

Bioactivity of Sodium Silicate Alkali-Treated Titanium Nanocomposites

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Research Article

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Additional Declarations:

The authors declare no competing interests.

Ethics Approval and Consent to Participate: Human red blood cells were obtained from healthy volunteers at the College of Science, University of Wasit, with informed consent obtained from all participants prior to sample collection. The experimental protocols were conducted in accordance with the institutional ethical standards and approved by the Ethics Committee of the College of Science, University of Wasit. Bacterial strains were provided by the Department of Biology, College of Science, University of Wasit.

Bioactivity of Sodium Silicate Alkali-Treated Titanium Nanocomposites

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Abstract. The lack of intrinsic bioactivity and antibacterial functionality in Titanium implant surfaces remains a critical limitation of current implant technologies. Accordingly, this study aims to engineer a multifunctional Titanium surface that promotes apatite formation while inhibiting bacterial adhesion. Hydroxyapatite (HAp) was synthesized using natural biological structures. X-ray diffraction analysis showed that the HAp was hexagonal with an average crystal size of 49.7 nm. X-ray fluorescence analysis of the HAp powder revealed a calcium-to-phosphorus ratio of approximately 1.67. A bio-ceramic composite was prepared from Titanium dioxide (TiO₂) and varying concentrations of HAp (3 and 6 wt.%). The samples were alkaline-treated with sodium silicate. Infrared spectroscopy of the ceramic composite revealed that the functional groups and bonding activity in the sample were characteristic of calcium phosphate phases. To evaluate the biological activity of the samples in vitro, they were immersed in Simulated Body Fluid (SBF). The Energy-dispersive X-ray spectroscopy (EDS) analysis post-immersion in the SBF, the biological activity increased significantly. This indicates the rapid formation of a normal apatite layer due to the alkaline treatment of the sample surface. The ceramic composite samples were imaged using a scanning electron microscope to study their surface morphology. The samples were tested using the inhibition zone method against two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and demonstrated antibacterial activity with inhibition zones ranging from 25 to 33 mm. Toxicity testing using hemolysis showed that the samples were safe, and therefore could be used in various medical applications. In conclusion, the TiO₂/HAp/Ag nanocomposites exhibit a superior combination of bioactivity and antibacterial efficacy. These results indicate that alkaline sodium silicate-treated surfaces are highly promising candidates for bone grafting, offering solutions to implant problems by promoting osseointegration while providing protection against postoperative bacterial infection.

Keywords: Titanium Implants, Hydroxyapatite (HAp), Bio-ceramic composite , Sodium Silicate, silver nitrate.

Introduction

Over the past few years, the development of biomaterials, including ceramics, has witnessed remarkable progress in the field of skeletal repair and reconstruction [1,2]. In recent decades, there has been significant interest in and increasing demand for bio ceramics in various medical applications as a metal alternative to enhance the biocompatibility of implants [3,4]. The need for their use to replace damaged parts resulting from tissue degeneration has grown. With age, bones are more susceptible to fracture due to loss of density and strength [5,6]. In many cases, the only treatment for bone diseases resulting from accidents is bone grafting to replace damaged parts. Therefore, significant bone defects require autologous bone grafting for repair [7]. Many studies have focused on tissue engineering strategies for bone regeneration [8,9]. Scaffolds are a key component in tissue engineering. Implantable biomaterials are also an essential method for bone treatment and regeneration. Bone grafting carries a risk of failure, so the main challenge lies in manufacturing a biomaterial that effectively replaces damaged tissue [10,11],[12]. Bone grafting is a process that carries a risk of failure, so the main challenge is to develop a biomaterial that effectively replaces damaged tissue [13,14]. It is essential to study the properties of bio ceramics to develop better materials with improved biodegradability and biocompatibility, surface properties, mechanical properties, and enhanced bone conduction, by understanding porosity, which plays a crucial role in tissue formation [15,16]. Calcium phosphate compounds are considered the ideal choice for bone grafting materials due to their natural bone components and, consequently, their excellent biocompatibility [17,18]. Hydroxyapatite is the most important calcium phosphate

compound and a promising candidate for bone repair and regeneration. All the Ca-p family compounds or phases listed in Table 1.

HAp is an inorganic biomaterial that forms a strong chemical bond with bone tissue because its chemical composition is similar to that of bone. Bones contain a high percentage of calcium phosphate in the form of HAp, which constitutes the hard part of bone [18-19]. Biologically, HAp has been shown to exhibit high bioactivity, enabling it to accelerate Osseo integration between artificial organs and body tissues. It is also a biomaterial that is non-toxic and non-inflammatory. Therefore, HAp is one of the best materials used in the process of replacing or repairing damaged bones because it reacts quickly with the living environment. Calcium and phosphate ions are not rejected by the human body, making it widely used in bone repair and as a metallic coating material, such as Titanium alloys [20].

On the other hand, Titanium, a bio inert metal used for many years in implant manufacturing and dentistry, is valued for its excellent mechanical properties, including high hardness, high compressive strength, and excellent corrosion resistance. It is also non-toxic to living cells. In numerous studies, Titanium has been used in conjunction with HAp to enhance biocompatibility and improve mechanical properties, either through composites or bio coatings. In this study, HAp was prepared by developing a novel, environmentally friendly, and cost-effective material via heat treatment of biological and natural materials, specifically bovine bones. These bones were used as raw materials to prepare a bio- HAp compound with a calcium-to-phosphorus ratio of 1.67. The aim is to utilize this compound in medical applications.

This study aims to prepare a bio-ceramic composite consisting of TiO_2 and HAp to improve the biocompatibility of implants. As a surgical procedure, bacterial infections can occur, leading to the need for repeated surgical interventions. Therefore, studies are being developed to apply bactericidal agents to the surfaces of implant materials. Silver nitrate (AgNO_3) has been used for this purpose, as silver can disrupt bacterial cell membranes [21], [22]. Surgical implant procedures involve risks of infection related to contamination of the implant itself/or the patient's condition, and the surgical procedure. Therefore, bacterial infection and biofilm formation are the two leading causes of postoperative complications related to implants, followed by rejection reactions. Biofilm formation involves the adhesion of bacteria to a surface, which may be biotic (animal or plant tissue) or non-biotic (minerals, polymers). This is a factor in 80% of infections associated with biomedical implants. Consequently, analyzing this parameter is essential during the development of new biomaterials [23]. In order to achieve high bioactive activity on the surface of bio inert Titanium, this study included an important addition, which is the alkali activation of the ceramic composites surface using sodium silicate, which reduces the activation energy and provides ideal nucleation sites, thereby accelerating the deposition of the bio apatite layer when immersed in simulated body fluid (SBF) [24,25].

Table 1. Calcium phosphate phases [26].

