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Biodegradable Elastic Sponge from Nanofibrous Biphasic Calcium Phosphate Ceramic as an Advanced Material for Regenerative Medicine

Yonggang Zhang, Jiaping Li, Mohammad Soleimani, Francesca Giacomini, Heiner Friedrich, Roman Truckenmüller, and Pamela Habibovic*

1. Introduction

In the past three decades, a variety of strategies has been investigated for treating large, clinically relevant bone defects that do not heal spontaneously, including natural bone grafts, synthetic bone graft substitutes, and cell- and/or growth factor-based tissue-engineered constructs. 3D porous scaffold materials play an imperative role in the majority of these strategies, mimicking the extracellular matrix properties of bone and providing a template that initiates and/or supports de novo bone tissue formation. Biodegradable porous calcium phosphate (CaP) ceramics, especially beta-tricalcium phosphate (β-TCP) and biphasic calcium phosphate (BCP, mixture of hydroxyapatite (HA) and β-TCP) have been extensively investigated as synthetic bone graft substitutes, owing to their similarity to the mineral component of natural bone, structural similarity to trabecular bone and related biocompatibility, and (controllable) degradation. Biodegradable CaP ceramics have also shown some excellent bioactivity features. Besides being bone-bonding and facilitating new bone deposition on the surface (osteoadhesion), a family of CaP ceramics with unique physico-chemical and structural properties, has been shown to be intrinsically osteoinductive. Thereby, uncommitted cells are triggered to differentiate into the osteogenic lineage, followed by the formation of new bone. Additional advantages of CaP-based bone graft substitutes are that they, unlike natural bone grafts, do not impose risks of morbidity or infection of the donor site, they can be produced in...
large quantities, at a relatively low cost, and easily sterilized and stored.[19] All these characteristics make biodegradable porous CaP ceramics stand out from the rest of the synthetic bone graft substitutes.

However, a limitation of nearly all existing CaP ceramics that cannot be underestimated in the context of bone regeneration, is their intrinsic brittleness.[10,11] Due to their brittle nature, biodegradable CaP ceramics, in particular in the porous form, exhibit poor processability and handling properties, and their clinical applications require additional instrumentation or are otherwise limited to non-load-bearing sites.[12–15] Considering that brittleness is inherent to CaP ceramics, this property seems impossible to change using conventional ceramic production methods.[16]

In the past few years, methods for developing advanced ceramic nanofibrous aerogels and ceramic nanofibrous sponges with exciting, untraditional properties have come under the spotlight, as promising alternative ways to overcome the brittleness of porous ceramics. These materials are fully inorganic, yet exhibit surprising elasticity, and even superelasticity. Examples are silica ceramic nanofibrous aerogels,[17–19] HA aerogels,[20] and nanofibrous oxide (titania [TiO$_2$], zirconia [ZrO$_2$], yttrium-stabilized ZrO$_2$, and barium titanate [BaTiO$_3$]) sponges.[21–23] Besides the unique elasticity, these materials feature highly interconnected porous architecture with open cell geometry and ultrahigh porosity of often above 99%. This unique combination of properties including the inorganic chemistry, elasticity, ultrahigh porosity, and large surface area has paved new avenues for application of porous ceramic materials in many fields including catalysis, thermal insulation, and air purification.[24,25] Nevertheless, the elastic ceramic aerogels and ceramic nanofibrous sponges developed so far are bioinert and/or non-degradable, limiting their use in regenerative medicine.

Herein, for the first time, we have developed a fully inorganic, nanofibrous, elastic BCP ceramic sponge that is moreover degradable and bioactive. This novel material consists of a self-supporting network of seamlessly interwoven HA nanowires and β-TCP nanofibers and possesses a highly interconnected porous structure with open cell geometry and ultrahigh porosity (>99% free volume). Owing to its unique physical properties, the sponge showed excellent processability into different shapes and dimensions, and water-triggered shape-memory behavior that enabled facile and minimally invasive application. Moreover, the nanofibrous sponge showed a significantly higher protein adsorption ability than a conventional porous BCP ceramic and it supported the proliferation and osteogenic differentiation of human mesenchymal stromal cells (hMSCs).

