particle dispersion. BGNs produced according to the M2-P1 method were observed in a size of around ~86 nm, forming aggregates with an average size of around 1–2 μm (Figure 1J), and presented mesoporosity (Figure 1K, inset). The surface characteristics of M2-P1 BGNs were determined by the analysis of nitrogen adsorption and desorption isotherms (Figure S1). The Brunauer–Emmett–Teller (BET) technique confirmed the mesoporosity of M2-P1 with an average pore diameter of 18 nm, the presence of smaller pore size (around 1.7 and 3 nm), and a surface area of 21.95 m²/g (Figure S1).

The decrease in H₂O concentration in solution B that was used in the M2-P2 A protocol (Figure 1M–O) yielded a trimodal distribution with an average particle size of around ~70 nm (52%), ~190 nm (39%), and ~500 nm (9%) (Table 1) and loss of mesoporosity compared to M2-P1. Particle size was also affected by increasing the stirring time before catalysis as it was applied in M2-P2 B (increase in X₁ stirring duration time before the addition of CaNT and solution B, Figure 1Q–S) and M2-P2 C (increase in X₂ stirring time after the addition of CaNT and before the addition of solution B, Figure 1U–W) in which both protocols produced BGN of ~20 nm that form aggregates of a size of ~1 μm. However, there was no significant difference in the particle size and aggregation size for nanoparticles formed by M2-P2 B and C protocols. This observation indicates that the increase in stirring time before

| protocols | M1-P1 | M1-P2 | M2-P1 | M2-P2 A | M2-P2 B | M2-P2 C |
|-----------|-------|-------|-------|---------|---------|---------|
| particle size (nm) | 438 ± 17 | 425 ± 17 | 86 ± 14 | 70 ± 13 | 18 ± 2 | 18 ± 5 |
| [60x202] Table 1. Particle Size of Synthesized BGN under Different Fabrication Protocols Based on TEM Image Analysis |
the addition of solution B is critical. However, no dependence was observed with regard to which step before catalysis in that the stirring time is prolonged (before or after CaNT (X₁ or X₂)). DLS and zeta potential were also performed to confirm the particle size and surface charge in M2 BGNs (Table S1). DLS tended to overestimate the sizes of the BGNs similarly to the effect observed by Greasley et al. and gave measurements of aggregate sizes rather than individual nanoparticles. However, most DLS measurements are within an acceptable range from those obtained in TEM.

2.2. Elemental Composition Analysis of BGNs. Control over the elemental composition of BGNs following a Stöber-like method has remained a challenge for years. The composition analysis of BGN particles was performed by SEM-EDS spectra. The spectra collected are presented in Figure 1D,H,L,P,T,X with the calculated values being summarized in Table 2. Nanoparticles fabricated by the reference protocol M1-P1 presented a significantly low incorporation of P and Ca in agreement with published data. The lack of phosphorous was attributed to the unbalanced hydrolysis rate between TEOS and TEP under basic conditions. The faster hydrolysis of TEOS caused nanoparticles to condense before the TEP had hydrolyzed, resulting in pure SiO₂ nanoparticles. This effect was overcome in the M1-P2 synthesis protocol by allowing both hydrolysis reactions to happen at comparable rates. Methanol served as a solvent in solution A for this purpose since shorter carbon chains were expected to allow TEP to dissolve faster. This effect was observed by de Oliveria et al. in whose work the P content significantly increased by applying methanol in BGN synthesis, although the nominal composition was still unmatched. The same effect was observed here in M1-P2 and M2 BGNs. Although EDS confirmed the incorporation of P within the desired range in the BGN structure and allows us to assess the effectiveness of the applied protocol toward incorporation, the errors associated with this technique challenge the ability to determine its exact composition. Future work will be performed using ICP analysis to measure accurately the composition.

