Abstract: Reconstruction and augmentation of the alveolar bone defects pose a challenge for the dental surgeons due to its complex structure. The primary objective of tissue engineering is to regenerate or replace damaged tissues or organs including damaged bone tissues with bone grafts, cells, and biological molecules. 45S5-bioglass (45S5-BG), with its superior osteoconductive and osteoinductive abilities, has been at the forefront of tissue engineering, alveolar bone regeneration, and periodontal regenerative surgical procedures for the past several years. With the aim of regenerating supporting alveolar bone, 45S5-BG was synthesized via sol-gel technique. 45S5-BG was characterized by X-ray Diffraction (XRD) and Transmission Electron Microscopy (TEM) analysis. In vitro bioactivity study was validated in simulated body fluid (SBF) and analysed by Fourier-Transform Infrared Spectroscopy (FTIR). In vitro cell compatibility was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using L929 cells. Further, in vivo alveolar bone regenerative potential of 45S5-BG bone graft was evaluated. XRD spectrum confirmed the formation of combeite crystalline phase after sintering. TEM images imparted ultra-structural features of the sample and proved the presence of a major crystalline phase embedded in a glassy matrix. In vitro bioactivity study proved the formation of hydroxy carbonate apatite (HCA) as confirmed by FTIR analysis. The in vitro MTT assay results confirmed the cell compatibility of 45S5-BG and histological analysis proved new bone formation. Within the limitations of this study, the results demonstrated that in addition to the observed bioactive and cell compatible properties, sol-gel synthesized 45S5-BG bone graft exhibited notable alveolar bone regenerative potential.

Key words: 45S5-bioglass, Alveolar bone regeneration, Biomaterial, Cell compatibility, Sol-gel

45S5-bioglass was originally synthesized through conventional melt-quenching technique, where melting oxides of precursors above 1300°C was done followed by the quenching process. Sol-gel based technique enabled synthesis of bioglass with enhanced bioactivity, greater bone-bonding properties, higher purity, homogeneity, higher dissolution rates and positive gene expression leading to accelerated osteogenesis as compared to melt derived glass.

The synthesis protocol for sol-gel bioglass is well documented and researched whereas studies involving sol-gel application for synthesis of Na₂O-containing bioactive glasses or glass ceramics are few, mainly due to high hydrolytic reactivity of sodium alkoxide in water. 45S5-bioglass was the first to be synthesized via modified sol-gel method.

Materials and Methods

Materials

Chemicals used as precursors for the synthesis of the sol-gel 45S5 materials: tetraethyl orthosilicate (TEOS) (Sigma Aldrich, St Louis, MO, USA), triethyl phosphate (TEP) (Sigma Aldrich, St Louis, MO, USA, 99.8%), sodium nitrate (Sigma Aldrich, 99%) and calcium nitrate...
tetrahydrate Sigma Aldrich, St Louis, MO, USA, 99%).

**Sol-gel process**

The molar ratios of TEOS, TEP, NaNO₃ and Ca(NO₃)₂ \(4\text{H}_2\text{O}\) were prepared as per the molar ratio of SiO₂, P₂O₅, Na₂O and CaO in 45S5. To attain a clear sol the molar ratio between water and the four precursor chemicals was set at 10. Each chemical was added at a slow rate into the HNO₃ aqueous solution of 0.3 molar at room temperature. Each compound was added only when the previous solution became clear, and was then stirred for at least 1 h. The resulting gel was dried at 60 and 200°C for 72 and 40 h, respectively, aged at 500°C for 5 h and sintered at 680°C for 2 h.

Characterization of the prepared 45S5-BG was performed using powder X-ray diffraction (XRD) analysis and high-resolution transmission electron microscopy (TEM).