Based on these unique properties, the nanofibrous BCP ceramic sponge developed here could be used as a synthetic bone graft substitute, providing a conducive environment for new bone formation. Additionally, the ceramic sponge could also be used as a guided bone regeneration (GBR) membrane to cover the bone defect, guiding new bone formation and preventing infiltration of the defect by undesired fibrous tissue. In general, the rapid appearance of connective tissue at the bone defect area prevents new bone formation, and often results in incomplete defect healing.[26–28] Taken together, this fully inorganic, highly porous, elastic and bioactive CaP ceramic sponge presents an appealing alternative to current brittle ceramics for bone regeneration.

2. Results and Discussion

The aim of this study was to develop a novel, fully inorganic, biodegradable, and bioactive CaP-based nanofibrous material with ultrahigh porosity, that is, in contrast to most conventional CaP ceramics, elastic (Figure 1). To this end, first, flexible single-crystal HA nanowires with the length of tens of micrometers and the diameter of tens of nanometers were synthesized via hydrothermal reaction (Figure S1A–C, Supporting Information). In addition, β TCP nanofibers were prepared by sintering the precursor ceramic consisting of octacalcium phosphate (OCP) and calcium-deficient HA (CDHA) at 800 °C for 3 h (Figure S1D–F, Supporting Information). The effect of heat treatment temperature on the crystal phase and morphology of the β-TCP nanofibers was investigated. At the sintering temperature of 700 °C, it was not possible to obtain phase-pure β-TCP nanofibers (Figure S2B, Supporting Information). When the sintering temperature was increased to 800 or 900 °C, both precursors, that is, CDHA and OCP (Figure S2A, Supporting Information) were transformed into pure β-TCP (Figure S2C,D, Supporting Information). Based on the combined results of XRD and TEM analysis (Figure S3A–F, Supporting Information), we selected 800 °C as the optimal sintering temperature since the obtained product was pure β-TCP phase while the nanofiber morphology was maintained. β-TCP nanofibers had a length of tens of micrometers and a thickness of tens of nanometers (Figure S3E, Supporting Information). In the second synthesis step, β-TCP nanofibers were added to the HA nanowire slurry and mixed to form a uniform suspension. The suspension was then transferred into molds with desired shapes, and frozen at −80 °C overnight. During the freezing process, ice crystal formation induced phase separation between liquid and solid phase resulting in the self-assembly of HA nanowires and β-TCP nanofibers into a seamlessly interwoven network. Finally, ice crystals were removed by freeze drying, and impurities were washed out (Figure 1A). To confirm the removal of oleates, Fourier-transform infrared spectroscopy (FTIR) analysis was performed (Figure S4, Supporting Information). The absorption bands at 2923 and 2858 cm$^{-1}$ in the FTIR spectrum of the BCP ceramic sponge, belonging to the asymmetric and symmetric C–H stretching vibration of the alkyl group of the oleates, disappeared after washing with hot ethanol (70 °C) and water for three times.

The resulting BCP ceramic sponge was not fragile but showed good elasticity and excellent flexibility, and its shape was tunable by simply choosing a proper mold (Figure 1B,C). Moreover, the ceramic sponge exhibited unique rapid water-triggered shape-memory property, and as a result could be folded and released from a syringe (Figure 1D). More information about the mechanical and handling properties are provided in the next section.

High porosity, interconnected pore structure, and appropriate pore size (often in the range 100–350 μm) are crucial factors for (synthetic) implants, for example, used as bone graft substitutes, to provide a conducive environment for cell attachment, migration, proliferation, tissue infiltration, oxygen and nutrients supply as well as waste removal.[29] The BCP ceramic sponge developed here had a homogenous, highly porous structure with a pore size in the range of 100–200 μm (Figure 2A,B and Figure S5A, Supporting Information). Besides macropores, resulting from the removal of ice crystals during the freeze-drying
process, the sponge structure also comprised pores at the (sub)micrometer scale, that were formed among the interwoven HA nanowires and β-TCP nanofibers, ensuring additional interconnectivity among macropores in the 3D structure (Figure 2C and Figure S5B, Supporting Information). The interconnecting pores of the BCP ceramic sponge had a size in the microscale range, up to 40 µm, as determined from the SEM images (Figure S6, Supporting Information). Due to the highly porous structure, the BCP ceramic sponge exhibited light-weight characteristics. A cylindrical BCP sponge with the dimensions of Ø 1 x 0.9 cm could be placed on the feathers of a dandelion flower without collapsing the structure (Figure 2D). The composition of the BCP ceramic sponge could be easily adjusted by changing the mass ratio between HA nanowires and β-TCP nanofibers during the fabrication process, which was confirmed by the XRD analysis in Figure 2E. The intensity of specific diffraction peaks of β-TCP increased with the increase of the amount of β-TCP compared to that of HA. The density of the BCP ceramic sponge increased with the increase of the β-TCP wt% from 30.9 mg cm⁻³ for 50 wt% β-TCP to 49.5 mg cm⁻³ for 70 wt% β-TCP (Figure 2F). The porosity of the BCP ceramic sponge was ultrahigh ranging from 99.02% for 50 wt% β-TCP to 98.42% for 70 wt% β-TCP (Figure 2G), which was higher than that of the other existing biodegradable BCP and TCP ceramics (Figure 2H and Table S1, Supporting Information). Moreover, the ultrahigh porosity with interconnected hierarchical pores endowed the BCP ceramic sponge with excellent water absorption ability ranging from 3688 wt% for 50 wt% β-TCP to 2382 wt% for 70 wt % β-TCP; this indicates that the BCP ceramic sponge could absorb an amount of water that was as high as 29 times its own weight (Figure 2I).