Name	Formula	Abbreviation	Molar ratio
Hydroxyapatite	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hap	1.67
Tricalcium phosphate	$\text{Ca}_3(\text{PO}_4)_2$	TCP	1.50
Octacalcium phosphate	$\text{Ca}_8\text{H}_2(\text{PO}_4).5\text{H}_2\text{O}$	OCP	1.33
Dicalcium phosphate dehydrate	$\text{CaHPO}_4.2\text{H}_2\text{O}$	DCPD	1.00
Amorphous calcium phosphate	$\text{Ca}_3(\text{PO}_4).3\text{H}_2\text{O}_9$	ACP	1.30-1.50

Tetracalcium phosphate	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	TTCP	2.0
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Materials and Methods

Hydroxyapatite nanoparticles were prepared using natural sources, specifically bovine bones. Femur bones from five different animals were used. The bones were thoroughly cleaned and then soaked in distilled water for six hours. All ligaments and cartilage were removed, and the bones were then re-boiled to ensure complete fat removal. A dryer was then used at 75°C for 24 hours. Calcination was carried out in an electric furnace at 700°C for four hours. This was followed by grinding with a DAMFOX rotary centre-blade crusher and sieving with an Azemoun vibratory screen to obtain HAp nanoparticles [27], (See Fig. 1).

Commercial Titanium dioxide, with a molecular weight of 79.6 g/mol, supplied by Nano Research USA Ltd., was used. The particle size was approximately 25 nm. Polyethelene glycol, with a molecular weight of 6000 g/mol, was used as a binder in the preparation of the samples. It was manufactured in Germany by BASF. To prepare the ceramic composite samples, the samples were weighed according to the weight ratio (3 and 6 % wt. HAp). Colloidal solutions of TiO_2 were then prepared, and replaced with different concentrations of HAp. The powders were dissolved in ethyl alcohol by magnetic mixing for 30 minutes at 30 °C. An ultrasonic cleaner was then used for 40 minutes at 30 °C. Finally, filtration was performed to remove residual ethyl alcohol, yielding a TiO_2 /HAp powder. The powders were dried using a dryer at 75°C for 8 hours. To obtain porous samples, paraffin wax was used to prepare them, which were then compressed to 3 tons using a semi-dry axial press. The ceramic composite samples were then heat-treated at 750°C for 1 hour. The resulting samples had good density and were safe for transport.

1. Alkaline Surface Activation using Sodium Silicate

Sodium silicate (Na_2SiO_3) solution was prepared using sodium silicate supplied by Thomas Baker, India, with a molar mass of 122.0632. To prepare a 0.002 M solution, 0.0366 g of sodium silicate was dissolved in 150 mL of distilled water. The ceramic composite samples were immersed in the sodium silicate solution for seven days at 36 °C, pH 7.4. This process represents the alkaline pretreatment stage of the ceramic composite samples prior to immersion in simulated blood fluid. Sodium silicate treatment of the samples is an alkaline preparatory step to enhance the surface bioactivity of the ceramic composites before the simulated body fluid (SBF) immersion test.

The alkaline ions activate the surface of (TiO_2) by partially dissolving its less stable surface fractions and promoting hydrolysis. This results in the development of active hydroxyl groups (Ti-OH) [25]. The silicate ions in solution are then deposited on the active surface, forming a protective and catalytic silica hydrogel layer. This layer, along with hydroxyl groups, provides negatively charged nucleation sites that efficiently and rapidly attract calcium (Ca_2^+) and phosphate (PO_4^-) ions from the simulated SBF (body fluid) solution [24-25]. Therefore, alkaline treatment with sodium silicate significantly accelerates apatite crystal deposition on the sample surface.

2. In vitro bioactivity evaluation protocol

To evaluate the biological activity of the ceramic composites after immersion in the simulated SBF, the SBF (body fluid simulation) immersion method was used according to the Kokobo protocol [25]. This fluid is prepared to mimic the ionic concentration of human blood plasma. The SBF solution was prepared in the laboratory by dissolving the salts listed in Table 2 at concentrations that reflect the ionic concentrations in blood plasma. Deionised water was used, with

the salts sequentially dissolved in 900 mL at 36.5°C to prevent premature precipitation. The pH of the solution was adjusted using 1.0 M sodium hydroxide to achieve the desired value of 7.4 at 36.5°C, which mimics the physiological pH of the body. Samples were washed and dried by placing them in distilled water, and then dried after 30 minutes at 30°C. The samples were put in different containers with a given quantity of the simulated body fluid. These containers were subsequently incubated at 36.5°C, body temperature, in sealed containers for 14 days. To prevent ionic oversaturation, which could slow apatite precipitation, the solution was replenished every 2 days. The samples were taken out of the incubator after 14 days, then washed using deionised water (to eliminate unbound surface salts) and dried using a low-temperature dryer at 45°C, after which they were subjected to analysis.

Table 2. Elements needed to prepare Simulated Body Fluid SBF [25].

Element	The weight
NaCl	7.996 g
NaHCO ₃	0.350 g
KCl	0.024 g
K ₂ HPO ₄ E3H ₂ O	0.228 g
MgCl E6H ₂ O	0.305 g
HCl	40 Cm ³
CaCl ₂	0.278 g
Na ₂ SO ₄	0.071 g
(CH ₂ OH) ₃ CNH ₂	6.057g

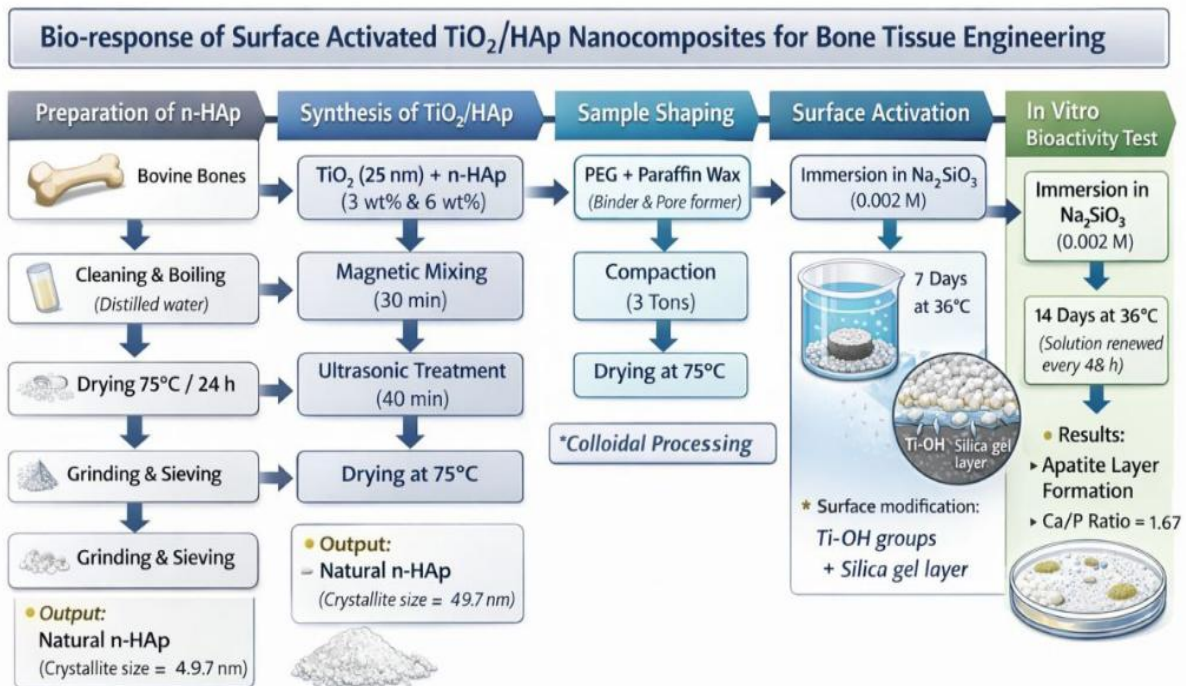


Fig. 1. Schematic diagram of the experimental methodology, including the extraction of biogenic Hydroxyapatite, compound synthesis.

