1. Introduction

Bioactive glasses and related materials have continued to receive immense attention for application as scaffold in bone regeneration [1]. Some of the promising materials currently used include 45S5 bioglass®, wollastonite, various forms of bioglasses and bioceramics. These materials have the ability to undergo surface reaction in the presence of physiological fluids to form biologically active apatite layer, which has close similarity in composition and structure to the mineral phase of bone. Apatite formation on the surface of a material is considered crucial for further cellular reactions to occur [2], which results in formation of a tenacious bond between the implant material and the host bone.

There is an evidence that nanostructured silica is required for the proper development of cartilage and bone in animals including mammals [3]. Furthermore, silicate-based glasses have become attractive thanks to studies showing that reactions leading to enhanced the new bone formation and growth are related to the controlled release of ionic dissolution products from the degrading bioactive glass, especially critical concentrations of biologically active, soluble silica and calcium ions [2]. Thus, ionic dissolution products from bioactive silicate resorbable glasses have been revealed to enhance osteogenesis by regulating osteoblast proliferation, differentiation, and gene expression [4].

The most successful silicate-based glass is the soda-lime 45S5 bioglass® [2], having the composition: 45% SiO2, 24.5% Na2O, 24.5% CaO, and 6% P2O5 by weight. 45S5 bioglass® is regarded as the gold standard in bone regenerative repair due to its ability to stimulate angiogenic properties, i.e., increased secretion of the vascular endothelial growth factor (VEGF) and the VEGF gene expression in vitro, as well as enhancement of the vascularization in vivo [5]. Additionally, 45S5 bioglass® possesses excellent bioactivity, biocompatibility and degradability, and shows a strong chemical bonding with the neighbouring tissues in vivo [6].

The silica utilized for preparing glasses may be obtained industrially or through other methods, which usually require harsh reaction conditions and high reaction temperatures. In most cases, silica is prepared from simple precursors. It is, thus, rather difficult to reproduce the quality at an industrial scale due to the batch alteration of the physicochemical characteristics. In this regard, biogenic silica may bridge this gap and afford a far superior quality, as it involves biomineralization of silica under ambient conditions by plants. Silica biomineralization on earth, however, is dominated by simple aquatic life forms including unicellular organisms like diatoms. These organisms produce silica-based exo- and endoskeletons that account for the majority of their body mass and
exhibit intricate cell wall patterns in the nano- to micro-meter range (biosilica nanopatterns). Diatom biosilica is mainly composed of amorphous, hydrated SiO₂ (silica) containing a small proportion of organic macromolecules, which have long been speculated to control silica deposition and nanopatterning [7].

The ubiquitous nature of diatoms coupled with their bio-inspired silica production under mild environmentally friendly and economically attractive reaction conditions might offer a convenient route to prepare silicate-based bioactive glasses commercially. Therefore, this work is focused on the use of diatom biosilica as an alternative to tetraethyl orthosilicate (TEOS) to prepare bioactive glass 45S5.

2. Experimental

2.1. Materials

The materials used for preparing the bioactive glass 45S5 include diatom biosilica, sodium metasilicate (Na₂SiO₃·9H₂O; LOBA), calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O; LOBA), citric acid (C₆H₈O₇; Sigma-Aldrich), sodium nitrate (NaNO₃; Sigma Aldrich), phosphoric acid and (H₃PO₄; BDH Laboratory). All chemicals were used as obtained without further purification.

2.2. Preparation of diatom biosilica

The cultured cells of Aulacosea granulata obtained from Lekki lagoon in Lagos, South-west Nigeria were extracted then transferred using a boreasilica micropipette for the culture initiation in series through droplets of sterile water culture (WC) media [8] until the medium surrounding the cells was free of contaminant organisms, thereby ensuring that the initial culture was unialgal. Isolation from this preliminary culture continued, until a bacteria-free stock culture was established. Approximately 80 L of stock culture was maintained in WC media, pH 7.0 within four 20 L glass containers at 12.5 °C on a 16:8 light/dark cycle at 200 µE m⁻² s⁻¹ to achieve an approximate density of 50,000 cells/mL. The cell density was determined by the direct enumeration of cultures via clove oil preparations [9] at St. Cloud State University, USA.