In the context of clinical applications of bone graft substitutes, important features are the material handling properties, processability as well as the mechanical properties. However, conventional porous CaP bioceramics, commonly fabricated by sintering ceramic powders at high temperatures, followed by post-processing to obtain bulk implants or particulates, are difficult to handle during surgical intervention, because of their intrinsic brittleness. Indeed, it is difficult to adjust the shape and dimensions of sintered ceramic implants to meet
the requirements of different patients. Similarly, handling particulate bone fillers to confine them within the defect area is often considered cumbersome. In contrast to these sintered ceramics, the BCP ceramic sponge developed here exhibited not only highly porous structure, but also very good structural stability and processability. The BCP ceramic sponge was not brittle, but instead, flexible and elastic. For example, after being compressed with a set of tweezers, it could recover to its original shape without an obvious loss of structural integrity (Figure 3A–C and Movie S1, Supporting Information). In order to evaluate the resilience to compression and the ability to recover the porous structure after the release of pressure, we performed a ten-times repeated compression test on the same samples in water by applying two different strains of 30% and 50%, respectively. As is shown by stress–strain curves in Figure 3D–F and Figure S7A–C, Supporting Information, the stiffness of the BCP ceramic sponge decreased after the first test, followed by a further small decrease in the next four tests, after which the stiffness remained relatively stable for the remaining five tests. These results were confirmed by the calculation of the compressive modulus of the BCP sponge during each test (Figure S8, Supporting Information). The compressive modulus of the BCP sponge during the first test was in the range of 35–40 KPa, then decreased significantly during the second test to about a half of the initial value at 30% strain and one-third of the initial value at 50% strain, and finally remained at a value of 10–15 KPa for the rest of the tests. Despite the change of modulus, there was no obvious loss of recovery ability when applying 30% or 50% strains, and the sponges retained their shape and elasticity, independent of the β-TCP amount (Figure S9, Supporting Information). It is hypothesized that the reason for the recovery behavior and structural stability lies in the flexible interwoven network structure of the BCP ceramic sponge. To test this hypothesis, an additional mechanical compression test was performed on a small piece of dry BCP ceramic on a mechanical testing setup that was integrated inside an SEM. First, we observed the deformation behavior of the porous structure of the BCP ceramic sponge at a relative low magnification (Figure S10 and Movie S2, Supporting Information). Under pressure, the porous structure of the BCP ceramic sponge