However, the modification of solution A to use methanol was still insufficient to reach the intended CaO content in M1-P2. The compositional gap observed for the calcium content is associated with their reported mechanism of incorporation into the SiO₂ structure. Calcination above 400 °C is necessary to activate the diffusion of Ca²⁺ ions into the SiO₂ network and consequently its modification. Because of this mechanism, most protocols of BGNs suggest immersing previously developed SiO₂ nanoparticles into a calcium nitrate bath in which Ca²⁺ ions would electrostatically attach to hydroxyl groups (OH⁻) at the nanoparticles’ surface. However, this electrostatic interaction is weak and limited by the number of OH⁻ available, which explains why CaO content has been rarely above 10 mol % for BGNs. The previously suggested approach to improve the incorporation of calcium in this type of protocol was reproduced here in M1 where less than 7 mol % CaO was detected. Synthesizing particles by the M2 protocol where CaNT has incorporated into solution A prior to catalysis and stirring for a long enough time allows cation interaction with SiO₂ tetrahedra to increase the CaO content up to 35 mol %. Particle size was also reduced below 100 nm.

2.3. Structure of BGNs. The structure of BGNs was further assessed by FTIR, XRD, and NMR analysis. The FTIR spectra in Figure 2 show the evolution of the bond vibrations for the applied fabrication protocols. All FTIR spectra present characteristic features of amorphous-like structures. The spectra of M1 BGNs present a dominant SiO₂ structure with vibration modes at 450, 805, 1000—1050, and 1200 cm⁻¹ for Si—O—Si bending and stretching. Additionally, the strong vibration of the Si—O—Si stretching mode at 1000—1050 cm⁻¹ overlaps with the P—O bending at 1040 cm⁻¹. The spectra of M2 BGNs present a modified SiO₂ structure as indicated by the development of a shoulder at the 900—1100 cm⁻¹ region. This shoulder band at 900 cm⁻¹, observed in the spectra of all

![Figure 2. FTIR spectra presenting nonmodified and modified SiO₂ networks by Ca²⁺ ions for BGNs fabricated by M1 versus M2 protocols, respectively.](image-url)
M2 BGNs, is attributed to Si–O non-bridging oxygen (NBO) bonds, which confirm the presence of modifier ions (e.g., Ca$^{2+}$) in the SiO$_2$ network. The formation of this vibration mode causes also the small shift of the peak at around 1050 cm$^{-1}$ to lower wavenumber.

Further structural analysis was performed by XRD. The BGNs fabricated by M1 and M2 protocols present XRD patterns of amorphous structures in agreement with FTIR spectra but with considerably different features in the patterns among the different protocols (Figure 3a). These features were analyzed by fitting the XRD patterns with Gaussian peaks for $R^2 = 0.99$. Five Gaussian peaks were identified to fit the XRD patterns with the maximum for each fitting peak at 2$\theta$: 21.9° ± 0.6, 27.4° ± 0.5, 31.3° ± 0.2, 52.3° ± 3, and 70.3° ± 0.03 (Figure 3b). The area under each fitting curve was calculated, and it was correlated with the evolution of the structure as ions were incorporated into the SiO$_2$ network (Figure 3c). As a general trend, the peak with a maximum at 21.9° 2$\theta$ decreases significantly in favor of the increase in the peak with a maximum at 27.4° 2$\theta$. This trend was obvious when the XRD pattern of M1-P1 BGNs was compared to that of M1-P2 BGNs where the only structural difference was the incorporation of P ions in the structure. Finally, the fifth peak with a maximum at 70.3° 2$\theta$ appears in the XRD patterns of all M1 BGNs, while it disappears in the patterns of all M2 BGNs, and two other peaks appear with maxima at 31.1 and 52.3° 2$\theta$ in the patterns of all M2-P2 BGNs (Figure 3c). These changes in the XRD patterns could be potentially assigned to the incorporation of Ca$^{2+}$ ions in M2 BGNs that modified the SiO$_2$ network compared to M1 BGNs where the network is barely modified due to the lack of calcium content.

