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SYNTHESIZE AND CHARACTERIZATION OF ARTIFICIAL HUMAN BONE DEVELOPED BY USING NANOCOMPOSITE

Pham Van Dong
Department of Science and Technology[1]

Santosh R. Patil
Department of Mechanical Engineering[2]

Shailesh S. Shirguppikar
Department of Mechanical Engineering[2]

Do Ngoc Tu
Department of Industry Tool and Equipment
Faculty of Mechanical Engineering[1]

Nguyen Huu Phan
Department of Industry Tool and Equipment
Faculty of Mechanical Engineering[1]

Le Thi Phuong Thanh
Department of Industry Tool and Equipment
Faculty of Mechanical Engineering[1]

Ngo Ngoc Vu ✉
Department of Mechatronics
Faculty of Mechanical Engineering
Thai Nguyen University of Technology
666 3/2 str., Tich Luong ward, Thai Nguyen, Vietnam, 24000
ngocvu@tnut.edu.vn

[1]*Hanoi University of Industry*
298 Cau Dien str., Bac Tu Liem District, Hanoi, Vietnam, 100000

[2]*Rajarambapu Institute of Technology, Rajaramnagar*
Shivaji University, Kolhapur
Rajaramnagar, Sangli District, Maharashtra, India, 400001

✉**Corresponding author**

Abstract

The combination of biopolymers with bioceramics plays vital role in development of artificial bone. Hydroxyapatite is extensively used as a material in prosthetic bone repair and replacement. In this paper synthesis of Hydroxyapatite-Polymethyl methacrylate-Zirconia (Hap-PMMA-ZrO_2) composite by using powder metallurgy technique. The mechanical, morphological, In-vitro biocompatibility and tribological properties were characterized by universal testing machine, micro-vickers hardness tester, high resolution transmission electron microscope (HR-TEM), MTT assay and pin-on-disc setup. In-vitro cytotoxicity test on HeLa cell lines shows cell viability constant when doses concentration increases so material found non-toxic. Results show that micro Vickers hardness i.e. 520 approximately matches with natural human bone i.e. 400. Compressive strength is less as compared to human bone because of powder metallurgy route used for fabrication and is 74 MPa. Density of proposed composite artificial human bone i.e. 1.52 g/cc is less as compared to natural bone i.e. 2.90 g/cc. The Hap-PMMA-ZrO_2 composite will be good biomaterials for bone repair and replacement work.

Keywords: biocompatibility, artificial bone, Hydroxyapatite, biopolymers, bioceramics, cytotoxicity.

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1. Introduction

Human body is made by bones, muscles, cartilage, ligaments and other tissues. The main part of human body is bone it provides structural support to the body with unique mechanical properties to perform the body functions. Also bone is performs as connecting tissue that gives support and connectivity for various parts of body. Bone is amazing composite which contains approximately 60 % hydroxyapatite 20 % collagen and remaining is water and non-collagenous proteins. Bone tissues loss or damage from accidents, surgery and trauma is a present medical challenge. More than 34 million bone injuries are reported in United States of America [1]. Current treatment on this medical challenge includes synthetic replacements of bones and tissues with composed of metals, ceramics and polymers.

In past years bioceramics are widely used in orthopaedic applications. Calcium sulphate, calcium phosphate and hydroxyapatite are clinically used for bone tissue engineering because attractive bioactivity and osteoconductivity [2–4]. Calcium silicate is used to improve osteoconductivity of ZrO_2 implant and it also reduced the toughness of ZrO_2 [5]. Hydroxyapatite is the most promising material in orthopaedic implants and dentistry [6]. Hydroxyapatite shows great bioactivity towards bone cells and therefore new bone formation, but it has poor resorb ability because of brittle nature. Metallic composites are using worldwide in orthopaedic applications nowadays. The size of the reinforcement reduced up to nanoscale is affects remarkable improvement in mechanical properties. Different metal matrix has been reinforced with ceramic compounds and intermetallic materials i.e. Mg, Al, Cu it shows high strength and stiffness for orthopaedic application after characterization [7].