Figure 2. A–C) SEM micrographs of a BCP ceramic sponge showing hierarchical interconnected porous structure. D) Image of a BCP ceramic sponge sample standing on a dandelion flower. E–G) XRD patterns, density, and porosity of BCP ceramic sponges with different amount of βTCP, respectively. H) A comparison of porosity between BCP ceramic sponges developed here and other BCP and βTCP ceramics described in the literature (more information and references can be found in Table S1, Supporting Information). I) Water absorption ability of BCP ceramic sponges with different amount of β-TCP.
compressed but did not collapse/fracture, and largely recovered its original shape when the load was removed. Subsequently, the mechanical behavior of the interwoven HA nanowires and β-TCP nanofibers was observed at a higher magnification. As is shown in Figure 3G–L and Movie S3, Supporting Information, a bundle of the interwoven fibrous network of the BCP ceramic sponge was observed to bend, and then recover during the loading and unloading process, indeed demonstrating its dynamic flexibility. Finally, to study the behavior of the HA nanowires and β-TCP nanofibers individually, the slurry containing 40 wt% HA nanowires and 60 wt% β-TCP nanofibers (as used for the preparation of BCP ceramic sponge) was washed with ethanol and water. The HA nanowires and β-TCP nanofibers were centrifuged and redispersed in water to form a suspension. Then, a flat BCP membrane was obtained by vacuum filtration of the suspension. As shown in Figure S11A–D, Supporting Information, the BCP membrane had a flat surface with HA nanowires and β-TCP nanofibers laid horizontally. The BCP membrane was then bent, and imaged using SEM at the bent state (Figure S11E,I, Supporting Information). In the concave area where bending of the BCP membrane occurred, some deformed β-TCP nanofibers (Figure S11G,H,J, Supporting Information) and HA nanowires (Figure S11K,L, Supporting Information) were observed, further demonstrating the ability of the HA nanowires and β-TCP nanofibers to bend. Occasionally, few fractured or cracked β-TCP nanofibers were also observed (Figure S11J, Supporting Information), in contrast to HA nanowires, where no signs of fracturing were seen (Figure S11K,L, Supporting Information). This suggests a comparatively more pronounced flexibility of HA nanowires. The observed fracturing of some β-TCP nanofibers may explain the decrease in compressive modulus that was observed after the first cycle of the repeated compression test (Figure 3). Taken together, the HA nanowires and β-TCP nanofibers have shown the ability to deform, thus making the BCP ceramic sponge tough and structurally stable, despite the fact that the CaP ceramic as a material is intrinsically brittle. This phenomenon has been observed before for other types of elastic nanofibrous ceramics. For example, it was suggested that due to the elasticity and bendability of single fibers, observed by in situ SEM during compression, silica [SiO₂] ceramic nanofibrous aerogels and nanofibrous oxide (TiO₂, ZrO₂, yttrium-stabilized ZrO₂, and BaTiO₃) ceramic sponges exhibited extraordinary elasticity and structural stability, rather than brittleness.[17,21]

Besides elasticity and structural stability, the BCP ceramic sponge also showed excellent processability. The shape of a cylindrical BCP ceramic sponge (Ø 15 × 2 mm) could be easily adjusted to desired structure (e.g., a star shape) by cutting it using a pair of scissors (Figure S12A,B, Supporting Information).

In many clinical situations involving replacement of hard or soft tissue such as bone and cartilage, minimally invasive application, such as by an injection, is very attractive since it

![Figure 3. A–C) Illustration of elasticity of BCP ceramic sponge in water. D) The uniaxial compression stress–strain curves of BCP ceramic sponge (50 wt% β-TCP) with compression strain of 30%. E) The uniaxial compression stress–strain curves of BCP ceramic sponge (60 wt% β-TCP) with compression strain of 30%. F) The uniaxial compression stress–strain curves of BCP ceramic sponge (70 wt% β-TCP) with compression strain of 30%. G–L) In situ SEM images of the BCP ceramic sponge during compressive loading and unloading, showing the bending and recovery of a bundle of fibers of the BCP ceramic sponge. Scale bar (A–C): 6 mm; (G–L): 1 µm.]
minimizes the risk of infection, scar formation, patient discomfort, and the cost of treatment.\textsuperscript{[30]} Because of the intrinsic brittleness, conventional porous bioceramics are difficult to process and are not injectable. To address this issue, CaP cements have been developed, however, optimizing setting time and introducing porosity still remain important challenges.\textsuperscript{[31]} Other approaches involve mixing of ceramic particles with a (degradable) binder/carrier, and development of putties, however, additional efforts are needed to control the degradation of the carrier material and ensure that the carrier does not affect the properties and bioactivity of the CaP ceramic.\textsuperscript{[32,33]} Interestingly, the BCP ceramic sponge developed here showed water-triggered shape-memory behavior. Moreover, it was possible to deliver the sponge from a syringe. As shown in Figure 4A,B, a wet, cylindrical BCP ceramic sponge was compressed to the fixed shape, while the water inside the sponge was squeezed out. The removal of the load did not result in the recovery of the original shape; nevertheless, upon wetting, the BCP sponge rapidly recovered its shape by reabsorbing water. The changes in microstructural properties of the BCP ceramic sponge at different stages of water-triggered shape-recovery process are shown in Figure 4C. The original BCP ceramic sponge exhibited an interconnected, cellular porous structure. Upon compression, the pore size anisotropically decreased in the direction in which the load was applied, resulting in a decrease of pore volume, while the water inside the pores was squeezed out. After reabsorbing water by placing the BCP ceramic sponge in water, the microstructure recovered to its original state. We propose the following mechanism for the water-triggered shape-memory mechanism: 1) during compression, the flexible HA nanowires and $\beta$-TCP nanofibers around the pore walls transform from relaxed state to bent state and a certain amount of energy is stored inside the sponge, like in the case of a compressed spring; 2) when the load is removed, the energy stored within the BCP ceramic sponge is not sufficient to overcome the internal material system resistance to return to the relaxed state; 3) when the BCP sponge is then placed in water, the inflow of water aids in overcoming the resistance, resulting in the recovery of the original shape of the BCP sponge. The quantitative results of the shape memory effect are shown in Figure S13, Supporting Information. The fixed ratio, swelling ratio, and recovery ratio during a five-times shape-memory test was calculated by measuring the dimensions of the BCP ceramic sponges in the original shape, compression-fixed shape, and recovered shape. The BCP ceramic sponges were able to recover their original shape within a few seconds upon wetting, further confirming their great flexibility and structural stability.

