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Evaluation on cross-linked nano microbial cellulose properties as bone scaffold

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ABSTRACT

Bacterial cellulose is produced by strains of Acetobacter and Acetobacter xylinum is the most important species producing it. Due to its nanostructure and morphological similarity to collagen, bacterial cellulose can be used for cell absorption and support. Consequently, this research presented a general description of bacterial cellulose, its applications in medicine, and utilization of hydroxyapatite/cross-linked bacterial cellulose composite scaffolds for bone tissue. Citric acid (20%) was used for cross-link, samples of bacterial cellulose were prepared and oven-dried, and hydroxyapatite (HAP) particles were then added at three concentrations (1, 2 and 4%). Using ATR-FTIR, XRD, FE-SEM, EDS and BET, the structure of the composites were compared. The results of ATR-FTIR confirmed that the HAP crystals contained a special carbonate that was similar to the apatite present in natural bone. XRD also indicated the characteristic peaks of HAP, and calculation of the size of the crystals using Scherrer equation demonstrated that the largest crystal size (81.42 nm) was that of the sample containing 20% citric acid with 4% HAP. Comparison of the FE-SEM images showed that the distribution and formation of apatite on the surface and cross-section were the best in the sample cross-linked with 20% citric acid and 4% apatite. The results of EDS analysis also indicated that all the precipitates contained calcium and phosphorus confirming the presence od HAP crystals, and the nanoparticles were also distributed uniformly in the scaffold structure. BET also showed that the sample cross-linked with 20% citric acid and 4% HAP had the lowest specific surface area and the highest porosity confirming the high uniformity of the 4% HAP distribution.

Keywords

Bacterial Cellulose, Crosslinking, Citric acid, Bone tissue scaffold, Hydroxyapatite (HAP).

1. Introduction

Bacterial cellulose is synthesized from saccharides (hydrocarbon) and bacterial sources. British scientist A.J. Brown first discovered bacterial cellulose in 1886 during fermentation of low molecular weight sugars with acetobacter xylinum to synthesize an extracellular gelatinous material similar to plant-derived cellulose in chemical structure and reactivity [1]. Acetobacter xylinum is the most important bacteria in synthesizing microbial cellulose, which is an anaerobic gram-negative rod-shaped bacteria that can convert 108 glucose molecules to cellulose [2]. Bacterial cellulose is a valuable biopolymer product used in various medical fields. The microfibrils of microbial cellulose are very similar to collagen, and are suitable for replacing or regenerating soft body tissue. They are also a suitable alternative to bone grafts, drug delivery carriers, drug release, and tissue generation. Microbial cellulose is used as scaffolding in tissue engineering and artificial skin, and is an effective adhesive for burn wound healing and dressing. Tissue scaffolds are porous, allowing cells to grow inside and migrate within the substrate, and must be body compatible and support the normal extracellular matrix (ECM). Due to its specific characteristics, including high water-absorption capacity, crystallinity, super-fine fiber mesh, high tensile strength (wet), optimal plasticity,

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biodegradability, high synthesis efficiency, lower production cost relative to other polymers, and body compatibility, microbial cellulose is a great candidate for tissue engineering scaffolds [3]. Meftahi et al. used citric acid as a crosslinking agent to prevent bacterial cellulose compression during rehydration. A bridge of carboxylic acid was formed between cellulose chains to avoid compression in drying. The results showed that the rehydration capacity was 5 times greater than normal. Furthermore, untreated samples had higher porosity, wettability, and expansion compared to the control sample. Their results suggest that citric acid can make the microbial cellulose tissue scaffolds more efficient with potential use in biomedicine [1]. Polymer-based scaffolds for bone tissue engineering often lose mechanical strength and require cross-linking for stability. Therefore, creating an ideal scaffold for bone tissue is a challenge. Kambar et al. crosslinked microbial cellulose and citric acid, and the scaffolds showed good mechanical characteristics for bones, and surface functionalization increased its bioactivity. Many researchers have created suitable composites with hydroxyapatite and organic nanofibers to imitate the basic chemical composition of normal bone [4]. Hydroxyapatite has been widely used for bone regeneration as a suitable substance for bone tissue engineering. They also discovered that hydroxyapatite crystals partially replace carbonate, and resemble normal bone. Collagen is covered with hydroxyapatite and forms an important part of the bone matrix. Guided bone regeneration is a medical practice using fillers and osteoporotic membranes to regenerate the intraosseous tissue in in-vitro tests. Bacterial cellulose fibers were used as a collagen-like substance to grow hydroxyapatite and ultimately produce GBR fillers [5, 6]. Zaborowska et al. also introduced the vascular endothelial growth factor (VEGF) into bacterial cellulose scaffolds to increase bone blood vessels in broken mice femurs, and the results showed new vascular formation, ossification, and growth of new bone [7]. Pang et al. (2019) showed that hydroxyapatite crystals increase cell adhesion and can induce vascular cells differentiation. Moreover, osteoblast adhesion, the first step in cell-substance interaction, can be enhanced by increasing the scaffold surface-roughness, which also affects cell ability to proliferate and differentiate on substance surfaces. For example, the two gelatin-hydroxyapatite composites have higher thermal stability and a very thick surface morphology, providing better adhesion and greater proliferation capacity [8].