3. Antibacterial Activity

The antibacterial effect of the prepared nanocomposites was tested against *Staphylococcus aureus* and *Enterococcus faecalis* using the agar well diffusion method. Bacterial isolates were obtained from the Department of Biology, College of Science, University of Wasit. A 100 μ L bacterial suspension was spread on Mueller-Hinton agar plates, and wells were filled with the test samples. A bacteria-free medium served as a control, and DMSO was used as a solvent control. Plates were incubated at 37°C for 24 hours, and the inhibition zones were measured in millimeters.

4. Cytotoxicity (Hemolytic Assay)

Human red blood cells were collected from healthy volunteers at the College of Science, University of Wasit with informed consent. RBCs were isolated by centrifugation at 3000 rpm for 10 minutes and diluted in PBS. Hemolysis was evaluated in two ways: Quantitative: Hemoglobin release was measured spectrophotometrically at 540 nm, and the hemolysis percentage was calculated. Qualitative: Cell morphology was examined under a microscope, comparing samples with a positive control (complete lysis) and a negative control (PBS).

Results and Discussion

1. X-ray fluorescence

(XRF) was conducted on the HAp powder that had been prepared to determine the chemical composition of the powder, verify the weight proportion of its constituent elements, and determine the calcium to phosphorus ratio to compare it with the normal ratio in the bone. The analysis of the

test revealed that the prepared compound is composed of major constituents of 55.1% wt, calcium and 32.9 % wt, phosphorus. The calcium to phosphorus ratio of the prepared HAp powder was determined as (Ca:P = 1.67), and this corresponds with the reference values of HAp [27,28],(see Table 3).

Table 3. XRF results showing the percentages of Hydroxyapatite powder elements.

Z	Symbol	Element	Concentration
12	MgO	Magnesium	3.105%
13	Al ₂ O ₃	Aluminum	<0.0038 %
14	SiO ₂	Silicon	<0.0011 %
15	P ₂ O ₅	Phosphorus	0 33.90 %
16	SO ₃	Sulfur	0.07199 %
17	Cl	Chorine	0.06529 %
19	K ₂ O	Potassium	<0.0012 %
20	CaO	Calcium	0 55.10 %
22	TiO ₂	Titanium	%<0.00034
23	V ₂ O ₅	Vanadium	0.00330 %
24	Cr ₂ O ₃	Chromium	<0.00015 %
25	MnO	Manganese	0.00085 %
26	Fe ₂ O ₃	Iron	<0.00014 %
27	CoO	Cobalt	<0.00039 %
28	NiO	Nickel	0.00031 %
29	CuO	Copper	0.00063 %
30	ZnO	Zinc	0.01316 %
31	Ga	Gallium	0.00032 %
32	Ge	Germanium	%<0.00001
33	As ₂ O ₃	Arsenic	<0.00007 %
34	Se	Selenium	<0.00005 %
35	Br	Bromine	0.00017 %
37	Rb ₂ O	Rubidium	0.00015 %
38	SrO	Strontium	0.03713 %
39	Y	Yttrium	<0.00005 %
40	ZrO ₂	Zirconium	<0.00014 %

41	Nb ₂ O ₅	Niobium	<0.00014 %
42	Mo	Molybdenum	0.00208 %
47	Ag	Silver	0.00243 %
48	Cd	Cadmium	0.00172 %
50	SnO ₂	Tin	0.00563 %
51	Sb ₂ O ₅	Antimony	0.00627 %
52	Te	Tellurium	0.00525 %
53	I	Iodine	0.00489 %
55	Cs	Cesium	<0.00040 %
56	Ba	Barium	0.03080 %
57	La	Lanthanum	<0.00020 %
58	Ce	Cerium	<0.00020 %
68	Er	Erbium	<0.00051 %
70	Yb	Ytterbium	<0.00020 %
72	Hf	Hafnium	<0.00010 %
73	Ta ₂ O ₅	Tantalum	<0.00853 %
74	WO ₃	Tungsten	<0.00013 %

2.X-ray diffraction

(XRD) was used to determine the structural properties of HAp using a 1.54 Å copper single-wavelength spectrometer at 45 kV. The results, shown in Table 4 , which present the structural parameters of HAp and compare them with the standard data on the card (JCPDS card No. 9-432), confirm that the prepared material is HAp , as illustrated in Fig.2. The X-ray diffraction patterns are also shown. The interatomic distance (d) values were calculated using Equation (1) [29], and the experimental values were found to be consistent with the standard values (confirming the complete crystallization of HAp with the chemical formula (Ca₁₀(PO₄)₆(OH)₂). The average crystal size (C.S.) was calculated using Equation (2) [30], yielding an average grain size of 49.7 nm.