Figure 4. A) Schematic representation of the shape-memory property of the BCP ceramic sponges. B) Images of a BCP ceramic sponge in the original shape, compression-fixed shape, and recovered shape. C) SEM micrographs of a BCP ceramic sponge in the original shape, compression-fixed shape, and recovered shape. D–F) Images of water-triggered, complete filling of a defect in chicken bone by using a precompressed BCP ceramic sponge.
To demonstrate the applicability of this property in a practical setting mimicking that of orthopedic clinical practice, we created an artificial defect (Ø 6 × 6 mm) in a piece of chicken bone (Figure S14A, Supporting Information). A dry BCP ceramic sponge of the same dimensions was compressed to a state that was smaller than the bone defect (Figure S14B,C, Supporting Information). This allowed for the sponge to be easily placed into the defect by using a pair of tweezers (Figure S14D, Supporting Information). Then, analogous to the sponge being filled with blood upon implantation, water was injected into the bone marrow cavity from the opposite side of the bone, reaching the defect and wetting the sponge. The precompressed BCP ceramic sponge swelled and gradually occupied the space of the bone defect (Figure 4D,E and Movie S4, Supporting Information). Finally, by applying gentle pressure, the ceramic sponge completely filled the defect (Figure 4F), making further adjustment or fixation unnecessary.

Due to the flexibility and shape-memory capability, the BCP ceramic sponge could be placed into and released from a syringe. As is shown in Figure 5A, a BCP ceramic sponge membrane (Ø 1.5 cm × 2 mm) was folded twice to a confined shape. The folded BCP ceramic sponge membrane maintained the folded shape and recovered to original shape rapidly like a flowing bud once it was again in contact with water (Movie S5, Supporting Information). Finally, by applying gentle pressure, the ceramic sponge completely filled the defect (Figure 4F), making further adjustment or fixation unnecessary.

The release of calcium and phosphate ions from the BCP ceramic sponge was determined by measuring the total calcium and phosphorus concentration of a Tris-HCl solution in time upon immersion of the ceramic. As shown in Figure 6A, the BCP ceramic sponge exhibited a relatively rapid calcium release during the initial 14 days, leveling-off up to day 45. The phosphate release profile was similar to that of calcium. At the end of the degradation test (45 days), a total release of 10.8 and 12.3 wt% was observed for calcium and phosphorus, respectively (Figure 6B). The XRD analysis of the BCP ceramic sponge after the degradation test (Figure 6C) showed that the intensity of βTCP peaks relative to the HA peaks decreased after immersion in Tris-HCl, indicating that the release of calcium and phosphate observed can be mainly attributed to the degradation of the βTCP phase. We also determined that the weight