Zinc ions have great osteoconductivity at low concentrations but zinc shows cytotoxicity at higher concentrations. Mainly stem cell shows different morphologies at different concentrations of zinc ions [8]. Polymers are also used for bone tissue engineering such as polyamide, polypropylene, polyethylene, ultra-high molecular weight polyethylene [9]. The biggest current problem in the health science field is post-surgical infections arising from recent implanted synthetic biomaterials, because these provides sites for bacterial adhesion [10]. There are some limitations for ceramic biomaterials i.e. stress shielding problems and metallic materials release the harmful ions through wear and corrosion and put the body in aggressive environment.

In the present investigation artificial human bone of composite Hap-ZrO_2-PMMA is developed by using powder metallurgy technique. Evaluation of tensile strength, compressive strength, hardness, coefficient of friction under wet lubrication condition and MTT assay by using HeLa cell line for cytotoxicity evaluation is carried out.

2. Materials and methods

Nano hydroxyapatite powder (average particle size 20 nm to 80 nm), Zirconia (average particle size 100 nm) and PMMA were purchased from Nano research lab, Zarkhand. Hydroxyapatite 40 % by volume, Zirconia 20 % by volume and PMMA 40 % by volume was mixed in ball mill at speed of 120 rpm ball to powder ratio is 10:1 for the time period of 60 minutes. The mixture of powder is dried and pressed quasi-statically in cylindrical die up to 400 MPa and kept at 5 minutes on compression machine. The obtained cylindrical specimen with the size of 10 mm diameter and 30 mm length is sintered in furnace up to 120 °C temperature and kept for 5 minutes then allowed to cool in furnace.

The biocompatibility test was carried out on MTT assay at Shreekrushna genetic lab, Rajkot. The cytotoxic ability of Hap-ZrO_2-PMMA was carried out at 20, 50, 100 up to 5000 ppm using HeLa cell lines (human cervical cancer cell line) [11–13]. National Repository of Animal Cell Culture, National Centre for Cell Sciences (NCCS), Pune, India, provided HeLa cell lines. Dulbecco's minimal essential medium (D-MEM) supplemented with 10 % FBS was used to grow the HeLa cells. The cells were cultivated for 24 hours in an incubator at 37 °C and 5 % CO_2. After seeding the cells for 72 hours in culture well plate, the plate was taken out from the incubator and the cells were exposed with D-MEM growth medium containing test compound and incubated for a further 48 hours at 37 °C and 5 % CO_2. Sample was tested in 11 different concentrations for next 24 hours. Thereafter 100 µl of MTT solution (5 mg/ml final concentration) was added to the wells and incubated for 4 hours at 37 °C and 5 % CO_2. The cell viability was calculated.

Hardness is a resistance offered by material to the plastic deformation which basically depends upon microstructure or structural arrangements of the atoms. The Vickers hardness

number (HV) is a value that is proportional to the applied force and the surface area of the permanent imprint created by a square-based pyramidal diamond indenter with a face angle of 136°. Vickers micro-hardness of Hp-ZrO_2-PMMA composite was evaluated. By using a diamond indenter, the load of 1 kgf was applied for period of 10 seconds. The specimen used for micro-hardness test is as shown in **Fig. 1**. After loading, the diagonals of indentation on the specimen are measured with the use of microscope arrangement. By measuring the average length of diagonal, d, the Vickers hardness can be calculated as,

$$HV = \frac{F}{A} \approx \frac{0.01819F}{d^2},$$

where F is in N and d is in mm.

The bone of composite which have L/D ratio is less than 10 was fabricated i.e. $L = 30$ mm, $D = 10$ mm. The specimen was then vertically positioned in such a way that loads were applied to the opposing sides of the pin after cleaning the bearing surface of the testing machine. Align the pin specimen in the centre of the machine's base plate. Hand-rotate the moveable part until it meets the specimen's top surface. Load was applied gradually and continuously till failure.