$$n \lambda = 2d \sin \theta \quad (1)$$

$$C.S = K\lambda / \beta \cos\theta \quad (2)$$

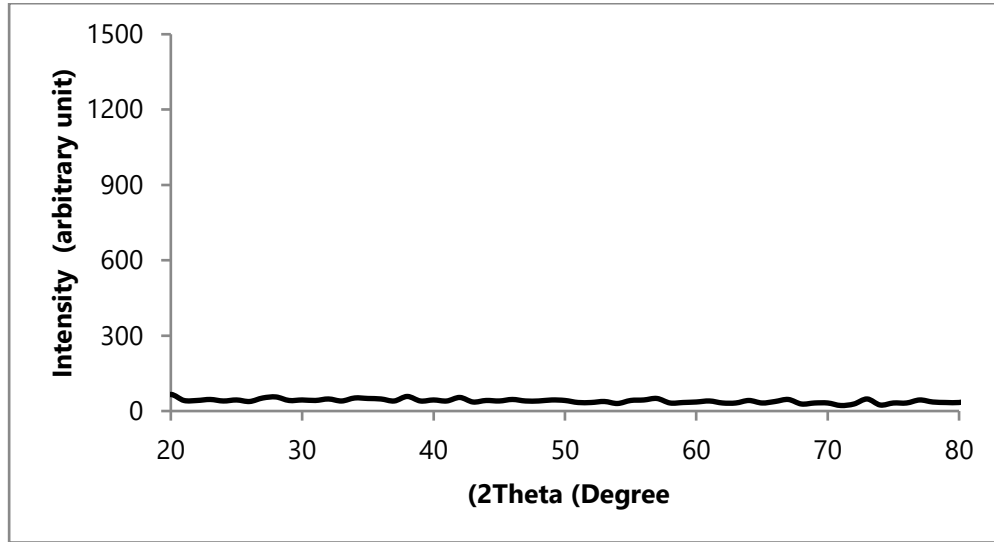


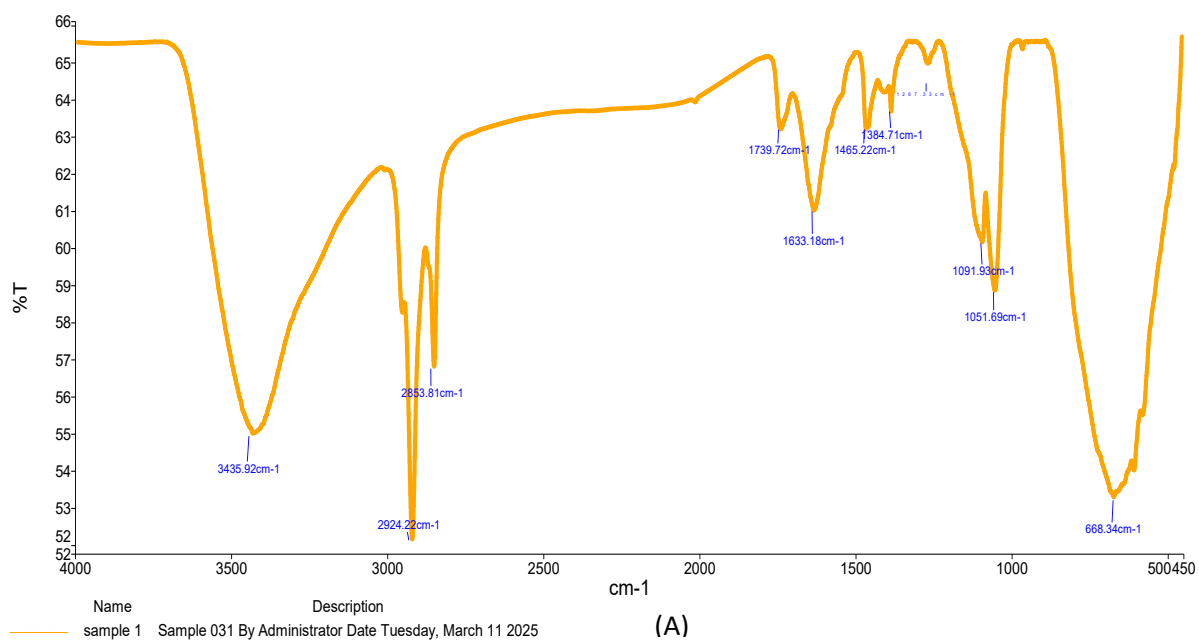
Fig. 2. X-ray diffraction spectrum of Hydroxyapatite Nano composite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$.

Table 4. Structural properties of HAp (Hex. $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), $a=b= 9.41 \text{ }^\circ\text{A}$, $c= 6.88 \text{ }^\circ\text{A}$.

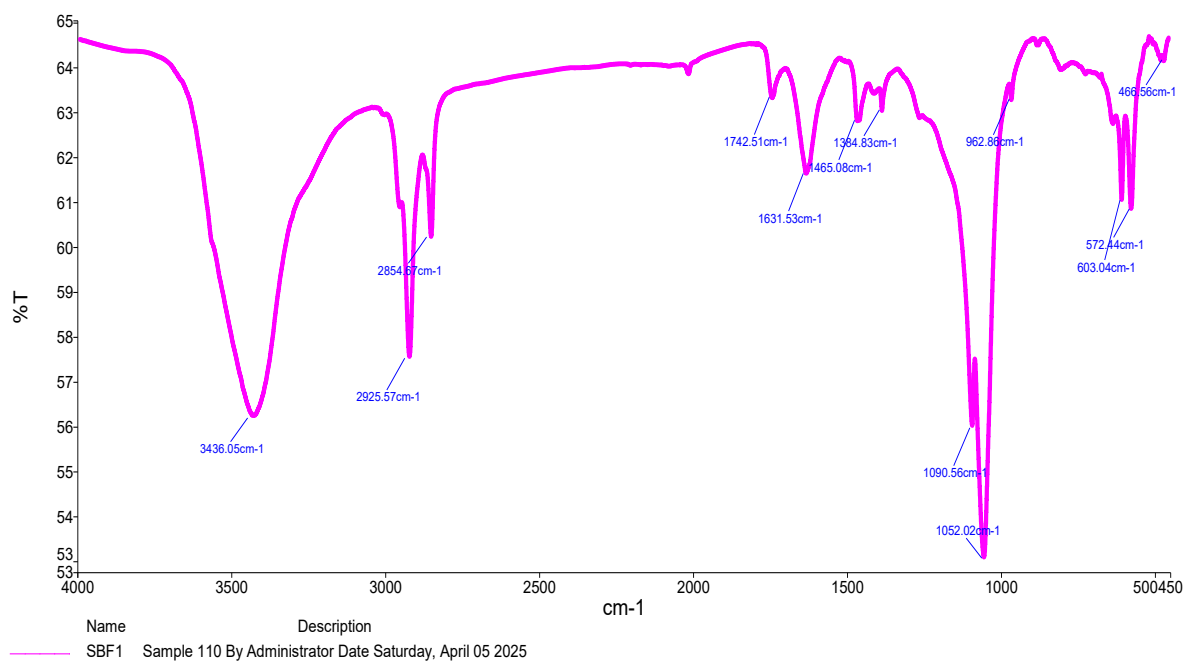
2 θ (Deg.)	C.S (nm)	hkl
31.78	48.53	(211)
25.85	50.79	(002)
32.79	49.58	(300)
39.76	49.92	(310)

3. Fourier transform infrared (FTIR)

Fourier transform infrared (FTIR) spectra were used for all composite ceramic samples (TiO_2/HAp) before and after immersion in simulated blood fluid (SBF), as shown in Fig 3 and Fig.4. FTIR spectra in the wavenumber range (400-4000) were used to identify the chemical bonds and functional groups in the prepared samples, which exhibit standard bands assigned to different vibrations. The FTIR spectra of all samples exhibit broad bands in the high-energy region. The peaks in the wavenumber range ($3640\text{-}3570 \text{ cm}^{-1}$) in the spectrum typically indicate the presence of a hydroxyl group (OH^-). On the other hand, the peaks at ($1025\text{-}1090 \text{ cm}^{-1}$) indicate the presence of phosphate bonds, which may indicate the presence of calcium phosphate or other compounds such as HAp. From the reference values for the structures of common calcium phosphate compounds, focusing on the position and bonds that appear in the FTIR spectrum, we find that the main peaks representing HAp are in the range ($1090\text{-}1030 \text{ cm}^{-1}$) representing (PO) bonds, and in the range ($602\text{-}630 \text{ cm}^{-1}$) which usually indicate vibrations of (P-O) bonds. As for di-calcium phosphate, the peaks appear in the range ($1000\text{-}1070 \text{ cm}^{-1}$) representing (P-O, PO) bonds, and in the range ($1450\text{-}1475 \text{ cm}^{-1}$) indicating the presence of (C-O) bonds. Tri-calcium phosphate appears in the peak range ($1000\text{-}1100 \text{ cm}^{-1}$), which indicates (P-O) bonds, and in the range ($1200\text{-}1300 \text{ cm}^{-1}$), which represents (C-O) bonds. Other phosphate compounds may also appear, such as anhydrous calcium phosphate. Anhydrous calcium phosphate is a chemical compound (salt) that does not contain water in its structure. It is considered a stable form of calcium phosphate that exists in anhydrous form (without water). In the FTIR spectrum, the main peak range is similar to tri-calcium phosphate, but differs from it in the absence of (C-O) bonds. The peak range ($1600\text{-}1650 \text{ cm}^{-1}$).