Figure 5. A) Schematic representation of the process of folding and unfolding of BCP ceramic sponge membrane. B) Images of BCP ceramic sponge membrane, syringe, BCP ceramic sponge membrane loaded in the syringe, and injected from the syringe. C) Images of BCP ceramic sponge membrane during different stages of injection. Scale bar: 6 mm.
loss of the BCP ceramic sponge at an intermediate time point of 21 days was ≈9.5%, which is higher than that of β-TCP reported in the literature under comparable conditions (less than 5%).[39,40] even though β-TCP is considered to have a higher degradation rate than BCP.[41] It should be noted that, although the Tris/HCl buffer is often used to study degradation of CaP-based materials in vitro, it does not represent the complex body fluids a material is exposed to in the body. Nevertheless, BCP ceramics have been shown to degrade in vivo, to different extents, depending on different physico–chemical properties.[42,43] It is therefore expected that the material developed here, having ultrahigh porosity and large specific surface area will also be degradable in vivo.

In vitro mineralization in simulated body fluid (SBF) is a common way to study the bioactivity of bone graft substitutes, in terms of their ability to mineralize. The results of in vitro mineralization test of BCP ceramic sponge developed here are shown in Figure 6D–F. A uniform mineral layer was observed on the surface of the pore walls of the BCP ceramic sponge as early as at day 4 of immersion in SBF, and its thickness further increased in time. At day 14, the nanofibrous structure of the BCP ceramic sponge was hardly visible underneath the newly deposited mineral layer (Figure 6F).

Protein adsorption from the surrounding body fluids is the first event that occurs once a biomaterial/implant is in contact with host tissue. The adsorbed proteins in turn play a key role in modulating subsequent cellular responses such as adhesion, spreading, and migration.[44–46] Therefore, we studied the protein adsorption ability of the BCP ceramic sponge by using hemoglobin (Hb) as a model protein. Conventional BCP ceramic, with the same chemical composition (40 wt% HA and 60 wt% β-TCP) and a high porosity of 93.7% (Figure S15A–D, Supporting Information) was used as a control. However, there was a large volumetric difference between the conventional BCP ceramic and the BCP ceramic sponge (Figure 7A). Visually, no differences were observed in the Hb solution appearance before and after immersion of the conventional BCP ceramic (Figure 7B). In contrast, the color of the Hb solution after immersion of the BCP ceramic sponge turned much lighter, for all Hb concentrations used (Figure 7C). SEM images of the ceramic surfaces after immersion in Hb solution showed no presence of proteins on the conventional BCP ceramic surface, whereas abundant proteins were observed on the BCP ceramic sponge (Figure 7D–G). Quantitatively, the amount of Hb adsorbed on the BCP ceramic sponge in a series of Hb aqueous solutions with different concentrations of 300, 600 and 1200 µg mL\(^{-1}\) was 52.7, 69.6, and 101.1 µg mg\(^{-1}\), respectively. These amounts were respectively 12.5, 14.5, and 38.8 times higher than was observed for the conventional BCP ceramic (Figure 7H). The equilibrium concentrations of Hb solutions after adsorption by BCP ceramic sponge were 52.8, 266.1, and 711.5 µg mL\(^{-1}\), respectively, which are considerably lower than the initial concentrations of 300, 600, and 1200 µg mL\(^{-1}\). These results are in contrast with those obtained for the conventional BCP ceramic, where the equilibrium concentrations of Hb solutions were comparable to the initial concentrations (Figure 7I), indicating a lower adsorption ability as compared to the BCP ceramic sponge. The adsorption efficiency of BCP ceramic sponge was as high as 82.4% when the original concentration of the Hb solution was 300 µg mL\(^{-1}\) (Figure 7J). The excellent protein adsorption ability of the BCP ceramic sponge was attributed to its hierarchical porous structure, with the HA wires and β-TCP fibers providing a large surface area for protein adsorption. Despite the high porosity of the conventional BCP ceramic, and the microporous granular surface resulting from sintering at a relatively low temperature, the available surface area for protein adsorption was lower. N\(_2\) adsorption–desorption isotherms of the BCP ceramic sponge and the conventional BCP ceramic are shown in Figure S16, Supporting Information. The specific surface area of the BCP ceramic sponge was 23.15 m\(^2\) g\(^{-1}\), which was ≈3.8 times higher than that of the conventional BCP ceramic (6.06 m\(^2\) g\(^{-1}\)). The high protein binding efficiency observed for the BCP ceramic sponge is not only relevant for the initial biological response to the material upon implantation, but can also be exploited for adsorption and delivery of compounds of interest, such as growth factors stimulating processes relevant to bone formation.
(e.g., osteogenesis, vascularization) or antimicrobial factors, further adding to the bioactivity of this material.\cite{47}