2. Experimental

2.1. preparation of BC

7-day BC pellicles was synthesized by komagataeibacter xylinus BPR2001 in Hestrin-Schramm (HS) culture medium (including 2g glucose, 0.5g peptone, 0.5g yeast extract, 0.27g disodium phosphate, and 0.115g citric acid, to 1 liter of distilled water). Just synthesized samples withinside the shape of pellicles have been purified in an alkaline condition (with the aid of using sodium hydroxide 0.1 N for 90 min). The neutralized pellicles have been immersed in citric acid (20 w/v %) for 24h at 30˚C. Then the specimens have been cured at 160 °C for 5 min and that they have been washed and rinsed with distilled water till the pH of samples became 5. Then they have been dried at room temperature and Hydroxyapatite (HAP) (TITRACHEM, Tehran, Iran) particles were then added at three concentrations (1, 2 and 4%).

2.2. Characterization techniques

2.2.1. ATR-FTIR analysis

The chemical shape of specimens turned into investigated through ATR-FTIR (Perkin Elmer model Frontier; USA) spectroscopy. The spectra had been recorded with a decision of 4 cm−1 and an accumulation of 16 scans in variety of 650 to 4000 cm^{-1}

2.2.2. XRD analysis

XRD (Model: X'Pert MDP, Philips, Netherlands); mirrored image method λ=1.540 56 A) turned into used to determine the crystallinity of

samples. The samples were scanned from 10° - 40°. Scherrer formula was used to determine the size of HAP crystals.

$$L = K\lambda / Bs.\ Cos\theta \qquad (Eq.1)$$

2.2.3. FE-SEM observation and EDS analysis

FE-SEM was conducted to observe the morphology and microstructure of samples and the particle size of HAP was investigated by FE-SEM (MIRA3, Czechia).

2.2.4. BET analysis

BET analysis (BELSORP Mini II, Japan) was employed at 77 K to assess the porosity and surface area of the specimens.

3. Results and Discussion

3.1. ATR-FTIR

Vibrational frequencies pertinent to bacterial cellulose are observed in the 3200-3500 cm^{-1} range. This region is a part of the hydroxyl group and the hydrogen bond [1]. The formation of a broad peak in this region is due to hydrogen bonding, justifiable due to the high number of alcoholic agents (O-H) along the cellulose chains and the establishment of bonding. This similarity is also seen in BC/HAP composites; however, these peaks in these composites have been weakened. This decrease indicates that the presence of HAP crystals affects and is impactful on the cellulose's hydroxyl groups and moreover proves that the crystals have grown on the surface of the bacterial cellulose and have covered them. However, the chemical interaction between HA and BC stabilizes the composite to maintain mechanical integrity, a requisite for bone replacement [9]. This peak is completely lost in the crosslinked sample with CA20%/HAP4%, indicating the extended thickness of the HAP crystal in this sample, consistent with the FE-SEM images. The second peak occurred in the range of 2800-3000 cm^{-1}. The adsorptions in this region are related to CH_2 and CH_3 groups as a consequence of C-H alkane tensile bonding [10]. Peak formation in the range of 1720-1745 cm^{-1} is observed in crosslinked samples, linked to the COO ester bond, induced by the crosslinking of the cellulose chain and polycarboxylic acid. The peak appearance in the region of 1030 cm^{-1} in BC/HAP composite is related to the vibrational state of ion PO_4^{3-} and the peak in the region of 874 cm^{-1} and 1405 cm^{-1} is also tied to the tensile state of ion CO_3^{2-}. Adsorptions in the 670-900 cm^{-1} are also connected to the C-H spectrum of off-plane bending, located in the cyclohexane ring [1, 11, 12]. ATR-FTIR findings reveal that HAP crystals formed on bacterial cellulose contain carbonate. The presence of carbonate ions is considered as an apatite, similar in structure and composition to apatite in normal bone. This induces the bone tissue to respond better to replacement.