The features observed in XRD revealed the presence of different SiO$_2$ coordinations being formed in the BGN structure. The network connectivity was evaluated in terms of Q speciation for two representative samples (M1-P2 and M2-P2 A) using $^{29}$Si MAS-NMR. Figure 4 shows the chemical shift (δ) for Q$^1$ (109–112 ppm), Q$^2$ (100–102 ppm), Q$^3$ (85–93 ppm), and Q$^4$ (76–79 ppm). Two different signals were identified for Q$^2$ species related to (1) silicon associated with hydrogen (≈93 ppm) and (2) silicon associated with network modifiers (≈85 ppm), in this case, calcium. The structure of M1 BGNs that was dominated by the presence of Q$^1$ and Q$^2$ species represents most of the intensity areas of the spectrum. However, M2 BGNs were dominated by Q$^3$ species and showed a significant increase in the total Q$^2$ speciation compared to M1 BGNs. Building on these facts and considering the composition in mol % detected in the synthesized BGNs, the network connectivity (NC) was
calculated from theoretical and experimental models. While phosphorous can appear in both orthophosphate and forming Si−O−P bridges, the most common status is the former. To account for the exact influence of the phosphorous status in the silicate network connectivity, \( {}^{31}\)P MAS-NMR studies would be required to determine the number of orthophosphate (Q\(_2^2\)) and Si−O−P bridges (Q\(_3^2\)) in the BGNs. Orthophosphates are associated with an increase in silicate polymerization,\(^{44}\) whereas Si−O−P bridges are known to decrease the network connectivity of the glass.\(^{45}\) Therefore, orthophosphates are accounted for the “no. of BO”, while Si−O−P bridges would contribute to the “no. of NBO” portion of eq 1. Because of the low level of P contained in the synthesized BGNs (62 Si/3 P) and its preferable chemical bonding to form orthophosphate units, the overall effect of Si−O−P bridges in the presented BGN system would be minimal. Thus, the theoretical model based on eq 1 assumed that phosphorous was present only as orthophosphate, neglecting the small percentage of phosphorous in Si−O−P bridges.\(^{36−38}\) Experimental network connectivity was obtained from the proportion Q\(_2^2/Q_3^2\) for M1-P2 and M2-P2 BGNs, where Q\(_2^2 = Q_H^2 + Q_{Ca}^2\).\(^{39,40}\) The network connectivity values obtained from the theoretical and experimental models are summarized in Table 3.

\[
NC = \frac{\text{no. of BO} - \text{no. of NBO}}{\text{no. of bridges}} = \frac{4 \left[ \text{SiO}_2 \right] - 2[\text{CaO}] + 6[\text{P}_2\text{O}_5]}{[\text{SiO}_2]} \quad (1)
\]

Table 3. Network Connectivity (NC) Based on Theoretical and Experimental Models

| protocol       | theoretical NC | experimental NC |
|----------------|----------------|-----------------|
| M1-P1          | 3.9            | N.A.\(^a\)      |
| M1-P2          | 4              | 0.73            |
| M2-P1          | 3.2            | N.A.\(^a\)      |
| M2-P2          | 3.3            | 0.36            |

\(^a\)N.A., not applicable.

These results allowed the correlation of the Q speciation observed in NMR and the amorphous structures observed in FTIR and XRD. The BGNs fabricated by any M1 protocol presented XRD patterns with a higher intensity at around 21.9° 2θ, while all M2 BGNs showed XRD patterns with a maximum intensity at around 27.4° 2θ. Thus, a highly connective SiO\(_2\) network, with mainly Q\(_2^4\) species, could be correlated with the highest intensity XRD peak at 21.9° 2θ in M1 BGNs, while less connectivity in the SiO\(_2\) network, with significantly higher Q\(_2^2\) species, is correlated with an increase in the XRD peak at 27.4° 2θ, as observed in M2 BGNs’ patterns.