Bone marrow-derived primary hMSCs, which are clinically relevant cells in the context of bone regeneration, were seeded on the BCP ceramic sponge and their attachment and morphology were studied after 24 h and 7 days. At 24 h, cells were abundant and homogenously distributed over the BCP ceramic sponge surface, as shown in Figure S17A–C, Supporting Information. The cells exhibited a spread morphology, with actin cytoskeleton taking the shape of the underlying nanofibrous material. At day 7, a similar observation was made, with more cells present and a more pronounced elongated actin cytoskeleton morphology comparable to the fibrous sponge surface structure (Figure S17D–F, Supporting Information). We then performed a cell penetration test to investigate whether cells could actively infiltrate the porous structure of the BCP ceramic sponge, using the conventional porous BCP ceramic as a control. First, hMSCs were seeded in wells of a non-adherent 24-well plate and after 4 h, ceramic samples were placed into the wells and incubated with the cells for 4 and 7 days, after which the samples were fixed, stained, and imaged using confocal microscopy. During the incubation, hMSCs were expected to migrate to the surface of the ceramic samples and infiltrate the pores. The cross-sectional view of the 3D reconstruction of fluorescence images of the hMSCs in the conventional BCP ceramic and BCP ceramic sponge are shown in Figure 8A–D. At day 4, a thin, flat layer of hMSCs was observed in the conventional BCP ceramic (Figure 8A), located predominantly on the sample periphery. In the BCP ceramic sponge, hMSCs exhibited a more pronounced distribution along the longitudinal axis (Figure 8B). At day 7, deeper cell penetration was observed in both materials. Quantitatively, the penetration in the BCP ceramic sponge was higher than in the conventional BCP ceramic at both time points (≈50 vs ≈20 μm at day 4, and ≈70 vs ≈40 μm at day 7). This may be attributed to the difference in pore size between the two materials (Figures S5A and S15A, Supporting Information). It should be noted that, in general, the penetration depth observed was not very high, which is plausibly due to the “challenging” experimental set-up selected, where cells were required to actively migrate from the tissue.
culture plastic and populate the materials. This is also supported by the results of cell attachment when cells were seeded on the material surface (Figure S17, Supporting Information), showing a homogenous cell distribution on the BCP ceramic sponge.

To further investigate the biocompatibility of the BCP ceramic sponge, a live–dead assay was performed by staining the cells cultured for 24 h on the BCP ceramic sponge with calcein-AM and ethidium homodimer-1 (EthD-1). As was expected, most cells showed intense green fluorescence (Figure S18A, Supporting Information), and the number of dead cells was negligible (Figure S18B,C, Supporting Information).

The quantification of metabolic activity and DNA amounts of hMSCs cultured on the BCP ceramic sponge showed an increase between 7 and 14 days (Figure 8E,F), further confirming that the material was able to support cell proliferation and maintenance of metabolic activity. These results were expected, since CaPs, including BCPs, are known to be biocompatible and already have a history of clinical use. Indeed, previous work has shown the ability of BCP ceramics to support attachment and proliferation of hMSCs.

Finally, ALP activity of cells, normalized for the DNA amounts, was investigated after 7 and 14 days of culture, as a marker of osteogenic differentiation, using the conventional BCP ceramic as a control (Figure 8G). Cells cultured on either material showed ALP activity, with an increase between 7 and 14 days. At both time points, the activity was higher for the BCP ceramic sponge as compared to the conventional BCP ceramic, and the difference was statistically significant at day 14. It should be noted that the cells were cultured in basic medium, that is, without soluble stimulators of osteogenic differentiation such as dexamethasone, indicating that it was the material itself that supported osteogenic differentiation of cells. These results are in accordance with previous studies, in which BCP was shown to support osteogenic differentiation of hMSCs. Taken together, the results of the in vitro cell culture confirmed that the BCP ceramic sponge developed here, with its unique mechanical and handling properties, supported attachment, growth, and differentiation of clinically relevant hMSCs.