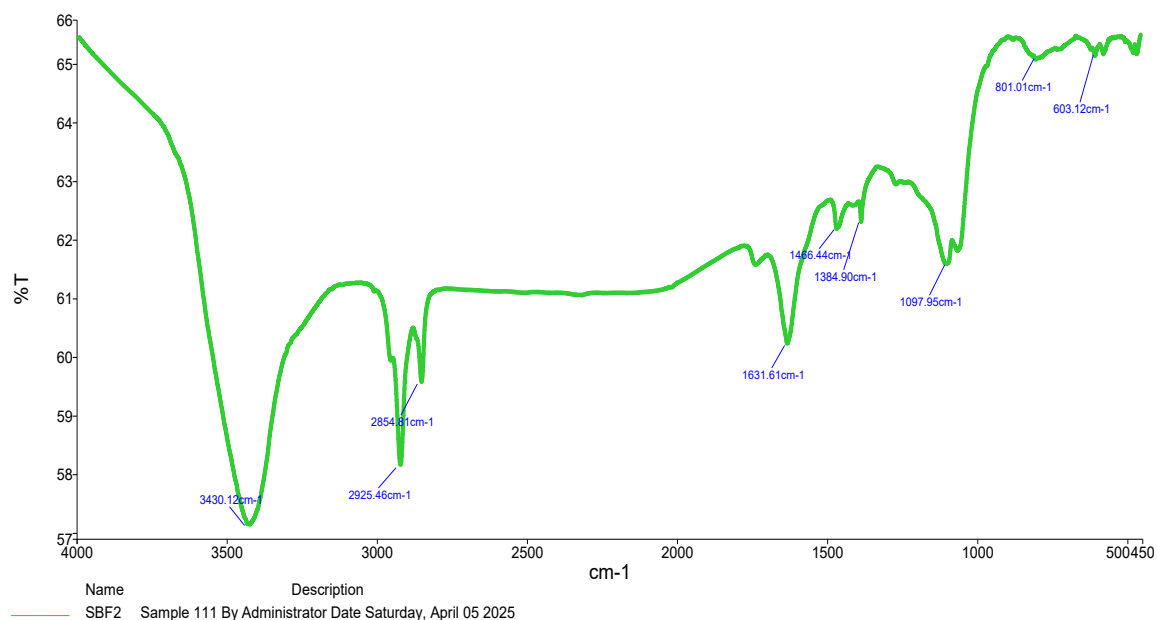
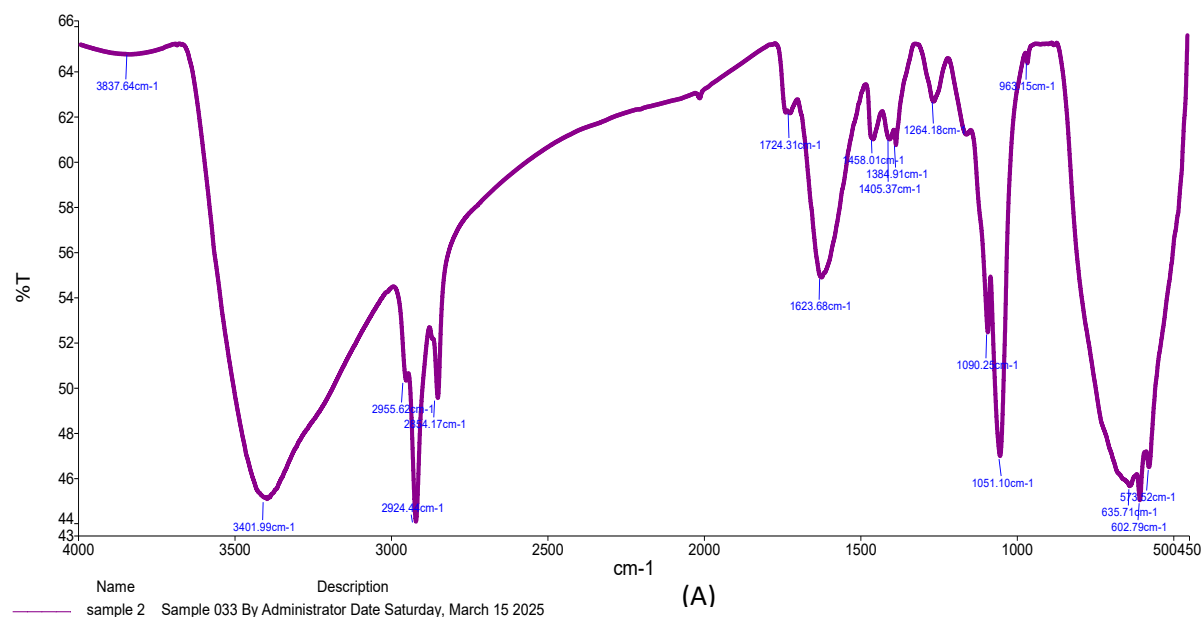


(A)



(B)

Fig. 3. FTIR spectra for the ceramic composite (TiO₂-3%HAp), A. before immersion in SBF, B. after immersion in SBF for 14 days.



(B)

Fig. 4. FTIR spectra for the ceramic composite (TiO_2 -6%HAp), A. before immersion in SBF, B. after immersion in SBF for 14 days.

4. The Energy-dispersive X-ray spectroscopy

The energy-dispersive X-ray spectroscopy (EDS) results for the prepared ceramic composite samples (TiO_2 /3%HAp) before immersion in simulated body fluids are shown in Fig 5 and Table 5. The samples were examined before and after immersion to determine their bioactivity and the effect of the immersion process on the elemental and phase ratios of the prepared composites. In the (3% wt HA) sample before immersion, the molar ratio of calcium and phosphorus was calculated to be 1.62, indicating that the predominant phase in this sample is HA. In contrast, the proportions of other calcium phosphate phases are negligible. After immersion of the same sample in simulated body fluids, we observed a significant increase in the calcium and phosphorus values, as shown in Fig 6 and Table 6. It means that natural apatite is developed through immersion in blood fluids,

which means that this ceramic composite sample is highly biocompatible and can be used in several medical practices.

Table 5. Graphical analysis of energy dispersive X-ray spectroscopy of the (TiO₂- 3% HAp) sample before immersion in the SBF.

