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Effect of Injectable Bone Substitute Preparation Formulation on Microscopic and Macroscopic Characteristics for Bone Graft

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Abstract

Biocompatible, mechanically stable, and easy-to-use injectable biomaterials are often needed for bone defects caused by trauma, disease, or surgery. Following tumor resection, injectable biomaterials are commonly required to fill the defects and stimulate tissue regeneration. This study aims to investigate the effect of polyvinyl alcohol (PVA) concentration on the microscopic and macroscopic characteristics of injectable bone substitute (IBS) formulations composed of hydroxyapatite (HAp), chitosan, and PVA. The biopolymer chitosan was extracted from pearl oyster shell waste (*Pinctada maxima*), and HAp was synthesized via precipitation. The formulations were prepared with varied PVA concentrations (5%, 10%, and 15%) and evaluated for their structural, physicochemical, and functional properties. Fourier-transform infrared (FTIR) spectroscopy was employed to determine the presence of functional groups and molecular interactions. Results showed that increasing PVA content enhanced the intensity and sharpness of phosphate (PO_4^{3-}) bands, with Sample C (15% PVA) exhibiting the strongest interaction, indicated by a peak shift to 1047.32 cm^{-1}. Organoleptic observations revealed stable color across all samples, with increasing viscosity and paste-like consistency observed in higher PVA concentrations. Sample C had the highest viscosity (82.2 dPa·s) and the lowest injectability (92.43%), while Sample A exhibited the highest injectability (98.33%) and the lowest viscosity (2.79 dPa·s). Sample B (10% PVA) showed balanced characteristics with a viscosity of 38.93 dPa·s and injectability of 97.26%, aligning closely with ideal ranges for injectable biomaterials. Density measurements indicated that all samples approximated or exceeded the minimum density of healthy bone, with Sample C reaching 1.18 g/cm^3. pH monitoring over 21 days revealed a consistent value of ~6, suggesting good chemical stability. These results demonstrate that the 15% PVA formulation achieves an optimal compromise between physicochemical properties and clinical applicability. This composite's injectability enables precise defect filling and promotes new bone formation, making it a superior and promising alternative as an injectable bone graft material in patients.

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Keywords: chitosan, hydroxyapatite, injectable bone substitute, polyvinyl alcohol, shell waste

Introduction

Bone tumors represent a serious pathological condition that often necessitates reconstructive intervention to restore the biomechanical function and structural integrity of the affected bone tissue. Following tumor resection, bone graft materials are commonly required to fill the defects and stimulate tissue regeneration. Although autografts and allografts remain the primary options in clinical practice, their use is associated with several limitations, including the risk of infection, immunological rejection, and limited donor availability. These challenges underscore the need to develop safer, more effective, and accessible synthetic bone graft alternatives.

One of the modern approaches in bone substitute development involves injectable bone substitutes (IBS) biomaterial formulations designed for direct administration into bone defects through minimally invasive procedures. In IBS formulation, biomimetic ceramics such as hydroxyapatite (HAp) are the principal component due to their close resemblance to the mineral phase of natural bone. HAp has been widely utilized in the biomedical field as a coating material for implants and a core constituent of synthetic bone grafts.[1] In Indonesia, various biological waste materials, including bovine bone and pearl oyster shells (*Pinctada maxima* sp.), have been successfully processed as calcium sources for HAp synthesis.[2,3] With its chemical formula $Ca_{10}(PO_4)_6(OH)_2$, HAp is recognized for its biocompatibility and bioresorbability, making it a promising candidate for bone regeneration applications.[4,5] Nevertheless, pure HAp is intrinsically brittle and lacks adequate mechanical flexibility, necessitating further modification by adding other materials to improve its overall performance.[6]

Numerous studies have demonstrated that the properties of composite biomaterials can be significantly enhanced by adding natural and synthetic polymers. Because of their biocompatibility and ability to support tissue regeneration, natural polymers such as collagen, chitin, fibrin, hyaluronic acid, and sodium alginate are frequently utilized.[7,8] In contrast, synthetic polymers such as polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), polyvinyl alcohol (PVA), and polycaprolactone (PCL) possess benefits in terms of chemical stability and ease of fabrication.[9] Chitosan is a promising natural polymer for use in biomedical applications. It has been successfully extracted from waste pearl oyster shells with a purity of over 70%.[10–12] Although instability and contamination risk are two potential limitations of natural polymers, combining them with synthetic polymers provides a synergistic way to get around these issues and enhance the overall qualities of the materials.[9]