Tensile test specimen (ASTM D-638) was fabricated by using sol-gel method as shown in **Fig. 2**. The specimen is fixed on universal testing machine by means of gripper. Load cell is also connected with gripper after all arrangements rotate the movable portion by hand so it touches top surface of specimen. Applied load gradually without shock and continuously up to failure.

A Pin-On-Disc configuration was used to perform the friction test at RIT, Islampur (DUCOM, Bangalore, India, model: TR20-LE). The tests were performed according to ASTM G-99. Before the test, the disc was thoroughly cleaned with solvent and secured to the support with four screws. Similarly, the sample was mounted in the holder against the rotating disk. The sample was placed above the wear disc using the height adjustment block. Sliding distance, track diameter, contact pressure and ambient temperature were kept constant. Wear, friction force was zeroed before the start of each test. Distilled water is used as lubricant. During the test the frictional force and wear were continuously recorded using load cell and LVDT sensor. The plots of wear, COF, and frictional force with time were displayed online during the test. Wear of the material in microns was noted down after each test.

Fig. 1. Sample bone for compressive test

Fig. 2. Specimen for tensile test

High-resolution transmission electron microscopy (HRTEM) is a kind of transmission electron microscopy that enables for direct imaging of the structure of individual atoms in a sample. HR-TEM is an excellent technique for studying atomic-scale characteristics of materials such as nanoparticles, metals, semiconductors, catalysis, corrosion, polymer science, and biology. HR-TEM is used to find the structure and particle size of composition. This facility is utilized in Sophisticated analytical instrument facility (SAIF lab), IIT Bombay. TEM uses high energy electron beam transmitted through a very thin section of the sample to image and analyze microstructure of material with atomic scale resolution as well as elemental composition. The Hap-ZrO_2-PMMA composite is dissolved in 5 ml of water (composite is soluble in water) and stirred up to 5 minutes. After that the container of sample and water solution is placed in a water sonicator under 12 °C at 20 minutes. After 20 min the container is removed from sonicator. The solution is injected on 4 mm dia. copper grid by use of injector. Then this copper grid is placed on paper and put this paper in glass cup. This glass cup is placed under light heater for drying for 15 minutes. The copper grid is removed from paper and is placed in HR-TEM setup by using double tilt holder and start taking the readings.

Density is the mass per unit volume of a material. ASTM-D792 was used to determine density and specific gravity. The mass ratio of a particular amount of material at 23 °C to the same volume of deionized water is measured using specific gravity. Specific gravity and density are particularly important because a lower density or specific gravity indicates that there is more material per mass or a greater variation in component weight.

Specific Gravity was obtained from using the following:

$$Specific\ Gravity = \frac{a}{a-b},$$

where a is the apparent mass of specimen in air and b is the apparent mass of specimen completely immersed in water.

3. Results and discussions

3. 1. Biocompatibility study

The MTT test revealed that the composite has a high metabolic activity. **Fig. 3** shows the MTT assay plots of the samples. Cells in direct interaction with produced composites: after 24 hours of interaction with HeLa cells in culture, Hap-ZrO_2-PMMA composites showed no evidence of toxicity effects. After interaction with the produced composites, no cytotoxic effect was found in the cells, even at the ultrastructural level.