Element	Apparent Concentration	k Ratio	Wt%	Wt% Sigma	Atomic %
O	2.43	0.00817	35.53	0.64	61.96
P	0.18	0.00103	0.66	0.12	0.59
Na	0.10	0.00089	0.56	0.12	0.48
Ca	0.24	0.00210	1.07	0.10	0.74
Ti	10.13	0.10135	62.18	0.63	36.22
Total:			100.00		100.00

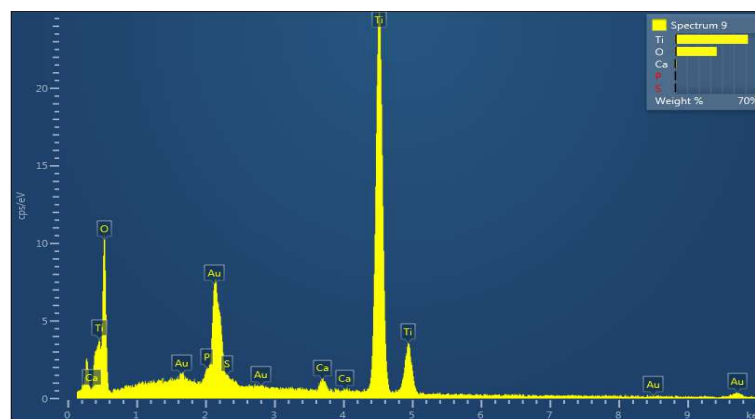


Fig. 5. Energy dispersive X-ray spectroscopy of the (TiO₂- 3% HAp) sample before immersion in the SBF.

Table 6. Graphical analysis of energy dispersive X-ray spectroscopy of the (TiO₂-3% HAp) sample after immersion in the SBF for 14 days.

Element	Apparent Concentration	k Ratio	Wt%	Wt% Sigma	Atomic %
P	4.18	0.02336	15.75	0.26	21.24
Ca	5.88	0.05253	31.30	0.32	32.61
Ti	7.59	0.07589	52.94	0.36	46.15
Total:			100.00		100.00

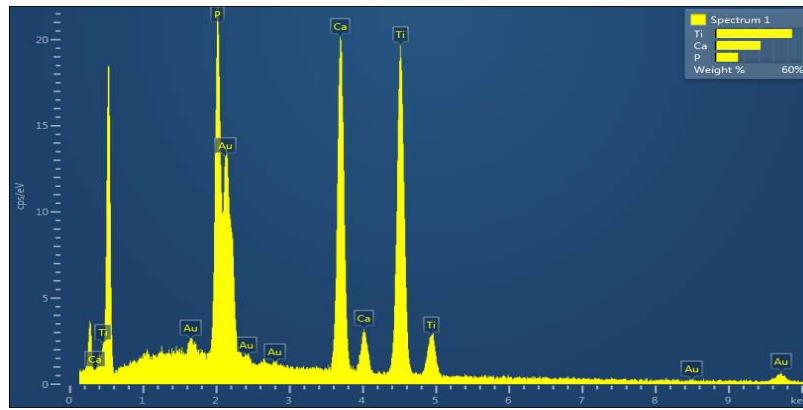


Fig. 6. Energy dispersive X-ray spectroscopy of the (TiO₂- 3% HA) sample after immersion in the SBF for 14 days.

In the sample (TiO₂/ 6%HAp), we found a molar ratio of calcium to phosphorus of 1.64, as shown in Fig 7 and Table 7. This ratio is consistent with the theoretical values for HAp. After immersion in a simulated body fluid for 14 days, the ratios increased significantly, as shown in Fig 8 and Table 8. This indicates the formation of natural apatite upon immersion in a simulated body fluid, resulting in excellent biocompatibility and good bone-conducting properties. These samples can be used in bone replacement procedures. The results demonstrate that the samples exhibit excellent ability to form a large layer of natural apatite and thus have good bone-conduction capacity.

Table 7. Graphical analysis of energy dispersive X-ray spectroscopy of the (TiO₂- 6% HA) sample before immersion in the SBF.

Element	Apparent Concentration	k Ratio	Wt%	Wt% Sigma	Atomic %
O	1.20	0.00403	26.48	0.46	50.99
P	0.47	0.00262	3.75	0.10	2.23
Na	0.07	0.00059	0.50	0.08	0.48
Ca	1.00	0.00891	6.18	0.12	4.75
Ti	7.81	0.07806	63.09	0.43	41.54
Total:			100.00		100.00

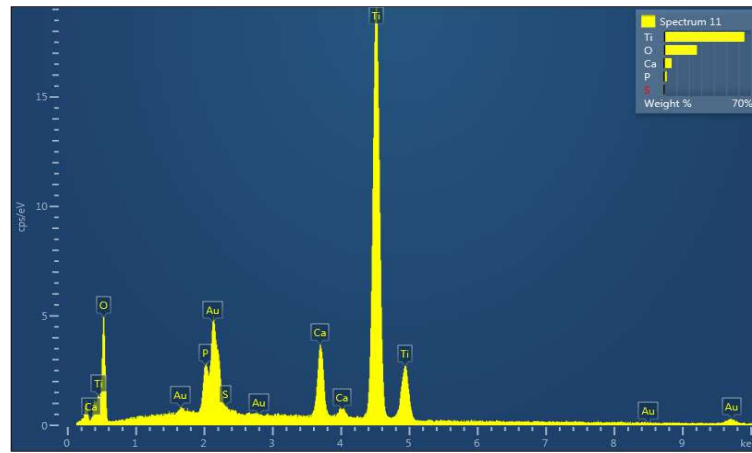


Fig. 7. Energy dispersive X-ray spectroscopy of the (TiO₂- 6% HA) sample before immersion in the simulated blood fluid.

Table 8. Graphical analysis of energy dispersive X-ray spectroscopy of the (TiO₂- 6% HA) sample after immersion in the simulated blood fluid.

Element	Apparent Concentration	k Ratio	Wt%	Wt% Sigma	Atomic %
P	1.36	0.00762	6.10	0.21	8.93
Ca	2.03	0.01816	11.70	0.22	13.24
Ti	10.64	0.10643	82.20	0.28	77.83
Total:			100.00		100.00

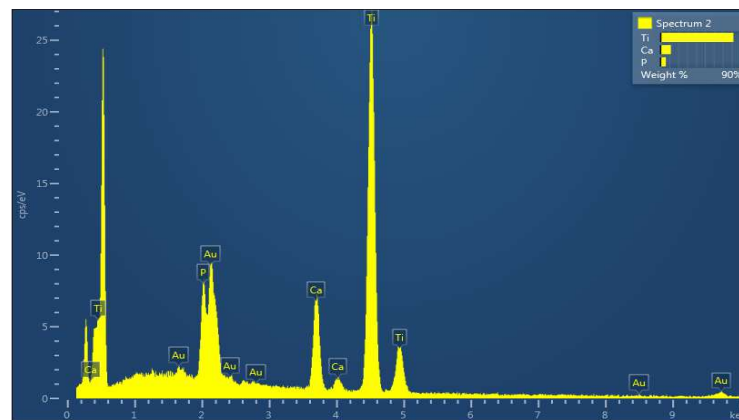


Fig. 8. Energy dispersive X-ray spectroscopy of the (TiO₂- 6% HA) sample after immersion in the SBF for 14 days.

5. Field Effect Scanning Electron Microscope

FESEM testing was performed on the ceramic composite samples (TiO₂/3% HAp), as shown in Fig 9, to determine the morphology of the samples before immersion in simulated blood fluid. On the whole, the samples had a particle size of around 50 nm. The grains were very tight and, at the same time, separated into individual grains without extensive aggregates. A distribution of pores between the grains was also observed. The samples were heat-treated at 750 °C in order to enhance

the grain density, but also to make the sample porous enough to enable bone cohesion and fluid flow and ion movement, thereby creating a layer of natural apatite. The heat treatment regime also resulted in particle fusion and cohesion, reducing interparticle distances and achieving a suitable density while maintaining porosity. Figure 10, shows FESEM images of the ceramic composite samples (TiO_2 /6% HAp) before and after immersion in simulated blood for 14 days. The images reveal small, white, scattered grains on the surface, which are deposits resulting from the accumulation of calcium and phosphate ions after immersion in the simulated body fluid.