2.4. Mechanism of Ion Incorporation. In this work, the sol−gel process with one-step basic catalysis was applied to synthesize BGNs in a ternary system without using polymeric templates. Previously reported protocols showed that the collected BGNs presented a significant drop in the incorporated P and Ca\(^{2+}\) ions compared to the nominal composition.\(^{56}\) Here, P was incorporated in the M1-P2 synthesis protocol by allowing the hydrolysis reactions of both TEOS and TEP to happen at comparable rates. In this work, extended stirring was the key to allow the incorporation of P into the silicate structure and achieve the nominal concentration for P. Modifying the stirring time \(X_t\) (after the addition of TEP) from 4 to 24 h proved that the nominal composition was only met in the latter, probably because the hydrolysis of all TEP was not completed after 24 h (Figure S2). Figure 5 shows the proposed mechanism of ion incorporation during particle formation. Monodispersed BGNs were obtained by providing a basic pH above the isoelectric point of the structure.\(^{52}\) This approach offered the ability of P ion incorporation without compromising particle size or dispersity. Particle diameter became slightly smaller (from 437 to 425 nm) by utilizing methanol as a solvent due to the shorter chain of alcohol.\(^{52}\) The chemical modification introduced in this protocol was insufficient to affect the surface charges caused by the increase in pH. Thus, particle size and dispersity will be still controllable by precisely tailoring water and ammonium hydroxide concentrations as reported in other Stöber-like protocols.\(^{37,54}\)

The incorporation of calcium was also achieved by introducing a major change in the synthesis process. Calcium nitrate was added into the solution and stirred for a long enough time to allow cation interaction with SiO\(_2\) tetrahedra. Catalysis of the solution after 24 h not only caused nanoparticle formation but also allowed trapping of Ca\(^{2+}\) ions within the structure. BGNs before calcination at 400 °C presented 35 mol % CaO content in SEM-EDS and lacked the Si−O−NBO vibration at 900 cm\(^{-1}\) in FTIR (Figure S3), demonstrating that Ca\(^{2+}\) ions were only trapped within the BGNs. After calcination, these trapped ions form Si−O−Ca NBO, modifying the SiO\(_2\) network, as observed in the FTIR and NMR spectra of M2 BGNs. Although the concentration of CaO was achieved at both stages, before and after calcination, their status in the silica network was different and leads to different behavior. For example, trapped Ca\(^{2+}\) will leach at an uncontrollable rate, while Si−O−Ca NBO not only allows the controlled release of Ca\(^{2+}\) but also accelerates the degradation of a silicate network since it is less interconnected. Following...
our approach, not only the CaO content was increased to the desired amount (35 mol %) but also the particle size was also reduced. It is also worth noting that although the concentrations of water and ammonium in the catalytic solution have previously shown a critical effect on the final particle size,7,36 here, the early addition of calcium nitrate before catalysis seemed to neutralize their overall effect on nanoparticles’ size. In fact, BGNs were collected after different stirring times $X_1$ (after catalysis) from 5 min to 6 h, and all presented similar sizes and composition (Figure S4).

This study also indicates a significant effect of stirring time prior to catalysis and condensation in both the composition and size of BGNs. Increasing the stirring time before catalysis allowed $P$ and $Ca^{2+}$ ions to position around $SiO_2$ tetrahedra. The time allowed for solution homogenization was at least 24 h, and further experiments would be required to determine the minimum time for optimal ion incorporation. In this regard, the significance of stirring time has been noticed in other works, although never highlighted before. In particular, Lukowiak et al. achieved 28 wt % CaO in europium-doped BGN in a two-step catalysis method by homogenizing the solution for 20 h,38 whereas only 12 wt % was obtained after 8 h.55 Stirring solutions for a total of 72 h before catalysis (as in M2-P2 B and C) yielded a significant reduction in particle size. In this case, the long stirring not only allowed ion incorporation but also affected the network connectivity. The hydrolysis in the methanol solvent for an extended period made $Si-O-Si$ bonds more susceptible to chain breakdown during catalysis. Furthermore, the additional stirring time allowed maximum utilization of TEOS, TEP, and CaNT precursors, as proved by the higher mass of material collected after calcination.