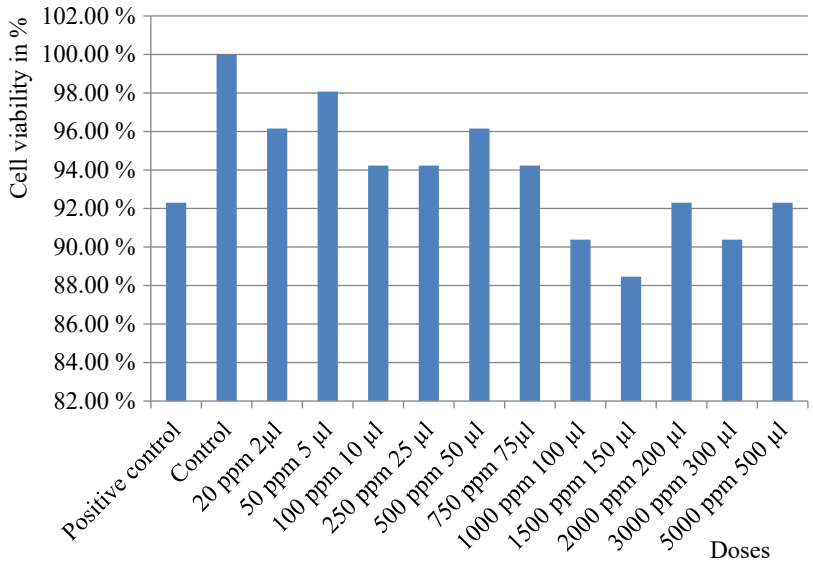


Fig. 3. Cell Viability (%) v/s Doses

In **Fig. 3** shows that with the injection of doses on HeLa cell line from 20 ppm in 2 µl up to 5000 ppm in 500 µl shows cell viability near about constant i.e. 94 % Exposure of different doses of processed material Sample was tested in Vitro on HeLa cell lines (Human cervical cancer cell line), result showing viability approximate (%) remains constant as the dosing concentration increases compared to non-exposed negative control standard.

3. 2. Hardness

Hardness greatly affects the resistance to wear, COF, strength of composite. Because of ceramics used in composition i.e. Hydroxyapatite and Zirconia hardness is quiet high as compared to natural human bone.

Comparison is shown in the graph in **Fig. 4**.

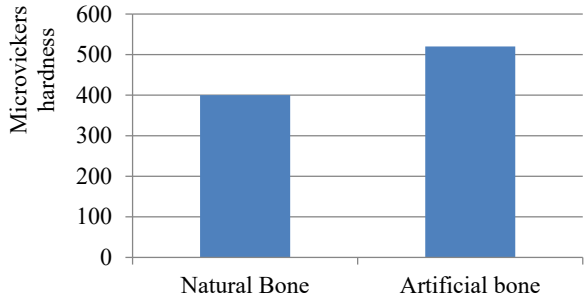


Fig. 4. Hardness of natural bone v/s artificial bone

The hardness of proposed composite bone is 23 % higher than natural human bone because material used for composite bone is brittle in nature. Manufacturing technique also affect the hardness of composite, because of powder metallurgy process the composite bone became porous so hardness increased. As the harness percentage of proposed composite bone increased the compressive strength will be decreased.

3. 3. Compressive and tensile strength

Compressive strength depends upon the properties of materials, fabrication methods, processing temperature. Because of powder metallurgy technique used for fabrication of artificial bone the compressive strength (73.4 MPa) is quiet less as compared to natural human bone as shown in **Fig. 5**.

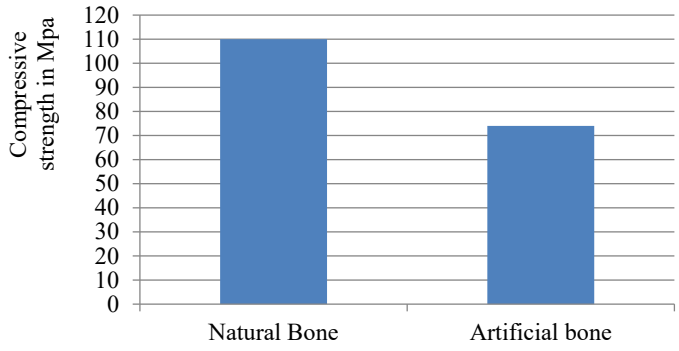


Fig. 5. Compressive strength of natural bone v/s artificial bone

Tensile strength also depends upon the properties of materials, fabrication methods, processing temperature. The tensile strength of natural human bone is 90 MPa to 120 MPa. In proposed artificial human bone tensile strength observed is less (around 84 MPa) than natural human bone as shown in **Fig. 6**.

