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Research Paper

# Evaluating the Potential of Polycaprolactone/Heparinized Nano Fluoro Hydroxyapatite Composite Scaffolds for Advancing Bone Tissue Engineering: A Comprehensive Analysis of Biodegradability and Water Absorption

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**Abstract**
The primary purpose of this study is to produce a composite scaffold using polycaprolactone (PCL) and heparinized nano-fluorohydroxyapatite for cancellous bone tissue engineering. The research investigated the impact of heparinized nano-fluorohydroxyapatite particles on various properties of the scaffold, including water absorption, biodegradability, and alkaline phosphatase activity. The scaffold was produced using the phase separation (solid-liquid) method in combination with freeze-drying, and two different concentrations of heparinized nano-fluorohydroxyapatite powder were utilized. Biodegradability was assessed by submerging the scaffolds in phosphate-buffered saline for 6 weeks, showing that increased nano-fluorohydroxyapatite content enhanced biodegradability. PCL/10%w(H-nFHA50) exhibited the highest biodegradability rate. Water absorption analysis revealed that PCL scaffolds had lower water absorption compared to composite samples with heparinized nano-fluorohydroxyapatite, with PCL/10%(H-nFHA50) demonstrating the highest water absorption. Alkaline phosphatase activity was assessed on day 14 of cell culture, showing higher activity in heparinized samples compared to heparin-free samples in the presence of nano-fluorohydroxyapatite. The presence of heparin and nano-fluorohydroxyapatite in the scaffold structure likely contributed to this difference. No significant difference was observed between heparinized scaffolds with different nano-fluorohydroxyapatite concentrations. The results emphasize that the constructed scaffolds possess the potential for utilization in cancellous bone tissue engineering.

**Keywords**
Scaffold, Water Absorption, Heparinized Nano Fluoro Hydroxyapatite, Regenerative Medicine

## 1. Introduction

Tissue engineering is a science that focuses on the creation and development of new tissues, intending to restore injured organs and replace lost parts caused by various factors [1]. In recent times, tissue engineering has gained significant recognition and has captured the interest of many researchers. Its

immense potential in treating different deficiencies and illnesses makes it an unprecedented therapeutic option, particularly for conditions that currently lack definitive treatment methods [2]. Tissue engineering techniques have been successfully employed in the recovery and treatment of various organs such as the skin [3], bones [4], cartilage, liver, bladder, ligaments, nervous system, and heart valves [5].

One of the major challenges in tissue engineering is the production of porous scaffolds, as they need to possess appropriate physical and mechanical properties, along with a unique morphology and microstructure, to fulfill the physiological and mechanical functions required by the extracellular matrix [6]. In tissue engineering applications, cells settle and multiply on these substrates. The porous scaffold gradually degrades within the body, while the cultured cells continue to grow and proliferate inside the scaffold until the bone tissue completely replaces it [5].

To fabricate biocompatible frameworks, a variety of biodegradable polymers and bioactive ceramics are employed. Among the synthetic polymers utilized in tissue engineering applications, polylactic acid, polycaprolactone, and other similar materials have exhibited promising results [7].

Bioceramics, known for their thermal stability, resistance to abrasion, appealing appearance, and excellent biocompatibility, have garnered significant attention in different forms such as crystals, polycrystals, and composites. The only drawback of these materials lies in their fragility, prompting extensive efforts to overcome this limitation and enhance their toughness [8]. Recently, calcium phosphate ceramics like hydroxyapatite, amorphous calcium phosphate, tricalcium phosphate, and tetra calcium phosphate have become the focus of extensive research [9]. However, fluoro hydroxyapatite can serve as a suitable alternative to hydroxyapatite, compensating for its shortcomings. Fluoride ions, naturally present in blood plasma and saliva, play a crucial role in the natural growth of teeth and bones. They enhance bone mass and can effectively treat osteoporosis. Furthermore, they stimulate bone cell activity in both in vivo and in vitro settings [10]. Due to its crystal structure, fluoro hydroxyapatite exhibits superior thermal and chemical stability compared to hydroxyapatite. One advantage of calcium phosphates is their exceptional biocompatibility, as they possess a calcium and phosphorus layer on their surface that aids in bone-implant chemical bond formation. Additionally, fluoride ions encourage calcium phosphate crystallization and mineralization in the bone formation process [11].

Polymers are typically flexible and lack the strength of bone. To address this, composites can be synthesized by blending them with bioactive bioceramics like hydroxyapatite and bioactive glasses, resulting in materials with optimized mechanical and biological properties derived from both components [12]. Thus, it is evident that producing composites from polymers and calcium phosphate ceramics can yield biodegradable substitutes with specifications similar to natural bone [13].

Polycaprolactone is known as a polymer which is a semi-crystalline hydrophobic ester with the molecular formula $(C_6H_{10}O_2)_n$. This polymer has appropriate biocompatibility, low antigenicity, simple processibility, a glass transition temperature of -60, small melting point (Tm=60 ℃), high hardness, solubility in most organic solvents, and low biodegradation rate [14].

Among polyesters, polycaprolactone has the slowest biodegradation. Additionally, its degradation residues are easily absorbed through the body's metabolism (citric acid cycle) and do not create an acidic environment, unlike polylactide and polyglycolic. In this research, the aim of using polycaprolactone is to achieve a proper degradation rate and mechanical properties. Ceramic

materials used in tissue engineering techniques enhance some properties such as biodegradability, pore sizes for suitable cell growth, mechanical stability, bone conductibility, and growth factor transportability [15].