The development of bone graft materials, such as the fabrication of PVA/HAp nanofiber composites that can compose scaffold structures that support the growth of new bone tissue, has been the subject of numerous studies investigating the potential of combining bioceramics and polymers.[13] Other investigations reported that a collagen–chitosan/PVA blend not only regulates the degradation rate of the material but also exhibits promising antibacterial activity.[14] Furthermore, the integration of PVA, chitosan, PED, and HAp has been shown to enhance the biodegradation rate and improve the biological response of host tissues.[15] Incorporating polymers into bioceramic matrices such as HAp has significantly improved mechanical strength, degradation control, pore formation conducive to osseointegration, and overall biological compatibility.[13–17]

Despite the considerable potential of local biomaterials such as pearl oyster shell waste, particularly abundant in regions like West Nusa Tenggara, where shellfish cultivation is prominent, research on injectable bone substitute (IBS) formulations derived from chitosan isolated from this waste remains limited. The combination of chitosan, hydroxyapatite (HAp), and polyvinyl alcohol (PVA) within a single formulation

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holds promise for producing injectable bone graft materials with enhanced physical, mechanical, and biological properties. However, several challenges still exist in developing bone filler, including optimizing viscosity for smooth injection, maintaining physiological pH, achieving appropriate density, and confirming chemical interactions through functional group analysis. The importance of injectable bone substitutes' macroscopic and microscopic characteristics must also be examined to assess their effectiveness in clinical applications. While macroscopic characteristics are necessary for ensuring the material can be applied successfully in situ and promote bone regeneration, microscopic characteristics are critical to biocompatibility and bioactivity. Therefore, this study intends to investigate the impact of formulation on the macroscopic and microscopic characteristics of pearl-shell-based chitosan IBS as sustainable and efficient substitutes for traditional bone grafts.

Method

Material

This study utilized several laboratory instruments, including a hot plate magnetic stirrer (IKA C-MAG HS 7, Indonesia), a digital analytical balance (Osuka, China), a pH meter with universal indicator strips, and a Brookfield-type viscometer for viscosity measurements. HAp, chitosan from pearl oyster shell waste (*Pinctada maxima*), polyvinyl alcohol (PVA), distilled water (aqua destillata) as the solvent, and 2% acetic acid solution (CH_3COOH) as the chitosan dissolving medium were the chemical ingredients used in the formulations. Hydroxyapatite and chitosan were obtained by extracting pearl oyster shell waste. The materials were extracted using hydroxyapatite precipitation and chitosan isolation methods.[2,18] Hydroxyapatite was synthesized using H_3PO_4 according to the method of Rahayu et al.[2] In contrast, chitosan was extracted by altering the method of Rahayu et al., which included demineralization, deproteinization, decolorization, and deacetylation.[18]

Procedure

Synthesis of Injectable Bone Substitute (IBS)

This study comprises numerous significant stages, including the preparation of hydroxyapatite (HAp) and chitosan biopolymer solutions, the combining of both components, and the synthesis of a PVA/HAp/chitosan for an injectable bone substitute (IBS) (Figure 1). The procedure began by dissolving chitosan in a 2% (v/v) acetic acid solution for 1 hour to produce a stable biopolymer. Separately, a 4% (w/v) HAp suspension was made by dispersing HAp powder in distilled water and stirring for 2 hours at 60 °C to establish homogeneity. Once both solutions were ready, HAp was gradually introduced dropwise into the chitosan solution in a 7:3 mass ratio, followed by 2 hours of homogenization with a magnetic hot plate stirrer at the same temperature. Adding polyvinyl alcohol (PVA) powder to the homogenized HAp/chitosan mixture was the last step in the formulation process. PVA was added to the composite paste in three different concentrations to optimize its viscosity and injectability: 5% (A), 10% (B), and 15% (C). After that, the paste was heated to 60°C for two hours to produce a uniform IBS composite formulation.