**XRD analysis**

The phase composition of the 45S5-BG after setting was analysed by X-ray diffraction (XRD), (GE, 3003TT, Germany), with CuKα radiation source \( (\lambda=1.54059\ \text{Å} ) \) operated at 40 kV. XRD patterns were recorded from 20° to 60° (2θ) with a step size of 0.04° and a counting time of 2s/step. The size of bioglass crystals in the cement samples were calculated from the characteristic peak fitting \( (20 = 34.09°) \), according to the Scherrer equation, (eq.-1).

\[
D = \frac{K \lambda}{\beta \cos \theta} \quad \text{(eq.-1)}
\]

where, \( D \) = size of crystal, \( K \) = crystallite constant = 0.9, \( \lambda \) = radiation wavelength = 1.54 Å, \( \beta \) = full width half maximum of the (002) peak.

**TEM analysis**

TEM investigation was employed for morphological evaluation and size distribution of the 45S5-BG with JEM-2010F (JEOL, Tokyo, Japan) and image J technical software (version 1.53).

**Evaluation of in vitro bioactivity**

In vitro bioactivity of the 45S5-BG in simulated body fluid (SBF) at pH 7.4 was evaluated using Fourier transform infrared spectrophotometry (FTIR), (Shimadzu, Tokyo, Japan) analysis frequency range 400 - 4000 cm⁻¹. SBF liquid was mixed with 50 ml SBF at 37°C for 7, 14 and 21 days. Samples were then dried at 60°C. FTIR was used to evaluate formation of hydroxy carbonate apatite (HCA) layer.

**In vitro cytotoxicity analysis**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the evaluation of cell proliferation (L929 fibroblasts). For the in vitro cytotoxicity analysis of 45S5-BG, cells were seeded in 96-well plate at a density of 3×10⁴ cells/well and incubated for 24 hours in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA).

All animals involved in the experiment were monitored after the surgical procedures for postoperative complications. Xylazine hydrochloride, 5 mg/kg administered IM, was used when needed for post-surgical analgesia.

**Histologic analysis**

All specimens were examined for features of bone regeneration (shown by osteoblastic activity and osteoid formation) and foreign body reaction (by the presence of giant cells).
Statistical analysis

In cytotoxicity analysis of 45S5-BG, the data was represented as mean±SD (n=3) with P value of 0.05 being significant using Graph Pad prism. The results were analysed by two-way ANOVA with Dunnett’s multiple comparison post-test. Comparisons were performed as Positive control vs other groups (ns-nonsignificant, *p<0.05, **p<0.01, ***p<0.001).

Results

XRD analysis

The wide-angle X-ray diffractogram of the sintered 45S5-BG is depicted in Fig. 1. Major crystalline phase was identified as Na$_2$Ca$_2$Si$_3$O$_9$ (Combeite crystalline phase) (PDF #22.1455). The crystallite size of the main phase in the 45S5-BG found out using Scherrer equation was 24.84 nm at full width half maximum (FWHM) 0.3198 corresponding to the 20 angle of 34.09°.

TEM analysis

Morphological evaluation of 45S5-BG performed using TEM, is represented in Fig. 2. The ultra-structures of the 45S5-BG clearly indicated the presence of crystalline particles embedded in an amorphous glassy matrix after sintering with each particle having an average size of 150 nm. Crystallinity was confirmed from the fringe patterns observed in the TEM images as well as from the selected-area electron diffraction (SAED) pattern.

In vitro bioactivity

FTIR spectra taken on the powder before and after SBF exposure (7, Figure 3. FTIR spectra of Bioglass scaffolds before SBF exposure (BG1) and after SBF exposure 7, 14 and 21 days (BG7, BG14 and BG 21).
In vitro cytotoxicity analysis

In the present study, *in vitro* toxicity profiling of 45S5-BG was performed and is represented in Fig. 4. Among the tested concentrations of 10%, 20% and 40%, exhibited similar cell viability to that of the control. The highest tested concentration of 45S5-BG (100%) exhibited 32.60% of cell viability in comparison to that of the positive control, 10% DMSO (22.33% viability). The cell viability associated with 10%, 20%, 40%, 60% and 80% of the 45S5-BG test concentration was found to be 90.33%, 73.46%, 62.39%, 56.92%, and 52.25% respectively \( (p < 0.001) \).