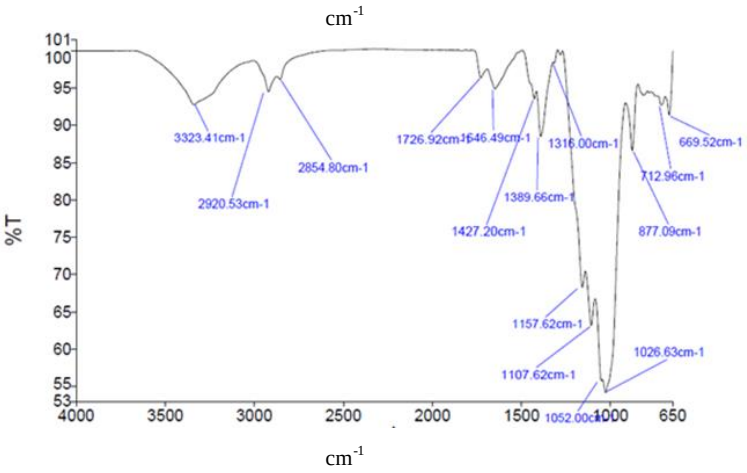

Fig1. Raw sample

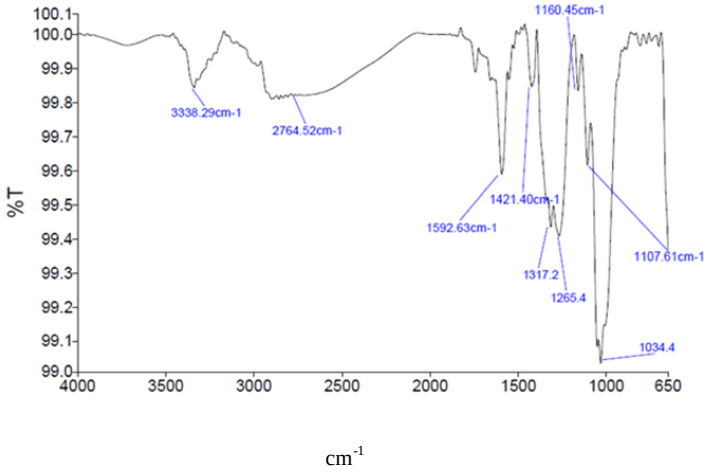

Fig2. CA20%

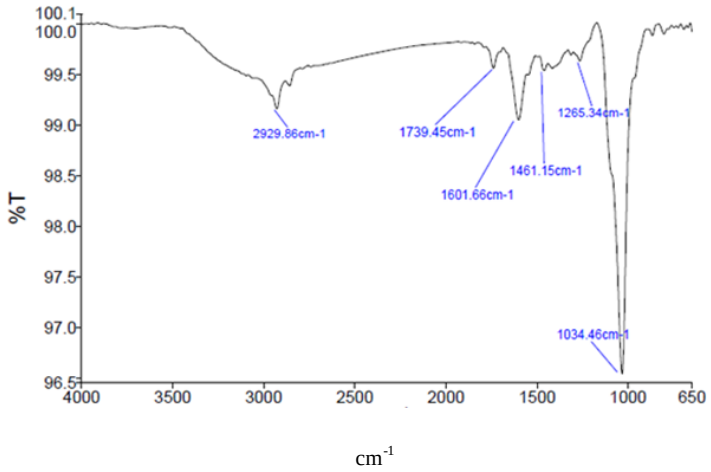

Fig3. Pure/ HAP4%

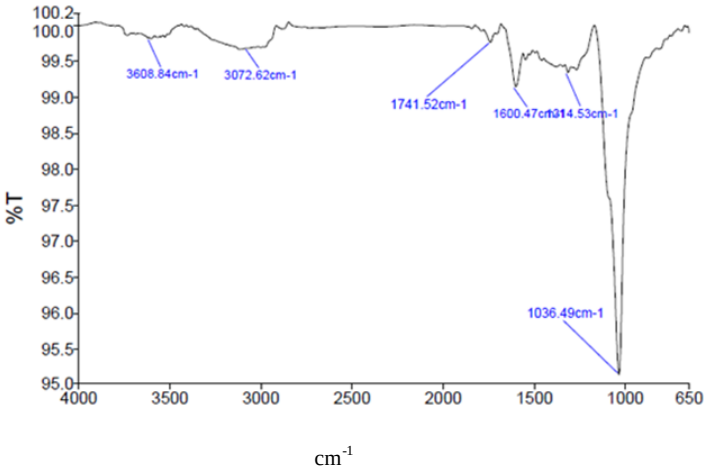

Fig6. CA20%/HAP4%

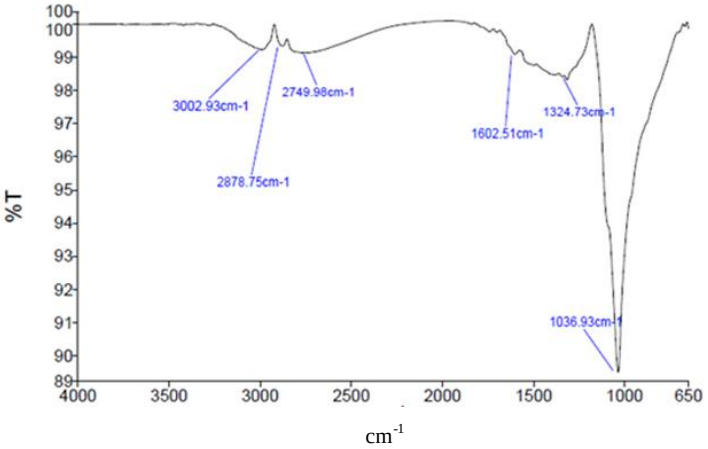

Fig4. CA20%/HAP1%

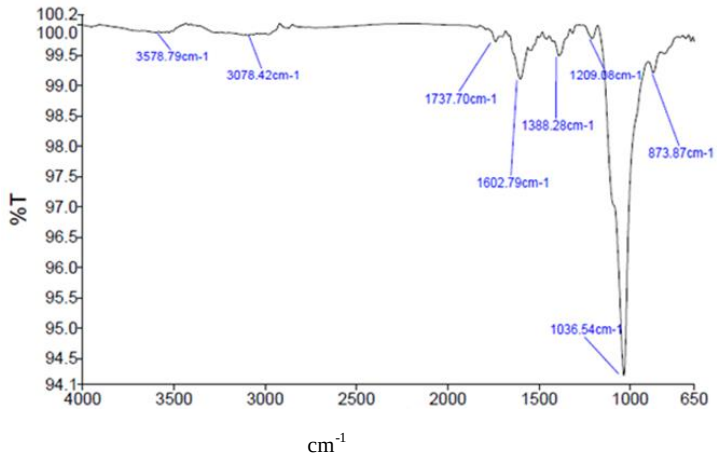

Fig5.CA20%/HAP2%

3.2. XRD

The initial findings obtained from XRD spectra indictes that crosslinking occurred in the amorphous part of bacterial cellulose. Plus, the crystal structures of crosslinked samples with polycarboxylic acid demonstrate changes. Sharp peaks additionally indicate that cellulose is highly crystalline. The diffraction pattern in the raw sample shows the index peaks at 14.7, 16.9 and 25.8 degrees [11], all characteristic of bacterial cellulose. Pursuant to the crosslink operation, the index peak is observed at the peak of θ=19.4. What's more, other peaks were recorded at 30.56, 32.6, 34.2, 37.5, 38.24, 38.5 degrees, which are the peaks of the HAP index [13]. By calculating the crystal size via the "Scherer Relation", the maximum crystal size (81.42) nm belonged to CA20%/HAP4%.