The 20 L stock cultures were used to inoculate the two 800 L cultures in the replicate 1000 L photobioreactors to an approximate density of 50,000 cells/mL at 12.5 °C on a 16:8 light/dark cycle at 200 µE m⁻² s⁻¹. Thereafter, replicate 800 L cultures were used to inoculate a single 25,000 L culture in a 35,000 L photobioreactor to an approximate density of 20,000 cells/mL at 12.5 °C on a 16:8 light/dark cycle at 200 µE m⁻² s⁻¹. Afterwards, approximately half the photobioreactor volume (12,500 L) was harvested using an Origin Oil AA4 high-voltage pulse assisted aggregation system, thus achieving a cell paste consisting of ~20% solids. The harvested culture volume was replaced with fresh WC media. The culture harvest continued, and each time a 20,000 cells/mL density was achieved, in every ~48 h. 100 mg of harvested cell paste was dried at 60 °C for 24 h, yielding ~10 kg of dried algal material. The dried algal material was “cleaned” in batches using concentrated HNO₃ [10]. The cleaned material was washed and centrifuged repeatedly with DDW until a pH of 7.0 was obtained. This process removed all unwanted materials, except A. granulata’s siliceous cell wall. The centrifuged siliceous material was dried at 60 °C for 24 h, yielding ~1 kg of dried A. granulata diatomite — diatom biosilica.

2.3. Preparation of 45S5 bioglass

The 45S5 bioglass with the composition (wt): 45% SiO₂, 24.5% Na₂O, 24.5% CaO, and 6% P₂O₅ was prepared by adding the diatom biosilica gradually to a 35% of freshly prepared citric acid solution under stirring at 120 °C [11]. This condition was maintained for 2 h to dissolve the silica. Then, stoichiometric amounts of H₃PO₄, NaNO₃ and Ca(NO₃)₂·4H₂O were added sequentially, while a 45 min interval between each of the additions was maintained. After adding the last reagent, stirring was allowed to continue for 60 min more. The obtained gel was treated according to the procedure shown in a previous report [12] and stabilized at 700 °C for 2 h, then sintered at 950 °C for 3 h in a muffle furnace operating at a heating and cooling rate of 10 °C/min.

2.4. Characterization

The sintered samples were tested for compressive strength in a universal mechanical tester (Mark-10, Model ESM301L, USA). Load was applied until failure at a cross-head speed of 5 mm/min on five cylindrically shaped samples labelled BG1, BG2, BG3, BG4 and BG5 measuring 8 mm × 4 mm (height × diameter). The compressive strength was determined using the relation:

\[ \sigma_c = \frac{F}{\pi r^2} \]  

(1)

where \( \sigma_c \) is the compressive strength, \( F \) is the applied load at failure and \( r \) is the sample radius. The compressive strength of the material was taken by averaging the values obtained on the five samples.

The microstructure of the samples was evaluated in a scanning electron microscope (SEM: Phenom ProX 800-07334) equipped with energy dispersive X-ray (EDX) analyser. The samples were attached to a sample holder via carbon adhesive for the visual observation at an accelerating voltage of 15 kV. The obtained micrographs were used to estimate the average pore diameter of samples with the software DIAMETER.