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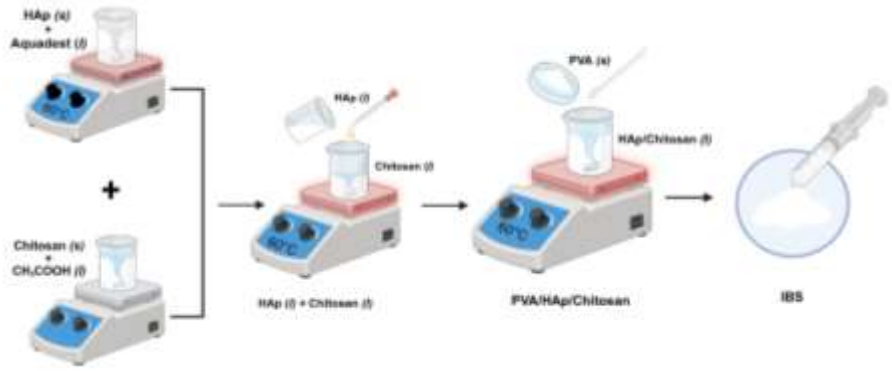

Figure 1. Schematic illustration of injectable bone substitute (IBS) preparation with PVA/HAp/Chitosan

IBS Functional Group Analysis

Functional groups in injectable bone substitute (IBS) formulations containing varying percentages of polyvinyl alcohol (PVA) (5%, 10%, and 15%) combined with hydroxyapatite (HAp) and chitosan were identified using Fourier Transform Infrared (FTIR) spectroscopy. Before analysis, each sample was dried at room temperature and scanned using a Bruker Alpha II FTIR spectrometer fitted with an ATR attachment, over a wavenumber range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1}, with 32 scans per spectrum. The resulting spectra revealed characteristic absorption bands for O−H stretching vibrations around 3200-3600 cm^{-1}, C=O stretching near 1650 cm^{-1}, and PO_4^{3-} stretching between 500-1100 cm^{-1}, indicating the presence of hydroxyl, carbonyl, carbonate, and phosphate functional groups, respectively. The comparison of spectra across PVA concentrations enabled the identification of potential molecular interactions and compatibility between the organic and inorganic components in the IBS composites.

Injectability Test

The injectability test assessed the material's ability to flow consistently when injected with a standard syringe. The injection process was performed at room temperature (25 ± 2°C) with a predetermined syringe size, allowing exact measurement of material flow. The material's flow was observed during the injection process to ensure no significant obstructions or changes in consistency when it was ejected. Digital photos taken at regular intervals to analyze flow behavior were utilized to document the injection process. The mass difference before and after injection was employed to estimate the injectability percentage. An analytical balance (Osuka, China) was utilized to measure the residual mass following injection, and the following formula was applied to determine the injectability percentage[19]:

$$Injectability\ (\%) = \frac{mass\ extruded}{total\ mass\ before\ injection} \times 100\% \quad (1)$$

Organoleptic Observation

Organoleptic evaluation and visual inspection were used to assess the physical characteristics of the injectable bone substitute (IBS) samples. Detected parameters included color, odor, and consistency. The examination was conducted at room temperature (25 ± 2°C) with standard laboratory lighting conditions. The consistency of the substance was tested by monitoring its uniformity and flow behavior with a sterilized glass spatula. Qualified personnel qualitatively assessed the odors and visually monitored color changes against a white background to identify off-odors that suggested

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contamination or degradation. These observations were taken at pre-arranged intervals throughout storage to evaluate the formulation's physical stability.