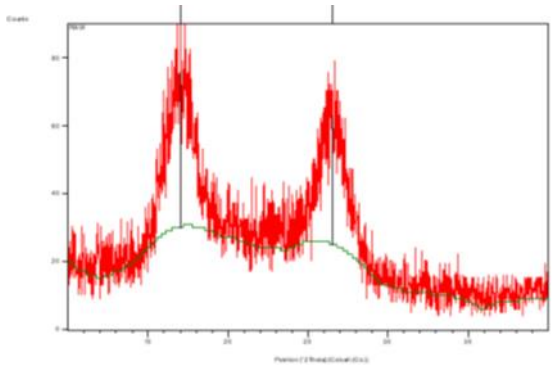

Fig7. Raw sample

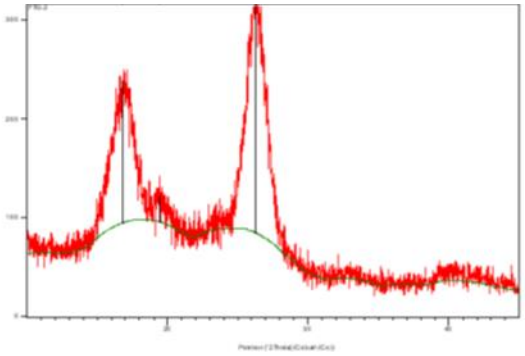

Fig8. CA20%

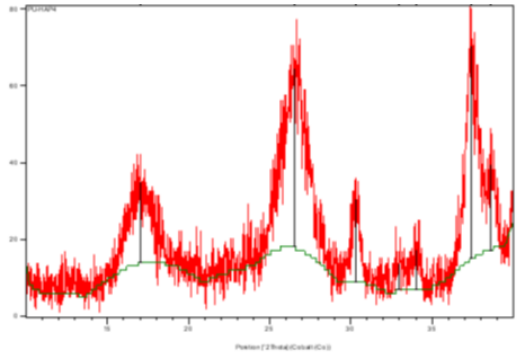

Fig9. Pure/HAP4%

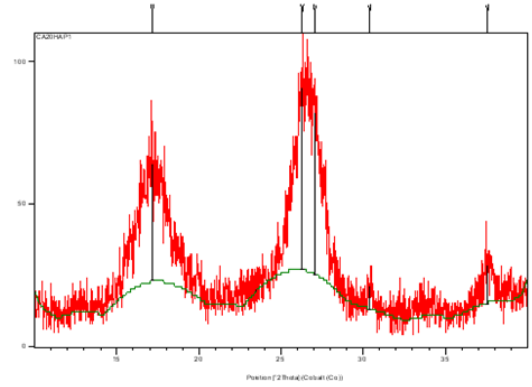

Fig10. CA20%/HAP1%

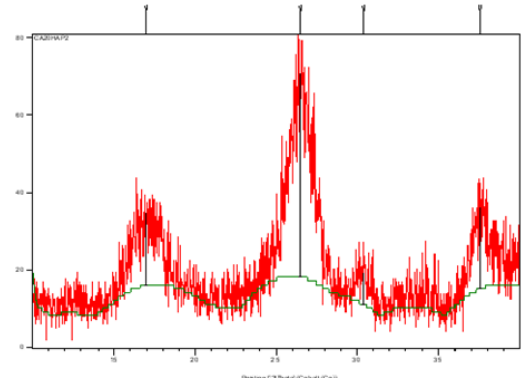

Fig11. CA20%/HAP2%

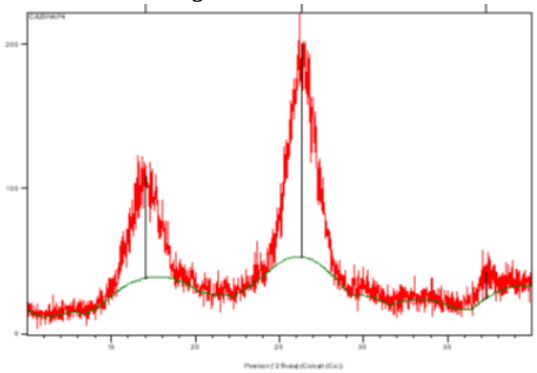

Fig12.CA20%/HAP4%

3.3. FE-SEM

In Figure 13, the surface and cross-sectional images of the raw sample are displayed. In this image, the bacteria on the surface of the pellicle and the bacteria among the various layers of bacterial cellulose are easily visible. Figure 14 reveals the purified samples (eliminated by further purification actions) reducing the amount of contaminant in the culture medium as well as acetobacter xylinum in relation with and compared to the raw sample. In addition, purification has induced the sample to turn white. In crosslinked samples with acid, discoloration is observed as a consequence of changes in the chemical structure of bacterial cellulose due to heat and the acidic environment. The formation of a porous surface is also quite evident. The porous surface increases the specific surface area and absorbs water higher than the raw sample. By comparing the cross-sectional images of the samples, the structure is bulkier and magnified porosity is observed via polycarboxylic acid [1, 10]. According to the BC/HAP composite images, it can be detected that HAP crystals are primarily situated on the surface of the membrane with a spherical morphology and provide complete coverage over the space between the bacterial cellulose. Moreover, HAP crystal spheres are directly attached to the BC surface and grow upwards in all directions. HAP particles have been observed in crosslinked samples, both on the surface and inside the BC, indicating that HAP formation is evenly distributed throughout the BC. In general, the images revealed