2.5. Bioactive Behavior. The osteoconductive potential of nanoparticles was assessed through in vitro biomimeralization studies. The formation of the biological apatite phase served as an indicator of bioactive glass behavior in a body simulated scenario. The capability of the BGNs to form this apatite phase was evaluated for M1, M2-P1, and M2-P2 A BGNs. Particles were immersed in SBF at 37 °C under constant agitation to reproduce body conditions. After 7 days, the formation of an apatite phase was observed by FTIR (Figure 6, solid line) and compared to that before SBF (Figure 6, dashed line). For both M1 BGNs, the vibration peaks that confirm the presence of a calcium phosphate phase after immersion in SBF are significantly lower than the respective peaks for the spectra of M2 BGNs. Nevertheless, the formation of this deposition was evidenced in both M1 BGNs by the development of a broad peak in the region of 575–620 cm$^{-1}$ commonly attributed to $P-O$ bending. Particles fabricated by M2 protocols presented the characteristic dual peak for $P-O$ bending at 575 and 620 cm$^{-1}$ that together with the carbonate group bands at $\sim$873 and $\sim$1450 cm$^{-1}$ confirmed the formation of a carbonated calcium phosphate phase. The band at $\sim$1050 cm$^{-1}$ was also slightly different in M2 BGNs before and after SBF, showing a better formed shoulder at $\sim$950 and $\sim$1200 cm$^{-1}$ and an increase in the sharpness of the peak at 1050 cm$^{-1}$. These features are attributed to a stronger $P-O$ bending vibration in the structure caused by the increase in $P-O$ bonds during apatite deposition.

The ability to develop the biological apatite phase was found weaker for M1 BGNs than for M2 BGNs as a consequence of nanoparticles’ composition and size. The mechanism of apatite formation in BG is attributed to the accumulation of dissolution products.56 Initially, $P$ and $Ca^{2+}$ ions are exchanged in solution, leaving an increased concentration of silanol bonds ($Si-OH$) at the surface of nanoparticles. Then, silanols are repolymerized, creating a silica-rich layer. Further ion migration of $P$ and $Ca$ species takes place from the core of the particle toward the surface and reacts to create an amorphous calcium phosphate layer. The supersaturated solution causes the deposition of hydroxy and carbonate groups as well as more $P$ and $Ca^{2+}$ ions and later the crystallization of the calcium phosphate phase to hydroxyapatite (HCA).56–58 The rate of HCA layer formation was greatly influenced by BG composition. The substitution of $Si$ by other ions such as $P$ and the modification of the network by $Ca^{2+}$ ions created a less connected network in which hydrolysis of $Si-O-Si$ is not necessary for the dissolution of silicate chains.57 BGNs fabricated by M2 protocols exhibited half network connectivity (NC) than M1 BGNs protocols as a consequence of higher calcium incorporation, and thus, they undergo a faster bioactive response. The NC was below the ideal reported by Edén (2 < NC < 2.6) and insufficient to generate a dense apatite phase after 7 days in SBF.59 Despite the low calcium content in M1 BGNs, a calcium phosphate deposition was observed. This result is in agreement with previous reports that proved bioactive behavior of sol–gel glasses with up to 90 mol % $SiO_2$.60 The bioactive response is also affected by the particle size.61 A lower particle size induces a higher ion dissolution rate due to the higher surface to volume ratio. Thus, ion release in M2 BGNs (particle size, <100 nm) is intrinsically higher than that in M1 protocols (~400 nm).