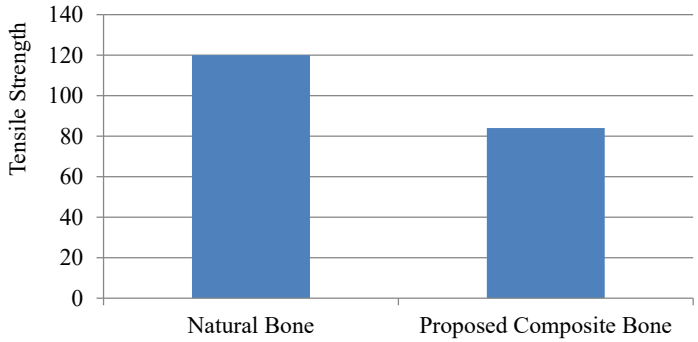


Fig. 6. Tensile strength of natural bone v/s artificial bone

3. 4. Tribological characterization

The coefficient of friction is calculated under various conditions are shown below **Table 1**. Ideal lubricant for the test is synovial fluid but because of cost and availability the distilled water is used as lubricant for the tests.

The coefficient of friction of various joints and artificial human bone is compared in above **Fig. 7**. The coefficient of friction in ankle, knee, and hip joint near about 0.02 but artificial human bone has coefficient of friction is 0.074. The coefficient of friction is high in artificial bone as compared to body joints because used lubrication medium is distilled water.

Table 1

Input parameters and coefficient under wet lubrication

Sr. Expt. no.	Speed in rpm	Load in kg	Time in min	Coefficient of friction (μ)
1	500	1	6	0.105
2	500	3	4	0.075
3	500	5	2	0.065
4	750	1	4	0.101
5	750	3	2	0.074
6	750	5	6	0.067
7	1000	1	2	0.080
8	1000	3	6	0.052
9	1000	5	4	0.038

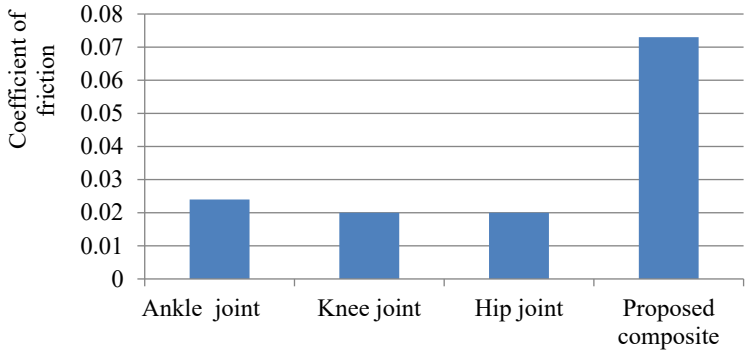
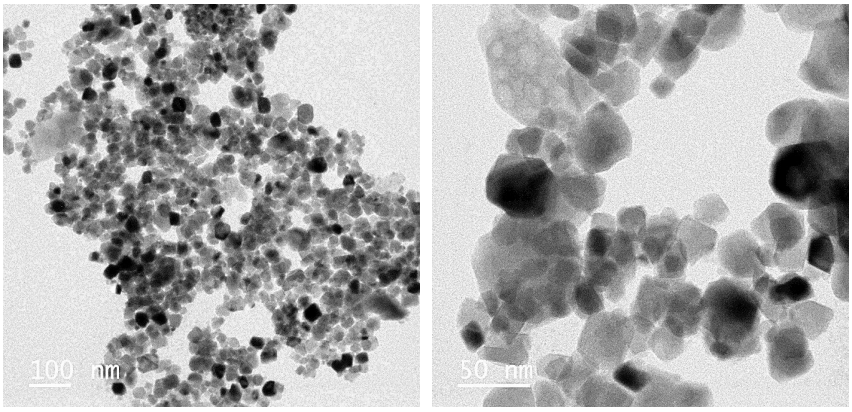