Density and Viscosity Measurements

The density of the sample was calculated by measuring the solution's mass and volume. A 10 mL solution sample was carefully measured with a volumetric pipette and weighed on an analytical balance (Osuka, China). The density was calculated using the following formula:

$$Density = \frac{mass\ of\ solution}{volume\ of\ solution} \quad (2)$$

These measurements were taken at room temperature (25 ± 2°C) to ensure consistency. Viscosity was measured using a Brookfield viscometer (Brookfield Engineering, USA) with a standard 50 mL sample placed in the viscometer chamber. The spindle was selected based on the expected viscosity range and immersed to a standardized depth. The measurement was conducted at a constant rotational speed of 100 rpm, and the viscosity was recorded in centipoise (cP) and then converted to dekaPascal-seconds (dPa.s) (1 cP = 0.01 dPa.s) for comparison purposes.[20] All tests were performed at 25 ± 2°C to minimize the effects of temperature variations. To ensure accuracy and reproducibility, each sample was measured three times, with at least 10 minutes of equilibration between measurements. The mean viscosity value from the three trials was used for the final analysis, providing reliable data to evaluate the flow properties of the injectable bone substitute material.

Acidity (pH) Test

The pH of the sample was measured daily over 21 days to monitor the chemical stability during storage at room temperature. The measurements were taken using a calibrated pH meter (Model, Manufacturer, Country), and the pH was recorded at regular intervals to observe any changes in acidity over time. The material was allowed to equilibrate at room temperature before each measurement. The acquired data was analyzed to determine potential changes in the material's acidic or basic properties that could affect its performance and stability. This method aids in determining the long-term durability of injectable bone substitutes under conventional storage settings.

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Result

IBS Functional Group Analysis

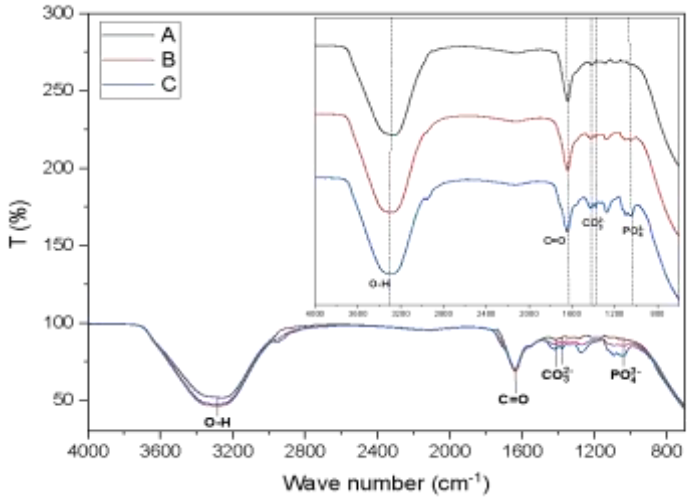

Figure 2. IBS IR Spectrum Analysis of Various PVA Concentration: 5% (Black), 10% (Red), and 15% (Blue)

Table 1. Analysis of Functional Groups and Vibrations of PVA/HAp/Chitosan Preparations

Functional Group	Wavenumber (cm^{-1})		
	A	B	C
O–H (stretching)	3256.84	3294.06	3293.83
C=O (stretching)	1637.62	1638.52	1640.04
CO_3^{2-} (stretching)	1416.04	1419.49	1418.63
PO_4^{3-} (stretching)	1368.33	1374.45	1379.76

Injectability Test, Organoleptic Observation, and Density and Viscosity Measurements

(a) (b) (c)

Figure 3. Analysis of IBS Injectability with Concentration Variations (a) PVA 5%, (b) PVA 10%, and (c) PVA 15%

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Table 2. Analysis of IBS Characteristics of PVA/HA/Chitosan preparations

Parameter	Sample		
	A	B	C
Consistency of being	Liquid	Gel	Paste
Color	White	White	White
Aroma	Odor	Odor	Odor
Injectability (%)	98.33	97.26	92.43
Density (g/cm^3)	0.99	0.99	1.18
Viscosity (dPa.s)	2.79	38.93	822

Acidity (pH) Test

Table 3. Results of pH Observations of IBS Preparations (PVA/HAp/Chitosan)

First week

Sample IBS	pH						
	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7
A	5	6	6	6	6	6	6
B	6	6	6	6	6	6	6
C	6	6	6	6	6	6	6

Second Week

Sample IBS	pH						
	Day-8	Day-9	Day-10	Day-11	Day-12	Day-13	Day-14
A	6	6	6	6	6	6	6
B	6	6	6	6	6	6	6
C	6	6	6	6	6	6	6