The X-ray diffraction patterns of the samples before and after the immersion experiment in SBF were obtained from a GBC eMMA X-ray diffractometer, previously calibrated using a pure standard silicon sample and operating with a CuKα radiation source of wavelength (λ) = 0.154060 nm at 40 kV and 40 mA in the 2θ range from 10°–70°. The samples were first ground to fine, homogeneous powders before they were analyzed. The strongest diffraction peak (at 2θ = 30°) was used to calculate the crystallite size of the main phase in the crystalline sample according to the Scherrer equation:

\[ \xi = (k·λ)/\beta·\cos \theta \]  

(2)

where \( \xi \) is the crystallite size, \( k \) the Scherrer constant (equal to 0.89), \( \lambda \) the wavelength of the CuKα X-ray (1.54060 × 10⁻⁴ m), and \( \beta \) the full width at half maximum of the diffraction peak.

The evaluation of the chemical bond characteristics in the glass network and confirmation of presence of apatite phase on the surface of the samples were performed using Fourier transform infrared spectroscopy (FTIR) with the attenuated total reflectance (Bruker-Alpha, Platinum ATR) operating in the wavenumber range of 4000–500 cm⁻¹.

2.5. In vitro bioactivity determination in simulated body fluid (SBF)

Bioactivity test on the apatite formation in the material was conducted by immersing the sample in SBF (pH 7.3) at 36.5 °C according to the standard in vitro procedure [13] using the following analytical-grade reagents: NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, CaCl₂, tris(hydroxymethyl)aminomethane [Tris—buffer (CH₂OH)₂CNH₂], and 1 M HCl to obtain a similar ionic concentration as found in human blood plasma [13]. The sample was immersed in the SBF solution at a concentration of 0.01 g/mL in a clean,
previously sterilized plastic bottle and then placed in an incubator for 7 days. During the immersion duration, the SBF was not refreshed in order to monitor the daily pH changes. After the sample was extracted from the SBF, it was rinsed with deionized water and left to dry at ambient condition in a desiccator for 4 days. Investigations of the apatite formation on the surface of the samples were then accomplished by SEM, EDX, XRD and FTIR analysis.

3. Results and discussion

3.1. Mechanical properties

Fig. 1 shows the compressive strength data of the five as-prepared glass samples. The representative compressive strength—deflection curve depicting the behaviour of the samples under load is presented in Fig. 2. As observed, there are 4 major stages: in stage I, the material maintains a positive slope while responding to load for a while, then decreases, giving a negative slope (stage II). This is due to cracking of the microstructure at stress-concentrating sites. This stage is followed by stage III where the curve rises again due to densification of the fractured structure, thus resulting in ability of the material to bear higher load. In stage IV, the material collapses completely, which is a typical behaviour of ceramics under load [14]. The average compressive strength of the glass was 3.75 ± 0.18 MPa, which falls in the region for the trabecular bone [15]. Hence, the material could serve as graft substitute for soft bone repair.

3.2. Morphology and composition

A SEM micrograph containing the EDX spectrum of the diatom biosilica is presented in Fig. 3. As observed, the particles are in the size range of 13–19 µm and possess petri-dish-like shapes, similar to those observed earlier by Sumpier and Kröger [15]. Furthermore, the particles are either separated or in clusters. The composition of the diatom biosilica as detected by EDX was 92.4% SiO₂, while the particles are either separated or in clusters. The presence of carbon in the material results from the existence of residual organic macromolecules which control the silica biogenesis [16]. Interestingly, no strange elements and phases as impurities were found.

Diagnostic peaks in the FTIR spectrum shown in Fig. 4 were identified confirming the presence of SiO₂ in the diatom biosilica. The peaks located at 1068 and 797 cm⁻¹ are associated with the siloxane (Si–O–Si) asymmetric stretching and symmetric stretching vibrations, respectively [17], while the deformation mode is observed as a shoulder around 530 cm⁻¹. The diffuse nature of the peak at 530 cm⁻¹ indicates that the sample was amorphous. In addition, the band near 947 cm⁻¹ is ascribed to the Si–OH stretching frequency for silanol groups, while the weak bands at 3450 and 1646 cm⁻¹ are due to O–H stretching and angular vibrations, respectively, of water molecules, an indication that the water content in the sample was low. The absence of carbon bands in the spectrum is due to the low concentration of organic molecules in the sample.