a cellulose network structure with a dense layer in the upper surface layer exposed to air, compared to the lower layer exposed to the culture medium during culturing. The structure of microcrystals, formed as thin sheets of granules, can also be observed in the images. However, in the purified sample, compared to the cross-linked sample, the HAP crystals inside the BC are hardly coated relative to its surface [1, 9, 13, 14, 15]. Additionally, the images show that the sediments in the crosslinked samples, unlike the purified sample, are evenly distributed throughout the BC, and this distribution is most optimal in the crosslinked sample with CA20%/HAP4%, and the formation layer becomes very thick after 14 days of immersion. Due to the fact that hydroxyapatite crystals with spherical morphology are more important in the ossification process than irregular morphologies, hence, the obtained composites can be utilized in bone tissue engineering applications. From the observations it can be stated that crosslinking is useful for the synthesis of BC/HAP composites. What's more, from the comparison of the images, HAP4% appears to be more suitable than other values for bone tissue scaffolding.

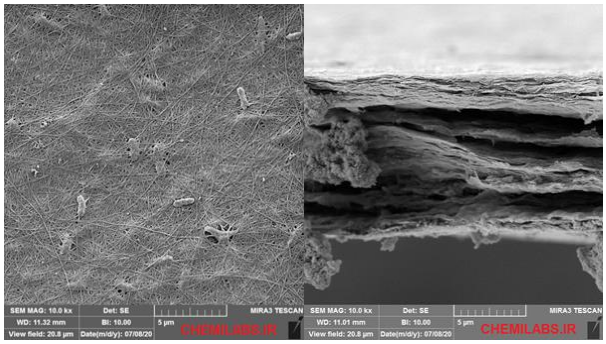

Fig 13. Raw sample

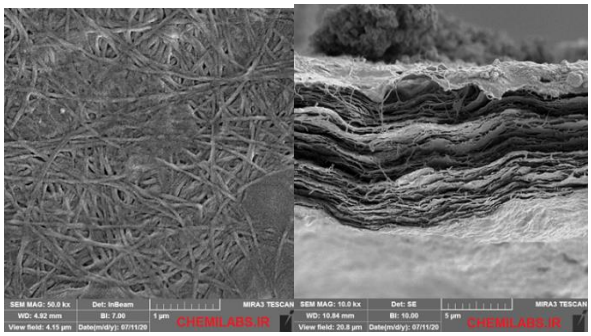

Fig14. Pure sample

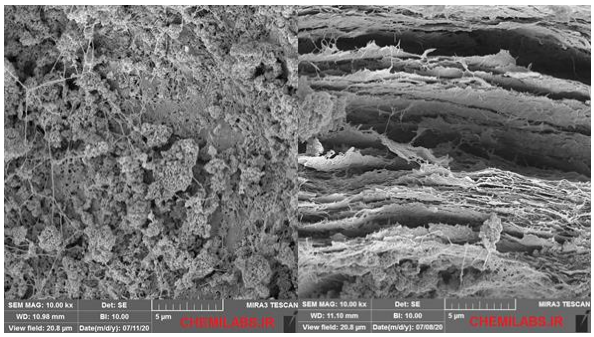

Fig15. Pure/HAP4%

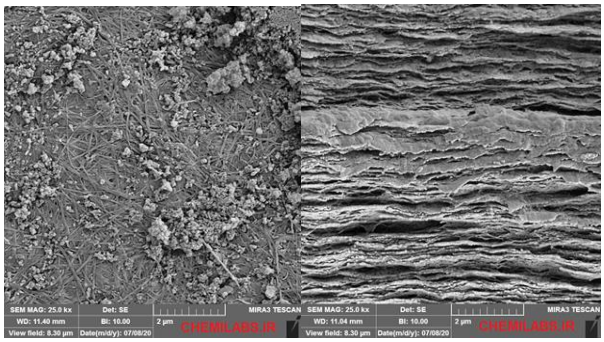

Fig16. CA20%/HAP1%

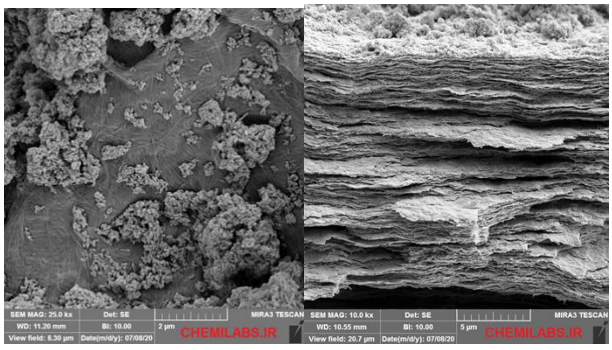

Fig17. CA20%/HAP2%

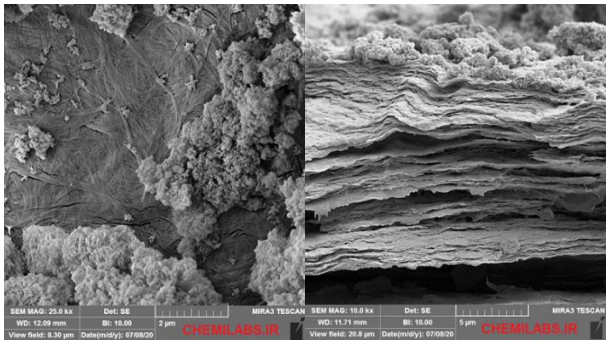

Fig 18.CA20%/HAP4%

3.3.1. EDS

BC/HAP composite samples were assessed and examined by this device and the structure and chemical components of the composite samples were analyzed. The analysis findings revealed that all these sediments contain elements of calcium and phosphorus, confirming the existence of HAP crystals [13].

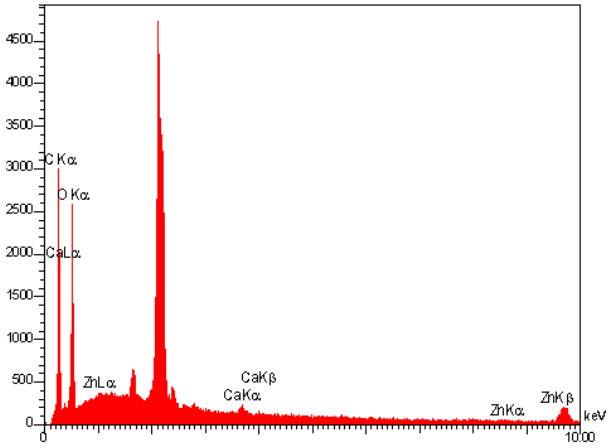

Fig19. Raw

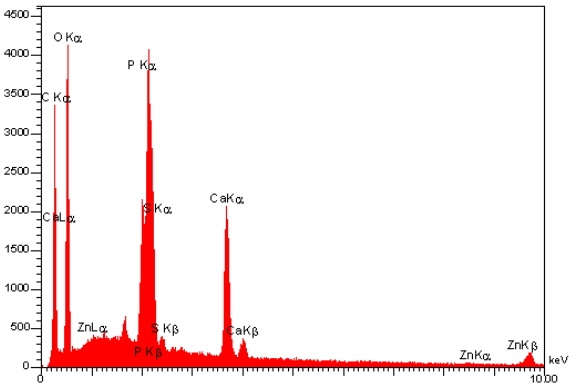

Fig20.CA20%/HAP4%

3.4. BET

Comparing the findings obtained from BET and FE-SEM images from the cross-sectional area of bacterial cellulose demonstrates that the raw sample has adhesive layers making penetration quite challenging and difficult. Furthermore, it also has extremely low porosity, however, the utilization of polycarboxylic acid and crosslinking has created a porous structure [1, 11].