Fig. 7. Comparison of COF between various joints and artificial bone

3. 5. High resolution transmission electron microscopy test

The TEM analysis determines the size and shape of Hap-ZrO_2-PMMA. **Fig. 8** shows TEM images of Hap-ZrO_2-PMMA composite. Below images shows that average particle size of Hap-ZrO_2-PMMA composite is 20 nm to 100 nm and composite shows crystalline behavior. The HA nanoparticles have a homogenous microstructure with a diameter of about 80 nm and numerous particles that appear to aggregate. However, the particle size of Zirconia and PMMA varies.



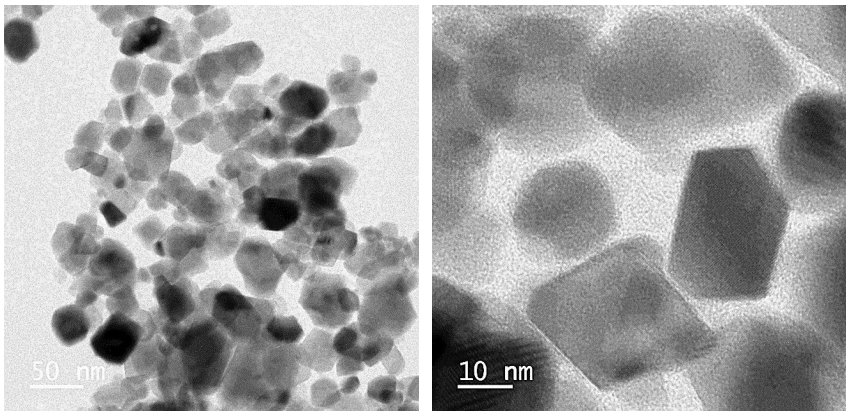
a *b*

Fig. 8. HR-TEM images of Hap-ZrO_2-PMMA composite: *a* – magnified at 100 nm range; *b* – magnified at 50 nm range

As shown in **Fig. 8** sample was magnified upto 100 nm range and 50 nm range average particle size of sample is below 100 nm. Sample shows crystalline behaviour.

As shown in **Fig. 9, *a***, ***b*** sample was magnified upto 50 nm range and 10 nm range with average particle size of sample is above 10 nm. Sample shows crystalline behaviour. The high resolution transmission electron microscopy (HR-TEM) images of the as prepared HAp-ZrO_2-PMMA used to provide details of the surface of the nanoparticles. The morphology of the synthetic HAp nanoparticles is square shape, whereas PMMA morphology is shown rod shape with a diameter of 10–20 nm and length of 100–150 nm. Similarly, an increase in particle size with an increase in the PMMA and ZrO_2 is also noticed.

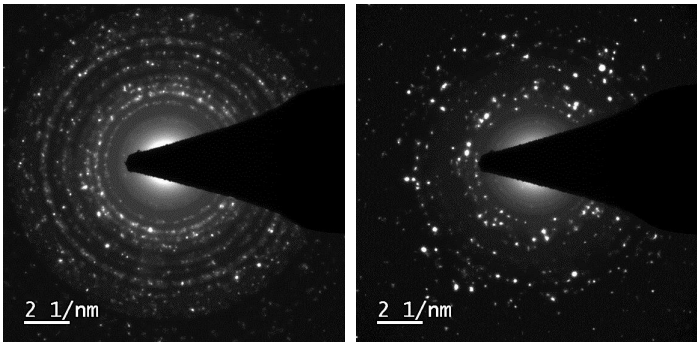
Thus, from the HR-TEM analysis the structural changes due to incorporation of PMMA and ZrO_2 in HAp structure are ascertained. The structural changes make the chemical and biological behaviour of materials to vary.



a *b*

Fig. 9. HR-TEM images of Hap-ZrO_2-PMMA composite: *a* – magnified at 20 nm range; *b* – magnified at 10 nm range