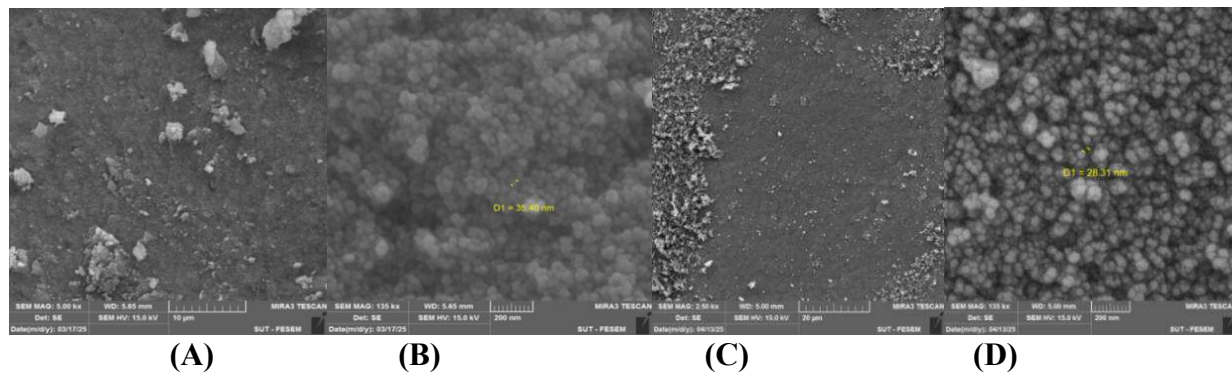


Fig. 9. FESEM images of the ceramic composite samples (TiO_2 / 3% HAp), (A, B) before immersion in the SBF, (C, D) after immersion in the SBF, with different magnifications.

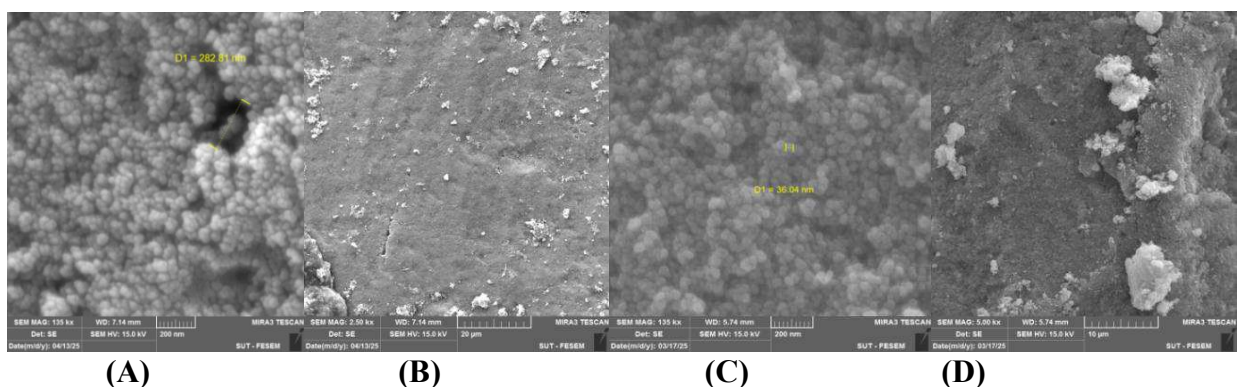


Fig. 10. FESEM images of the ceramic composite samples (TiO_2 / 6% HAp), (A, B) before immersion in the SBF, (C, D) after immersion in the SBF, with different magnifications.

6. Antibacterial Test Evaluation

The biological activity of the prepared ceramic composites, composed of (TiO_2) replaced with varying weight percentages of HAp (3 and 6% wt. HAp) and saturated with 0.005% silver nitrate (AgNO_3), was evaluated after immersion in a simulated body fluid (SBF) for 14 days. This experiment sought to examine how the structural and compositional characteristics of the composites influenced the antimicrobial activity of the composites. The obtained results presented in Table 9, demonstrate that TiO_2 and HAp control samples had no antibacterial activity because these materials are biologically inert. Figure 11, demonstrated that the ceramic composite samples (3,6 % wt) exhibit antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*), with inhibition zone diameters of 25 to 33 mm.

The formation of transparent halos around holes on Mueller-Hinton plates suggests the potential of the composites to prevent bacterial growth, with wider halos indicating stronger antibacterial activity. The three ceramic composite components also present a significant effect in this antibacterial action. The silver nitrate particles are also useful in the formation of silver ions, which can react with the bacterial cellular components, e.g. proteins and DNA, thereby interfering with the

essential processes in the cells and causing their death. TiO_2 also has photo-catalytic and oxidative characteristics, which generate reactive oxygen species (ROS), which disrupt the cell wall of bacteria and enhance membrane permeability [22]. HAp is a biocompatible, porous material that enhances surface adhesion, providing a larger surface area for the gradual release of silver ions and thereby enhancing the antibacterial effect over time [31]. Immersion in simulated body fluid (SBF) for 14 days stabilised the surface structure of the ceramic composite, forming a bioactive layer rich in calcium and phosphate. This controlled the rate of silver ion release and ensured long-term antimicrobial efficacy without affecting biocompatibility [32].

Table 9. shows the biological activity test measured by the inhibition diameter around the prepared compounds (TiO_2/HAp) against Gram-positive bacteria.

Sample	Activity-Inhibition zone of Ag against bacteria measured by mm	
	<i>S- aureus</i>	<i>E- faecalis</i>
TiO_2	11	9
Hap	3	7
$\text{TiO}_2/3\% \text{ HAp}$	25	25
$\text{TiO}_2/6\% \text{ HAp}$	33	27

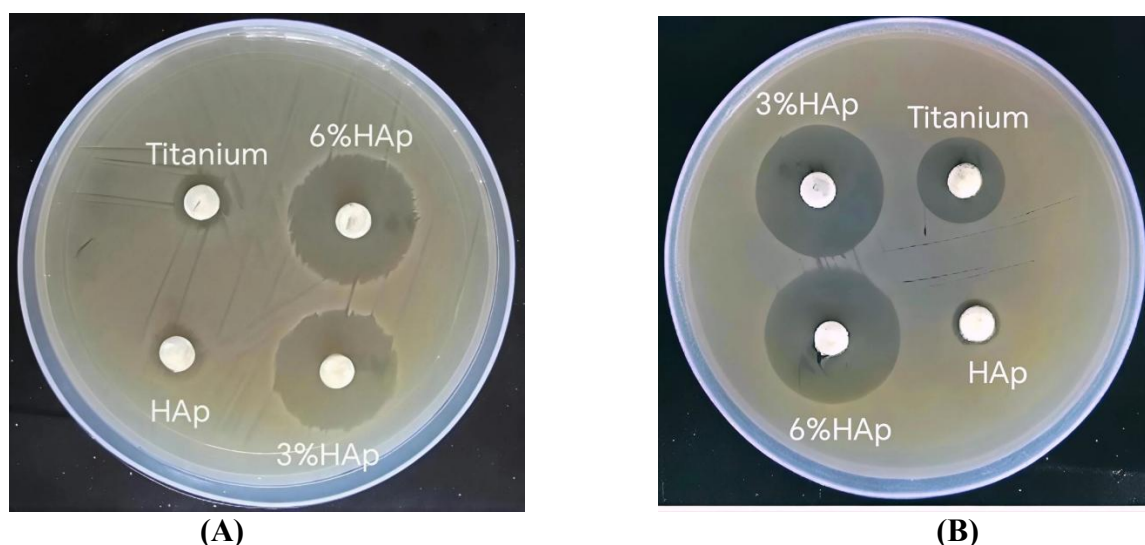


Fig 11. (A,B) Images of antibacterial tests for A- *Enterococcus faecalis*, B- *Staphylococcus aureus* for ceramic composite samples (TiO_2/HAp) doped with 0.005 silver nitrate with different concentrations of hydroxyapatite.