14 and 21 days to analyse its *in vitro* bioactivity is shown in Fig. 3. Bioglass before SBF exposure (BG1) exhibited characteristic peaks at 460.58 cm\(^{-1}\) attributed to Si-O-Si bending vibrations peak at 1020 cm\(^{-1}\) is due to Si-O-Si stretching and absorption peaks at 513 cm\(^{-1}\) and 584 cm\(^{-1}\) belong to the respective Si-O-Si and P-O bending, originating due to the presence of crystalline phase. SBF treated bioglass exhibited peaks at 558 and 604 cm\(^{-1}\) attributing to P-O bending vibration. Intensification of peaks at 558 and 604 cm\(^{-1}\) are in accordance with the increase in time of SBF exposure to the bioglass.

Figure 5. Group A - Figure A and B - retained intrabony defect where black arrow indicates tooth structure, blue arrow indicates periodontal ligament and white arrow indicate bone margin of retained defect. Group B - Figure C - indicate completely restored defects by the regeneration of periodontal ligament with new bone formation, black arrow indicates tooth structure, blue arrow indicates fully regenerated periodontal ligament and green arrow indicates fully restored intrabony defect with new bone formation. Figure D - yellow arrow indicates resting and reversal lines and red arrow indicates formation of new blood vessels. Figure E - water blue arrows indicate new bone formation with osteoid, yellow arrow indicates resting and reversal lines, red arrow indicates formation of new blood vessels and violet arrow indicates osteocytes.

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In vivo bone regenerative potential of 45S5-BG

Subjective observations - 45S5-BG grafts were easily packed into the intrabony defect. Test and control sites healed uneventfully without any postoperative complications

Histologic evaluation

At 42th day, histological analysis by H and E staining of test and control group animals were devoid of inflammatory infiltrates. There was a significant difference between test and control groups in terms of lamellar and trabecular bone pattern. In Group B, Fig. 5C the osteotomy defect was fully restored with new bone formation with complete restoration of periodontal ligament. Bioglass particles had undergone cellular mediated excavation with the resulting space being filled by newly formed bone and osteoid tissues as presented in Fig. 5D. Group A animals retained the intrabony defect with minimal periodontal ligament regeneration and new bone formation depicted in Fig. 5A, B.

Discussion

Biomaterials capable of inducing specific cellular responses when they come into contact with tissue fluids are considered to be bioactive\(^\text{13}\). Alloplastic graft materials should possess biocompatibility, bioactivity, osteoconductive, osteoinductive properties and provide space for new bone formation. Bioactive glass has the ability to elicit intracellular and extracellular responses at graft and host bone interface and hence has been categorized as Class A material\(^\text{14}\).

Higher the specific surface area, leading to increase in the contact surface between the material and biologic fluid, greater will be the glass bioactivity\(^\text{15}\). Larry Hench in 1982, clinically used bioglass for first time to reconstruct the ossicles of middle ear\(^\text{20}\). US Food and Drug Administration (FDA) approved melt-derived compositions of 45S5-BG for clinical application in regenerative surgical procedures\(^\text{16}\).

Sol-gel synthesized bioglass has bioactivity and degradation rate significantly higher than conventional melt-derived bioglass of the same composition\(^\text{21,22}\). Sol-gel technique enables synthesis of bioglass by altering processing parameters in a highly versatile way\(^\text{9}\). Sol-gel derived bioglass particles possess an increase in pore volume and specific surface area twice magnitude higher than the melt-derived ones\(^\text{20}\). Due to its bone regeneration potential and antimicrobial properties, bioglass is the material of choice for alveolar bone regeneration.