The corresponding selected area electron diffraction patterns for the HAp-ZrO_2-PMMA nanoparticles obtained from HR-TEM analysis are shown in **Fig. 10, *a*, *b***. The spotty ring formation shown in **Fig. 10, *a*** indicates random orientation of the crystalline phase of the HAp-ZrO_2-PMMA nanoparticles. In this image, it is observed that HAp-ZrO_2-PMMA sample shows clear diffraction pattern, indicating highly crystalline nature of the sample. However, the pattern corresponding to HAp with ZrO_2-PMMA concentration presented in in **Fig. 10, *b***, shows a reduction in the spotty ring density, indicating that increase in ZrO_2-PMMA concentration shows a reduction in the crystalline phase.



a *b*

Fig. 10. Diffraction patterns on HR-TEM: *a* – Spotty ring formation; *b* – PMMA concentration

3. 6. Density

The density of composite material depends upon the molecular interaction, porosity induced, and molecular configuration. The density of artificial human bone and natural bone is shown graph in **Fig. 9**. Density of artificial human bone is less (**Fig. 9**) than natural human bone because of porosity. Density of proposed composite artificial human bone is less as shown in **Fig. 11** than natural human bone because of porosity. The quantity of matter per cubic centimeter of bones is known as the bone density. Osteoporosis is caused by a loss of bone density. According to reports and statistics, one in every two women and one in every four males over the age of 50 has osteoporosis.

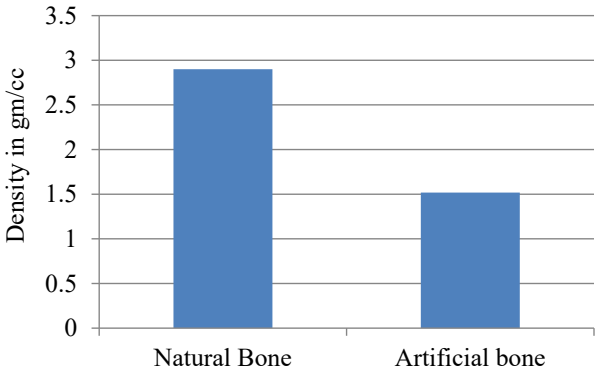


Fig. 11. Density of natural bone v/s artificial bone

The reported study covers the development of artificial bone using various combination of Hydroxyapatite-Polymethyl methacrylate-Zirconia (Hap-PMMA-ZrO_2). The results reported that this would be the one of the best materials in the application of prosthesis. The composite was prepared and tested as per desired characterization but it is quite difficult to mold the expected material in the exact scale and shape of bone or joint. The performed results based on various combinations of composite materials, and it is novel and unique work in the field of nano composite. The experimental work confirms the exact combination of Hydroxyapatite-Polymethyl methacrylate-Zirconia (Hap-PMMA-ZrO_2) as per desired characterizations.

4. Conclusions

The artificial bone had been developed and tested for their biocompatibility characteristics. The cell viability remains approximately constant as the dosing concentration increases compared to non-exposed negative control standard. The results indicate that the composite found non-toxic and it will be used for prosthetic applications. The mechanical properties viz. hardness, compressive strength and tensile strength have been determined for proposed composite material. The micro Vickers hardness i.e. 520 approximately matches with natural human bone i.e. 400. Compressive strength is less as compared to human bone because of powder metallurgy route used for fabrication and is 74 MPa. Tensile strength matches with human cortical bone and is 84 MPa.

Under tribological investigation the coefficient of friction of proposed composite artificial human bone slightly different from the ankle, knee and hip joints coefficient of frictions so proposed composite artificial joint is the probable candidate in human joint replacements.

Density of proposed composite artificial human bone i.e. 1.52 g/cc is less as compared to natural bone i.e. 2.90 g/cc because proposed composite artificial bone have porous structure, some residual gases is present in structure but improvement in fabrication technique it will be covered.

High resolution transmission electron microscopy test, X-ray diffraction test results shows that the composite have average particle size 20 to 80 nm and have crystalline nature.

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