The morphology of the bioactive glass after sintering as observed by SEM is shown in Fig. 5. The surface of the material is covered by well distributed tiny glass particles of average crystallite size of 32 nm, as obtained from Scherrer equation (Eq. (1)). The EDX spectrum presented along with the micrograph confirms the elemental composition of the as-prepared glass. A wide central pore measuring 154 µm in size is clearly seen in the material. A minimum of 100 µm pore diameter has been suggested [18] as adequate for cell infiltration, nutrient delivery and waste removal if a material must serve as a temporary scaffold to guide bone cells formation. Furthermore, the surface of the glass, as observed in the micrograph, appears rough. This feature has been identified as useful for enhanced protein adsorption, incorporation of collagen fibrils, attachment of bone progenitor cells, cell differentiation and excretion of extracellular matrix that precede the process of bone mineralization [19]. Importantly, the morphology exhibited by the glass is excellently comparable with that of the 45S5 bioglass obtained using TEOS as silica source [20].

The morphology and composition of the sample after immersion in SBF for 7 days is presented in Fig. 6. The SEM micrograph shows tiny balls considered as hydroxyapatite, which are in separate clumps. The presence of the apatite is supported by the EDX spectrum. The peaks located at 1068 and 797 cm⁻¹ are associated with the siloxane (Si–O–Si) asymmetric stretching and symmetric stretching vibrations, respectively [17], while the deformation mode is observed as a shoulder around 530 cm⁻¹. The diffuse nature of the peak at 530 cm⁻¹ indicates that the sample was amorphous. In addition, the band near 947 cm⁻¹ is ascribed to the Si–OH stretching frequency for silanol groups, while the weak bands at 3450 and 1646 cm⁻¹ are due to O–H stretching and angular vibrations, respectively, of water molecules, an indication that the water content in the sample was low. The absence of carbon bands in the spectrum is due to the low concentration of organic molecules in the sample.

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The morphology and composition of the sample after immersion in SBF for 7 days is presented in Fig. 6. The SEM micrograph shows tiny balls considered as hydroxyapatite, which are in separate clumps. The presence of the apatite is supported by the EDX spectrum which shows an increase in the concentration of phosphorus and calcium. Also, there is a decrease in the silicon concentration due to the loss of Si to the surrounding SBF, in support of stage 2 of the reactions of a glass immersed in physiological fluids [2]. This stage equally indicates the ability of the glass to degrade in body fluids. It is worth mentioning that the Ca/P ratio of the apatite formed on the surface of the glass during this period was 1.71, which is close to the stoichiometric ratio of natural hydroxyapatite (1.67) [21] and similar to those of sol–gel glasses obtained from TEOS [20,22]. The presence of the carbon peak in the EDX spectrum signifies the crystalline nature of the apatite (carbonated-hydroxyapatite, HCA), while the chlorine is due to the insufficient rinse of the material in deionized water after extracting from the SBF.
3.3. Diffraction patterns

The XRD patterns of the sample after sintering and the phases identified are depicted in Fig. 7(a). The diffractogram contains sharp peaks, most of which match those of the combeite (Na$_2$Ca$_2$Si$_3$O$_9$) crystals, both at the angular location and intensity according to the standard PDF #22.1455 [23]. There are also minor wollastonite (CaSiO$_3$) peaks (JCPDS 42-0547) [24] at very low intensity. Except for the peak located at $2\theta = 30^\circ$, the majority of the combeite peaks are observed at much lower intensity, suggesting semi-crystallization. The formation of the Na$_2$Ca$_2$Si$_3$O$_9$ crystal phase is consistent with that of the sintered 45S5 bioglass, which has been reported to help improve the mechanical properties [14], and hence agrees with the 3.75 MPa value of the compressive strength of the glass.