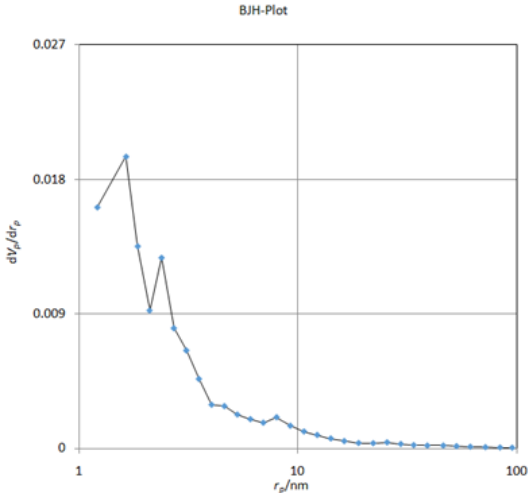

Fig21. Raw

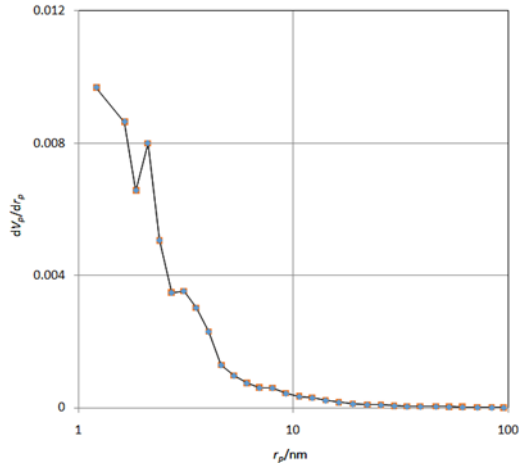

Fig22.CA20%

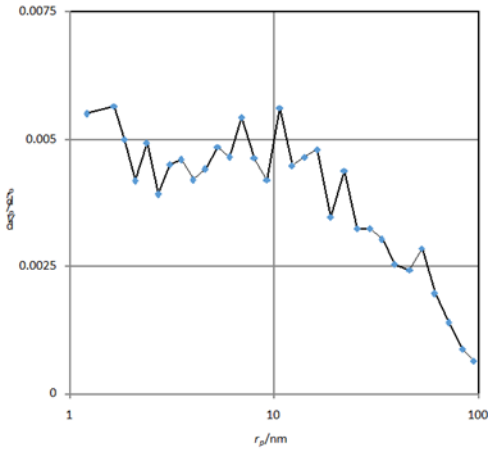

Fig23. Pure/HAP4%

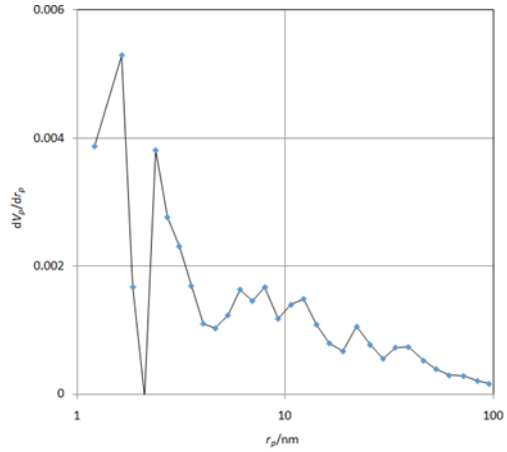

Fig24.CA20%/HAP4%

Table1: Porous characteristics of the BC samples (nm)

7	Raw
4	pure
4.175	CA20%
19	CA20%/HAP4%

4. Conclusions

Bacterial cellulose is a valuable biopolymer product utilized in diverse scientific fields. The microfibrils of microbial cellulose are very just like collagen, and are appropriate for changing or regenerating soft body tissue. It suggests many potential residences and additionally it is able to be acquired from wasted materials with carbohydrate sources. But after drying process, BC pellicle could to begin with be collapsed right into a dense layer with much less rehydration. For the primary time on this study, citric acid became proposed as a cross-linking agent to save you BC from condensing all through rehydration. Citric acid (20%) was used for cross-link, samples of bacterial cellulose were prepared and oven-dried, and HAP particles were then added at 3 concentrations (1, 2 and 4%). Then pure and cross-link sample with 3 concentration of HAP were compared. The results confirmed that the HAP crystals contained a special carbonate that was similar to the apatite present in natural bone and the sample cross-linked with 20% citric acid and 4% HAP had the lowest specific surface area and the highest porosity confirming the high uniformity of the 4% HAP distribution. What's more, from the comparison of the FE-SEM images, HAP4% appears to be more suitable than other values for bone tissue scaffolding.

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