3. Conclusion

In this work, a fully inorganic, biodegradable, nanofibrous BCP ceramic sponge with unique elasticity and flexibility, water-triggered shape-memory ability and excellent handling properties was developed by using HA nanowires and βTCP nanofibers as building blocks. The BCP ceramic sponge had an ultrahigh porosity (up to 99%), and an interconnected, hierarchical porous structure with pores ranging in size between 100 and 200 µm down to (sub)micrometer. Unlike intrinsically brittle BCP ceramics, traditionally fabricated by sintering BCP powders at high temperatures, the BCP ceramic sponge exhibited extraordinary elasticity and structural stability, enabling water-triggered...
shape memory behavior and a minimally invasive application, for example, from a syringe. The BCP ceramic sponge can be easily processed into desired shapes and dimensions according to the specific application requirements, which is much more difficult if not impossible for conventional BCP ceramics.

Besides these superior physical properties, the BCP ceramic sponge also presented attractive behavior in an in vitro biological environment, including degradation, a highly pronounced mineralization ability, and a high protein adsorption efficiency. The protein adsorption ability of BCP ceramic sponge was an order of magnitude higher than that of a conventionally produced BCP ceramic. Furthermore, the BCP ceramic sponge was biocompatible, and supported attachment, proliferation, and osteogenic differentiation of bone-marrow derived hMSCs. Taken together, these results demonstrated that the BCP ceramic sponge developed here forms an appealing alternative to current brittle CaP ceramics for various bone regeneration applications, including bone fillers and GBR membrane.

4. Experimental Section

Materials: Materials used were anhydrous calcium chloride (96%, thermo Fisher), sodium oleate (≥82% fatty acids), Sigma-Aldrich), ammonium phosphate dibasic (≥99%, Sigma Aldrich), calcium nitrate tetrahydrate (≥99%, Sigma Aldrich), urea (99.5%, thermo Fisher), ethanol absolute (99.5%, VWR), hydrochloric acid (VWR), Hb (Sigma Aldrich), HA nanopowder (Merck), βTCP (Kuros Biosciences), tris(hydroxymethyl) aminomethane (Tris, VWR), and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, Sigma Aldrich).

Preparation of Hydroxyapatite Nanowire Slurry: In a typical procedure, 17.5 mL of sodium oleate aqueous solution (0.58 M) and 17.5 mL of anhydrous calcium chloride aqueous solution (0.16 M) were mixed for 3 h using a magnetic stirrer. Then, 14 mL of ammonium phosphate dibasic aqueous solution (0.34 M) was added into this mixture and vigorously stirred for 30 min. Next, the obtained reaction suspension was poured into a 100 mL Teflon-lined stainless steel autoclave and placed in oven at 200 °C for 24 h. HA nanowire slurry was then collected from the Teflon bottle. 5 mL of HA nanowire slurry was washed with ethanol and Milli-Q water, and dried. The solid fraction of HA in the slurry was calculated by dividing the volume of the HA nanowire slurry by the weight of HA nanowires within the slurry.

Preparation of Calcium-Deficient Hydroxyapatite and Octacalcium Phosphate Precursors: 100 mL of calcium nitrate tetrahydrate aqueous solution (0.1 M) and 100 mL of ammonium phosphate dibasic aqueous solution (0.0667 M) were mixed for 30 min using a magnetic stirrer. Then, 100 mL of urea aqueous solution (1 M) was added. The pH value of the suspension was adjusted to 3.0 using 0.05 M HCl solution, the suspension was transferred into a 500 mL Teflon-lined stainless steel autoclave, and placed in oven at 180 °C for 135 min. The product, a mixture of CDHA and OCP, was collected, washed with ethanol and Milli-Q water, and dried.

Preparation of Beta-Tricalcium Phosphate Nanofibers: βTCP nanofibers were obtained by sintering the CDHA and OCP precursors in a muffle furnace (Nabertherm L16/14, Germany) at 800 °C for 3 h at a heating rate of 5 °C min⁻¹.

In the manuscript, the term BCP ceramic sponge is used for the BCP ceramic sponge with 60 wt% βTCP, unless otherwise stated. Conventional porous BCP ceramics were prepared to serve as a control. Briefly, HA and βTCP powders (40 wt%:60 wt%) were mixed with 2 wt% chitosan aqueous solution to form a suspension with a solid content of 15 wt%. The suspension was then freeze-dried and sintered in a muffle furnace (Nabertherm L16/14, Germany) at 1100 °C for 3 h at a heating rate of 3 °C min⁻¹.

Further experimental details can be found in the Supporting Information.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

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