3. CONCLUSIONS

In this study, a sol–gel method was optimized to incorporate $P$ and $Ca^{2+}$ ions in the structure of BGNs, achieving for the first time the nominal composition. The role of the order in which
reagents were added, the concentrations in the catalytic solution, and the stirring time before catalysis were evaluated in terms of the final particle size, composition, and structure. The incorporation of P ions was achieved by utilizing methanol for the hydrolysis of TEP and long stirring time. The incorporation of calcium in amounts higher than 20 mol% was accomplished by adding calcium before catalysis and the SiO2 condensation reaction. This process also causes particle size reduction below 100 nm. Long stirring times were required to ensure the reaction between ionic species and SiO2 tetrahedra. Despite their aggregation in micromized clusters, BGNs below 100 nm proved nanoscale properties as evidenced by the faster bioactive response. This faster reactivity, a consequence of the high surface area, and the bioactive properties emphasize the potential of these particles for tissue engineering application.

4. EXPERIMENTAL PROCEDURE

4.1. Materials. Particle synthesis was performed with analytical grade tetraethyl orthosilicate (TEOS), triethyl phosphate (TEP), calcium nitrate tetrahydrate (CaNT), and 28–30% ammonium hydroxide (NH4OH) solution purchased from Sigma-Aldrich. The solvents used were distilled water, 200 proof ethyl alcohol, and methanol. All reagents were used as received without further purification.

4.2. Preparation of Bioactive Glass Nanoparticles (BGNs). Bioactive glass nanoparticles with a nominal composition of 62 SiO2, 34.5 CaO, 3.2 P2O5 (in mol %) were prepared using the sol–gel process with one-step basic catalysis. Various experiments were conducted to investigate how different processing parameters, such as (1) the type of solvent, (2) the addition order of the CaNT, and (3) the relative concentrations of the components in the catalytic solution (solution B), affect the fabricated nanoparticles. The layout of the synthesis protocols is illustrated in Figure 7.

Initially, two solutions were prepared. Solution A containing 41.6 mL of solvent (ethanol (M1-P1) or methanol (M1-P2 and M2)), 5.55 mL of TEOS, and 0.5 mL of TEP in a Teflon beaker was stirred for a specific time (X). The catalytic solution was named as “solution B” and prepared by mixing distilled water and 28–30% ammonium hydroxide in ethanol. The ratios of the concentrations (in molarity, M) of the reagents (H2O and NH4OH in ethanol) used for solution B are summarized in Table 4. All processes were performed at room temperature under vigorous stirring (~500 rpm). All solutions were covered in beakers with parafilm.

Method 1 (M1) has been previously reported by Zheng et al. and is utilized here as a reference for the later systematic modifications. Briefly, solution B was incorporated into solution A and stirred for 30 min before the addition of 3.14 g of CaNT. Particles were collected after 2 h of stirring duration. The effect of the type of the solvent was also investigated by using ethanol (M1-P1 as described by the Zheng et al. protocol) or methanol (M1-P2) as an alternative solvent in solution A.

Method 2 (M2) studies the effect of changing the order in which CaNT is added in solution A by incorporating this reagent before the incorporation of solution B. Methanol was used as the solvent constantly in M2 because of the advances shown in M1-P2. After the addition of 3.14 g of CaNT into solution A, the solution was under stirring duration for X1 amount of time and then, the collection of particles was happening 24 h after solution B was incorporated into solution A.

The effect of the catalyst (solution B) was studied by modifying the concentration (in M) of H2O and consequently the relevant ratios of H2O/TEOS and H2O/ethanol in all M2-P2 protocols from that of M1, as reported in Table 4. Additionally, the effects of the stirring durations (X1 and X2) before the addition of solution B in the size of the collected particles were evaluated for M2-P2 protocols as it is presented in Figure 7. The effect of the stirring duration time after catalysis on particle composition and size for M1 protocols is not reported in this work as it has been previously explored by other research groups, while no effect on particle size and composition was observed for all M2 protocols with different stirring duration times after catalysis (data presented for M2-P1 in the Supporting Information).

All particles were collected by centrifugation at 3000 rpm for 3 min. The collected particles were then heat-treated at 60°C for 6 h, calcinated at 700°C for 2 h with a 2°C/min heating rate, and cooled down to room temperature with 5°C/min. The collected powder was additionally mortar pulverized, washed with ethanol twice to remove calcium-rich areas, and air-dried before characterization.