Third Week

Sample IBS	pH						
	Day-15	Day-16	Day-17	Day-18	Day-19	Day-20	Day-21
A	6	6	6	6	6	6	6
B	6	6	6	6	6	6	6
C	6	6	6	6	6	6	6

Note: PVA = Polyvinyl Alcohol; HAp = Hydroxyapatite; IBS = Injectable Bone Substitute

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Discussion

The process for producing the injectable bone substitute (IBS) from pearl oyster shell waste was successfully carried out. The formulation of IBS employing a PVA/HAp/chitosan combination with various PVA concentrations allowed for the examination of both macroscopic and microscopic structural properties. This research significantly focused on optimizing Polyvinyl Alcohol (PVA) concentration to achieve specific characteristics in PVA/HAp/Chitosan injectable composites. While the study of injectable bone substitute has been explored previously, this study investigates varying PVA concentrations to develop an Injectable Bone Substitute paste that exhibits ideal viscosity and injectability for effective bone application.

The first microscopic analysis performed was infrared (IR) spectroscopy, which was used to investigate the presence of O–H, PO_4^{3-}, and CO_3^{2-} functional groups in the PVA/HAp/chitosan-based injectable bone substitute (IBS) formulations (Figure 2).[21] A broad absorption band observed between 3256.84 and 3294.06 cm^{-1} was attributed to O–H stretching vibrations, indicating extensive hydrogen bonding among hydroxyl groups in HAp, chitosan, and PVA (Table 1). Notably, the highest wavenumber recorded in sample C (3293.83 cm^{-1}) suggests enhanced hydrogen bonding and hydroxyl group dominance with increased PVA content.[22] Meanwhile, the characteristic C=O stretching vibrations associated with amide I from chitosan were consistently observed in the range of 1637.62–1640.04 cm^{-1} across all samples, indicating the presence of stable amide bonds formed during the gel formulation process.[23,24]

Carbonate functional groups (CO_3^{2-}) were identified within 1416.04–1419.49 cm^{-1} and 1368.33–1379.76 cm^{-1}, likely reflecting either minor carbonate substitution or environmental incorporation during synthesis. The phosphate (PO_4^{3-}) stretching vibrations, indicative of hydroxyapatite presence, were observed at 1039.22 cm^{-1} in sample A, 1042.39 cm^{-1} in sample B, and became increasingly distinct in sample C at 1047.32 cm^{-1}.[21] This shift and sharpening of PO_4^{3-} absorption bands in sample C suggest better dispersion and integration of HAp particles within the polymeric matrix at higher PVA concentrations. These findings imply that the formulation with 15% PVA (sample C) facilitated stronger intermolecular interactions and more effective HAp incorporation, producing a structurally stable and homogeneously distributed IBS gel with potentially enhanced mechanical and biological performance.[13–17]

The molecular interactions observed through FTIR analysis align with the organoleptic observations and physical properties observed. Organoleptic observations demonstrated that all formulations retained a consistent white coloration throughout the 21-day storage period, indicating stability in visual appearance. However, the formulations observed notable differences in texture and odor, reflecting the influence of PVA concentration on physical properties. Sample A (5% PVA) exhibited a semi-liquid consistency and a distinct sour odor characteristic of natural biopolymers, which persisted beyond 14 days of storage. Meanwhile, Sample B (10% PVA) showed a thicker, slightly viscous consistency with a subtler aroma, suggesting better matrix integration and chemical balance. In contrast, Sample C (15% PVA) developed a dense, paste-like texture accompanied by an adhesive-like odor that became dominant around day 13, likely due to the elevated PVA content affecting the volatility and interaction of molecular components.

Injectability testing revealed that all samples could be successfully administered through a standard syringe, suggesting potential as minimally invasive injectable materials. However, resistance varied among samples, as evidenced by differences in injectability percentage. Sample A, being the least viscous (2.79 dPa.s), exhibited the highest injectability rate (98.33%), whereas Sample C, with a significantly higher