In the present work, we have synthesized, characterized and investigated the preclinical properties of 45S5-BG. XRD analysis of bioglass samples revealed specific crystalline phase for 45S5-BG identified as Na\(_2\)Ca\(_2\)Si\(_3\)O\(_9\) (Combeite crystalline phase) (PDF \#22.1455). The same was identified by the other studies of sintered bioactive glass of same composition\(^\text{23,24}\). There are reports which claim that the crystallinity of the sintered bioglass cannot be 100%\(^\text{25}\). Fringe and SAED pattern confirm presence of crystalline phase.

Bioglass before SBF exposure (BG1) exhibited characteristic peaks at 460.58 cm\(^{-1}\) attributed to Si-O-Si bending vibrations. Peak at 1020 cm\(^{-1}\) is due to Si-O-Si stretching corroborating with the earlier report\(^\text{26}\). In addition, absorption peaks at 513 cm\(^{-1}\) and 584 cm\(^{-1}\) belong to the respective Si-O-Si and P-O bending, originating due to the crystalline phase\(^\text{27}\). SBF treated bioglass exhibited peaks at 558 and 604 cm\(^{-1}\) attributing to P-O bending vibration arising due to surface minerals and amorphous calcium phosphate crystallized to HCA. Intensification of peaks at 558 and 604 cm\(^{-1}\) are in accordance with the increase in time of SBF exposure to the bioglass, highlighting the bioactive characteristics of synthesized 45S5-BG\(^\text{28}\).

Toxicity of biodegradable scaffolds are mainly attributed to release of their degradation end products, which in turn will stimulate or inhibit metabolic activities in the cells\(^\text{29}\).

In vitro cell compatibility was assessed by exposing L929 cell lines to 45S5-BG to analyse for cytotoxicity. L929 Cell lines treated with the 45S5-BG extract (10, 20 and 40%) did not show any significant change in their viability even after 24 h of exposure, in comparison with the control. As a matter of fact, after treating with the highest test concentration (100%), 32.61% of the cells remained viable in comparison to the positive control. As per ISO 10993-5 guideline, a reduction in cell viability greater than 30% is considered a cytotoxic effect\(^\text{30}\).

At 42th day, histological examination of mandibular bone of Group B animals exhibited complete restoration of the intrabony defect with new bone formation in Fig. 5C. Implanted 45S5-BG grafts were completely resorbed, providing space for new bone formation. In group B animals, the intrabony defect showed new bone formation with osteoid, regeneration of periodontal ligament, newly formed blood vessels and increased number of osteocytes. In addition, resting and reversal lines were also noted in Fig. 5D, E indicating remodelling of bone\(^\text{9,20}\). These observations confirm the bone regenerative potential of 45S5-BG.

Group A animals, at 42th day retained the intrabony defect with minimal periodontal ligament regeneration and new bone formation in Fig. 5A, B.

Studies have shown that dissolution products of 45S5-BG will promote differentiation of stem cells into osteoblast and also accelerate gene expression of osteoblast leading to bone regeneration, and influence neovascularization effect promoting the formation of blood vessels \textit{in vitro}\(^\text{9,31}\). As indicated in our \textit{in vitro} bioactivity studies, 45S5-BG graft had reacted with body fluid to form a HCA layer leading to a tight bonding between the bone graft, bone and the soft tissue providing a suitable surface for osteogenic cell attachment and proliferation\(^\text{32}\). It is proposed that this might be the possible mechanism for the observations associated with bioglass assisted bone regeneration.

In the present study, 45S5-BG has been synthesized through modified sol-gel method to investigate its role in alveolar bone regeneration. FTIR analysis confirmed the \textit{in vitro} bioactivity and MTT results proved the \textit{in vitro} cell compatibility of 45S5-BG. Histological analysis proved new bone formation and bone maturation validating its osteoinductive behaviour, demonstrating the potential flour of 45S5-BG bone graft to be used for alveolar bone regeneration material.

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Conflicts of Interest

The authors have declared that no conflicts of interest exist.

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