All fabrication protocols were applied three times, and the number of samples under characterization from each group was three.

4.3. Morphological and Elemental Evaluation. The morphology of the BGN was observed using a scanning electron microscope (ZEISS FIB-SEM) operated at 3 kV. Elemental analysis was performed at 15 kV using another SEM instrument (MIRA3 TESCAN FEG-SEM) equipped with an EDS detector. Powder samples were spread on carbon tape to avoid interference from the substrate in the elemental analysis. All SEM samples were Pt sputter coated for 30 s. The compositions reported here are the average result of three scans at different regions of the samples.

4.4. Particle Size and Distribution. The particle size and the size of distribution were investigated using transmission electron microscopy (JEOL 100 TEM) operated at 100 kV. Ethanol was used to disperse the BGNs through sonication, and 5 μL of the solution was pipetted in a 200 mesh Cu grid.

4.5. Structural Assessment and Surface Charge. Structural analysis was performed with Fourier transform infrared (FTIR) spectroscopy for wavenumbers in the range of 400–2000 cm−1 in the transmission mode. Additionally, the microstructure of the BGNs was examined by X-ray diffraction.
analysis (Rigaku Smartlab XRD) using Cu Kα radiation at 40 kV/40 mA. Data were collected in the 2θ range of 15−70° with a step size of 0.1°. The evolution of the amorphous structure was approached by curve fitting the experimental spectra with Gaussian peaks for an R2 value of 0.99. The coordination of silicon in the synthesized samples was evaluated with 29Si magic angle-spinning (MAS) solid-state nuclear magnetic resonance (NMR). The NMR spectra were recorded on a Varian Infinity Plus 400 spectrometer. Samples were spun in a 5 mm probe at 5 kHz for a spectrometer frequency set to 79.49 MHz. All spectra were evaluated using FTIR.

4.6. In Vitro Formation of an Apatite Phase. The bioactive behavior of the particles was assessed in terms of the apatite-forming ability with an immersion test in Kokubo’s simulated body fluid (SBF). Samples were prepared with a BGN/SBF weight ratio of 3.33:1 and then placed in an incubator at 37°C under constant shaking (175 rpm). After 7 days, the solution was centrifuged, and particles were rinsed with 100% ethanol, dried at 37°C and stored for analysis. The presence of the hydroxycarbonate apatite (HCA) layer was evaluated using 1H→29Si magic angle-spinning (MAS) solid-state nuclear magnetic resonance (NMR). The NMR spectra were recorded on a Varian In

| Protocol | M1-P1 | M1-P2 | M2-P1 | M2-P2 A | M2-P2 B | M2-P2 C |
|----------|-------|-------|-------|---------|---------|---------|
| Solvent Type (in Solution A) | Ethanol | Methanol | Ethanol | Methanol | Ethanol | Methanol |
| H2O (M) (in Solution B) | 12.7 | 12.7 | 12.7 | 7.3 | 7.3 | 7.3 |
| Ratio of H2O (in Solution B) / TEOS (in Solution A) | 55.9 | 55.9 | 55.9 | 32.2 | 32.2 | 32.2 |
| Ratio of NH4OH (in Solution B) / TEOS (in Solution A) | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 |
| Ratio of H2O /EtOH (in Solution B) | 1.1 | 1.1 | 1.1 | 0.56 | 0.56 | 0.56 |
| Stirring Duration X1 (h) | 24 | 24 | 24 | 24 | 48 | 24 |
| Stirring Duration X2 (h) | 0.5 | 0.5 | 24 | 24 | 24 | 24 |
| Stirring Duration X3 (h) | 2 | 2 | 24 | 24 | 24 | 24 |

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c00180

Author Contributions
N.P.-C. performed the experimental work, collected the data, contributed to the design of the experiments and analysis, and drafted the manuscript. X.C. supervised the experimental work, contributed to and supported the experimental design and data analysis, provided critical feedback, and revised the manuscript.

Notes
The authors declare no competing financial interest.

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