viscosity of 822 dPa.s, showed a reduced injectability of 92.43%. Sample B balanced both attributes, with an injectability of 97.26% and viscosity of 38.93 dPa.s, which closely approximates the optimal viscosity range for injectable bone materials (~40 dPa.s) as reported by previous studies.[22] The density values of the samples were also within the ideal range to simulate the physical characteristics of the human bone. Samples A and B both demonstrated a density of 0.99 g/cm³. In comparison, Sample C reached 1.18 g/cm³, which surpasses the density of osteoporotic bone (<0.648 g/cm³) and closely aligns with the average density of healthy bone (>0.833 g/cm³).[24] These results imply that the formulations, especially Sample C, can provide mechanical support comparable to native bone. However, its higher viscosity may present challenges in injectability. In contrast, Sample B, with its favorable density, near-optimal viscosity, and adequate injectability, appears to represent the most balanced formulation for practical application as an injectable bone substitute, capable of conforming to defect sites while maintaining mechanical integrity and ease of administration.[22,25]

The pH evaluation of the injectable bone substitute (IBS) formulations was conducted over 21 days to assess their chemical stability and compatibility with physiological conditions. As is well established, bone biomaterials should maintain a pH close to the physiological range (6.8–7.4) to avoid triggering inflammatory responses or pain upon application. In this study, all IBS samples consistently maintained a pH of approximately six throughout the monitoring period. Although slightly acidic, this value remains tolerable for biological applications. It aligns with the average pH of hydroxyapatite suitable for bone-setting applications, which is approximately pH 6.[25] This stability suggests that the PVA/HAp/Chitosan formulation did not undergo significant chemical degradation or ionic imbalance during storage, thereby preserving its structural integrity for short- to medium-term biomedical use.

However, the uniform pH readings across all samples may also be influenced by the limitations of the measurement method employed. In this investigation, a universal pH indicator strip was used, which provides only approximate readings and lacks the precision of digital pH meters. As a result, minor pH fluctuations due to chitosan hydrolysis, ionic interactions involving HAp, or partial degradation of PVA may have gone undetected. This methodological limitation should be acknowledged when interpreting the results, and it is recommended as an area for refinement in future studies by employing high-sensitivity digital pH meters with continuous monitoring capabilities.

Nevertheless, the apparent pH stability indicates good chemical compatibility among the components of the IBS system. The slightly acidic pH of 6 is likely attributed to residual acetic acid used during chitosan solubilization and ionic interactions between phosphate groups and calcium in hydroxyapatite. The absence of significant pH drift over the 21 days implies resistance to hydrolytic degradation, meeting the chemical stability requirements for injectable biomaterials and supporting their safe use in bone repair, particularly in conditions such as osteopenia or mild osteoporosis.[19]

Conclusion

This study successfully developed and evaluated an injectable bone substitute (IBS) formulation based on a combination of PVA/HAp/chitosan synthesized from pearl oyster shell waste, focusing on the effects of varying PVA concentrations on the material's properties. The formulation containing 15% PVA (Sample C) demonstrated the most favorable characteristics in terms of hydroxyapatite (HAp) integration, as evidenced by FTIR analysis through the phosphate peak shift at 1047.32 cm^{-1} and the intensified O–H stretching band at 3293.83 cm^{-1}, indicating stronger hydrogen bonding. The viscosity of Sample C reached the highest value of 822 dPa.s, which led to a reduced injectability of 92.43%, although still within the injectable range. Furthermore, the density

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of Sample C was measured at 1.18 g/cm³, closely approximating that of healthy human bone (>0.833 g/cm³), thus offering potential for mechanical support. The pH monitoring over 21 days revealed consistent values around 6, indicating chemical stability and limited hydrolytic degradation during storage. These findings suggest that Sample C exhibits a structurally stable and chemically compatible formulation, with strong molecular interactions and bone-like density. However, the elevated viscosity highlights the need for further optimization to enhance injectability while maintaining its favorable mechanical and biological characteristics.

Additionally, using pH indicator strips as the measurement tool represents a significant limitation that must be acknowledged. The low accuracy of this method may have failed to detect subtle pH fluctuations that are critical in evaluating the chemical stability of the material. Therefore, future studies are recommended to employ high-sensitivity digital pH meters further, along with extended evaluations of in vitro and in vivo biocompatibility, to validate this IBS formulation's clinical potential. However, this research provides a valuable contribution to the sustainable utilization of local biomaterial waste in developing next-generation injectable bone substitutes.

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