7. Cytotoxicity test by hemolysis assay

A hemolysis assay was performed to evaluate the compatibility of the synthesized bioceramic composites ($\text{TiO}_2/\text{HAp}-\text{Ag}$) with blood, as shown in Table 10, which represents the percentage of human red blood cell hemolysis by these composites. The symbol (N.H) indicates no hemolysis, indicating the absence of cytotoxicity. This was confirmed by observing the color change in the test tubes containing the material and a suspension of purified human red blood cells, compared to the positive control sample (^+C). After two hours of incubation, neither sample showed hemolysis, as

shown in Table 11, shows the change in UV absorbance values at a wavelength of (540 nm). This is seen in the lack of the dark red color in the upper liquid whereas the positive control sample had intense redness as a result of total cell hemolysis. Microscopic analysis of blood samples was done to establish that the compounds did not influence cell morphology. The red blood cells did not exhibit toxic or hemolytic effects of the compounds and their biconcave shape was maintained and no evidence of membrane rupture or shrinkage was detected. This good compatibility with blood is explained by the synergistic physicochemical interaction of the constituents of the compound [33]. The HAp creates a non-toxic, stable and inert surface, which limits the direct contact with red blood cells [34,35].

Table 10. The percentage of human red blood cells being lysed (hemolytic rate) by compounds (1), (TiO₂/3%HAp- AgNO₃) (2), (TiO₂/6%HAp -AgNO₃).

Concentrations	Hemolysis rate of samples HR%		+ Control	- Control
	1	2		
1/10	N.H	N.H	100%	N.H
1/100	N.H	N.H	100%	N.H
1/1000	N.H	N.H	100%	N.H
1/10000	N.H	N.H	100%	N.H
1/100000	N.H	N.H	100%	N.H

Table 11. Represents the change in absorbance values with respect to concentrations.

Concentrations	Absorption
1/10	0.6
1/100	0.6
1/1000	0.6
1/10000	0.6
1/100000	0.6
+ ve control	1.25 nm
- ve control	0.6 nm

Discussion: Synergistic Bio-activity and Antibacterial Efficacy.

Despite the body's capacity for self-healing, the treatment of significant segmental bone defects still requires bone grafting using biocompatible bone implants. These implants must combine sufficient mechanical strength with a micro-porous structure to promote tissue growth. Therefore,

despite the successful use of metals in orthopaedic surgery and their replacement, several major long-term challenges remain for metal implants within living tissue. Thus, current research focuses on developing multifunctional materials that simultaneously offer high mechanical integrity, enhanced biological properties, and biocompatibility to ensure long-term implant integration and minimise postoperative damage. In this study, one of the bone components (HAp) was prepared from a natural biological structure, namely bovine bone. TiO₂ nanoparticles (anatase phase) were used to synthesise a bioceramic composite consisting of TiO₂ replaced by varying concentrations of HAp. X-ray diffraction showed that the composite is hexagonal HAp, with a crystallite size of 49.7 nm. The X-ray fluorescence analysis of the HAp composite indicated that the primary constituents of the powder were calcium and phosphorus, and the molar ratio of calcium and phosphorus was 1.67 Ca/ P.

IR analysis of the samples of ceramic composites prior to immersion in SBF indicated the presence of chemical bonds that represented various stages of calcium phosphate. The outcomes revealed the development of new peaks of HAp after 14 days of immersion in SBF. The samples were analyzed prior to immersion in SBF using X-ray energy dispersion spectroscopy to establish the chemical composition and estimate the molar ratios of calcium and phosphorus. These ratios were in line with reference values. Analysis of the same samples after immersion in SBF indicated high levels of calcium and phosphorus. The bioactivity of the 3% sample was higher than that of the 6% HAp sample. Such behavior suggests that the sample can form a natural apatite layer potentially enabling its use in orthopedic surgery.

The rapid apatite layer formation can be attributed to the synergistic effect of alkaline treatment with sodium silicate and the use of high-purity, biologically derived HAp containing Na, Mg, and other elements not present in commercially available HAp. Furthermore, the silicate treatment significantly contributed to the formation of silanol (Si-OH) groups on the Titanium surface, which act as active sites for crystal nucleation. This gives the surface a negative charge that attracts calcium ions (Ca²⁺) from the SBF solution [36-37].

The ceramic composite samples were analyzed under a scanning electron microscope before and after exposure to simulated blood fluid to determine surface morphology. The findings suggest that the samples contain nanoparticles no larger than 50 nm. To increase the samples' antibacterial activity, 0.005 M silver nitrate was added. The antibacterial activity of the samples was then examined using the inhibition zone technique, and the method was applied to the two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*). The samples were antibacterial, with inhibition zones ranging from 25 to 33 mm. The improved functional performance of TiO₂/HAp-Ag ceramic composite is primarily due to its regular porous structure and the balanced distribution of silver nanoparticles within interconnected pores. These pores act as retention zones (reservoirs), allowing the diffusion of biological fluids and ions. This behavior enables the stable release of silver ions (Ag⁺) rather than a rapid and sudden release. The surface porosity creates a controlled pattern of silver ion release, thus maintaining an effective concentration for a longer period [38]. Cytotoxicity testing using hemolysis did not show any hemolysis of the ceramic composite samples. Microscopic examination of blood membranes confirmed that the compounds did not affect cell morphology, indicating the absence of cytotoxic or hemolytic effects.[39-40]

Conclusion

According to the results of this study, it can be concluded that the silver-doped TiO₂/HAp/Ag ceramic composite exhibits promising physicochemical and biological properties. The synthesis of HAp from natural bovine bone, combined with silver nitrate, resulted in a biocompatible material with significant antibacterial activity against *S. aureus* and *S. faecalis*. These findings suggest that the prepared composite is a viable candidate for medical applications as a bone graft that promotes self-healing of bone tissue through a micro-bio-mimicry mechanism.

While the current study successfully demonstrates the bioactivity and antibacterial potential of the TiO₂/HAp/Ag nanocomposite, it serves as a preliminary foundation. Future research will focus on evaluating the long-term mechanical fatigue behavior and conducting in vivo clinical trials to fully assess the integration of these implants within living bone tissue.

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