The XRD spectrum of the glass incubated in SBF for 7 days (see Fig. 7(b)) reveals the emergence of hydroxyapatite peaks ([25] and JCPDS PDF #9-0432). In addition, some of the Na$_2$Ca$_2$Si$_3$O$_9$ peaks originally present in the sample (Fig. 7(a)) disappeared from the spectrum after immersion in SBF, a further testament that the material possesses the ability to degrade in body fluids.
Fig. 6. SEM micrograph and EDX spectrum of the glass after soaking in SBF for 7 days showing increase in Ca and P due to apatite formation.

Fig. 7. XRD diffraction patterns of the 45S5 bioactive glass (a) before immersion in SBF showing the phases present and (b) after soaking for 7 days in SBF showing emergence of apatite peaks.
3.4. Confirmation of bioactivity

The reactivity of the glass sample in the SBF leading to the apatite formation, which was assessed by the changes in pH during the period of immersion in SBF is presented in Fig. 8. During the first 3 days, there is a sharp rise in pH from 7.3 to 8.1. This is attributed to the ion exchange reactions occurring between the Ca\(^{2+}\) and Na\(^{+}\) ions on the surface of the glass and the H\(^{+}\) or H\(_3\)O\(^{+}\) ions of the SBF solution [14], which is enhanced by a good surface topography. After this period, the pH rises slowly, signifying the redesorption of Ca\(^{2+}\) from the SBF solution onto the glass surface to form the apatite [14]. As a consequence, the ion release rate on the glass surface slows down to eventually stabilize over 7 days at a pH of 8.4. This property agrees with a ceramic or glass material undergoing surface reaction in a physiological fluid to form apatite [26].

The FTIR spectra of the as-prepared glass (i.e. at day 0) in Fig. 9(a) show peaks located at 1060, 1010, 931, 682, 644 and 566 cm\(^{-1}\). The peaks at 1060 and 1010 cm\(^{-1}\) are assigned to the asymmetric stretching vibration of Si–O–Si bonds [27], while the peak at 931 cm\(^{-1}\) is attributed to its symmetric stretching mode. The peaks at 682 and 644 cm\(^{-1}\) are due to the vibration of the Si–O bonds in silicate tetrahedra with a bending mode at 566 cm\(^{-1}\), all of which are sharp, suggesting some measure of the crystallinity (as observed earlier in the XRD result, in Fig. 7(a)). After incubation for 7 days in the SBF, as seen in the spectrum in Fig. 9(b), the peak at 566 cm\(^{-1}\) becomes more intense and shifted to 563 cm\(^{-1}\), which is characteristic of the P–O bending mode in PO\(_4\)\(^{3-}\), confirming the formation of hydroxyapatite. Furthermore, the spectrum shows evidence of the presence of carbonated hydroxyapatite at low concentration. The twin bands at 1469 and 1427 cm\(^{-1}\) are due to the asymmetric C–O stretching vibration of the CO\(_2\)\(^{3-}\) group in carbonated apatite [28], which is supported by the small peak located near 601 cm\(^{-1}\). Similar properties have been observed in the previous analysis of 45S5 bioglasses prepared from TEOS [20,29].

4. Conclusion

A 45S5 bioactive glass has been prepared by using diatom bio-silica as the silica source. Analysis has shown that the diatom bio-silica contained mainly SiO\(_2\) and a very small amount of carbon. The mechanical properties indicate compressive strengths in the trabecular bone range, while the morphology revealed well-distributed glass particles giving a favourable topography due to the presence of nanostructured diatom biosilica, which ultimately resulted in an appreciable bioactivity in a simulated body fluid when immersed for 7 days. Importantly, the bioactivity of the 45S5 bioactive glass formed in this work compares with those obtained in previous studies from commercial TEOS. Additionally, the design of the 45S5 composition was possible because there was no interference from unwanted ions in the diatom biosilica. We therefore conclude that diatom biosilica has potentials for utilization as an alternative precursor for the fabrication of bioactive glasses and ceramics for application as scaffold in bone regeneration.

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