Proteoglycans serve as the primary polysaccharide components in the extracellular matrix (ECM). The polysaccharide portion of proteoglycans is referred to as glycosaminoglycan (GAG). Among the members of the GAG family is heparin. Heparin finds utility as an anticoagulant medication, effectively preventing the formation of blood clots [16]. It is an acidic and highly hydrophilic polymer, possessing a strong negative surface charge in each of its monomer units. The structure of heparin is illustrated in Figure 1. Because of the presence of carboxyl and sulfate groups, heparin holds a negative surface charge of -75, facilitating an electrostatic interaction with various proteins, including growth factors. Many proteins contain regions that can bind with heparin. Consequently, heparin can interact with numerous proteins, such as adhesive proteins (fibronectin, vitronectin), cell growth proteins (FGF growth factor), and bone morphogenetic proteins (BMP2) [17].

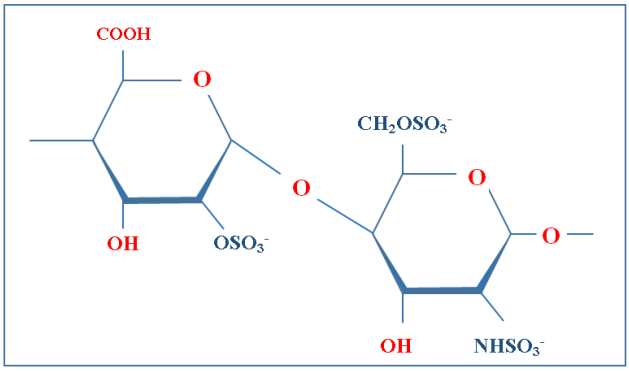


Figure 1. Chemical structure of heparin

One of the challenges in using bone composites is the inadequate distribution of ceramic nanoparticles, such as hydroxyapatite, which leads to poor mechanical properties. The absence of effective methods to control the distribution of these nanoparticles within polymer matrices greatly restricts their commercial production [18]. Zhao et al. conducted a study demonstrating that the colloidal stability of hydroxyapatite nanoparticles would be improved with the presence of heparin in composite structures [19].

Regarding the literature, obtaining a scaffold with proper mechanical properties, biodegradability, and bioactivity is desirable. According to the results of our recent study, it is known that the addition of heparin to the composite scaffolds enhances their mechanical and biological properties for use in the applications of bone tissue engineering [20]. In this research, the effect of the presence of heparinized nano fluoro hydroxyapatite particles on water absorption, biodegradability, and alkaline phosphatase activity of heparinized polycaprolactone/nano fluoro hydroxyapatite composite scaffold is assessed.

## 2. Materials and Method

### *2.1 Modification of nano fluoro hydroxyapatite particles with heparin*

In this research heparinized nano fluoro hydroxyapatite/polycaprolactone composite scaffolds were produced. For this aim, the first nano fluoro hydroxyapatite particles (with the molecular formula of [FHA: $Ca_{10}$ $(PO_4)_6$ $OH_{2-X}$ $F_x$] in which x is the proportion of hydroxyl groups in hydroxyapatite composition substituted with fluorine ions) were heparinized. For immobilizing heparin on nano fluoro hydroxyapatite particles, first $nFHA_{25}$ (25 percent of OH value in hydroxyapatite structure replaced with F) was and $nFHA_{50}$ (50 percent of OH value in hydroxyapatite structure replaced with F) powders were added to deionized water. The mixture was then placed in an ultrasonic homogenizer for 30 minutes until the nano fluoro hydroxyapatite-deionized water suspension was obtained. After adding heparin to the suspension, the mixture was placed on an orbital stirrer for two hours until the reactions between heparin and nano fluoro hydroxyapatite particles were done. It was then centrifuged for 30 minutes until the fluoro hydroxyapatite powder was separated from the suspension. After drying, the attained powder was completely ground until reaching nano size. Eventually, the powder was passed through a 100 nm sieve to ensure that the final particles were on the nanometer scale.

*2.2 Producing heparinized nano fluoro hydroxyapatite/polycaprolactone scaffolds*

The scaffolds containing 5 and 10-weight percentages of $nFHA_{25}$ and $nFHA_{50}$ immobilized with heparin (H- $nFHA_{25}$ and H-$nFHA_{50}$) were prepared using phase separation (solid-liquid) along with the freeze-drying method. 1,4- dioxane of 5% (w/v) was utilized to solve the PCL polymer. A homogeneous solution was obtained after 24 hours of standing on the stirrer at 1000 rpm. H- $nFHA_{25}$ and H- $nFHA_{50}$ were added to polycaprolactone and 1,4 dioxane solutions, which were then stirred for two hours and in the next step placed in an ultrasonic bath for 20 minutes. They were then cooled down up to -5 ºc. After freeze-drying for 72 hours under vacuum circumstances (0.1 mbar) until complete sublimation of the solvent. Finally, a vacuum oven was used to dry the porous samples at room temperature until their weight became constant. The protocol used in this study is following our previous research [20].

The produced scaffolds are shown in Figure 2. In all scaffolds, the amount of polycaprolactone was a constant equal to 5w/v%.

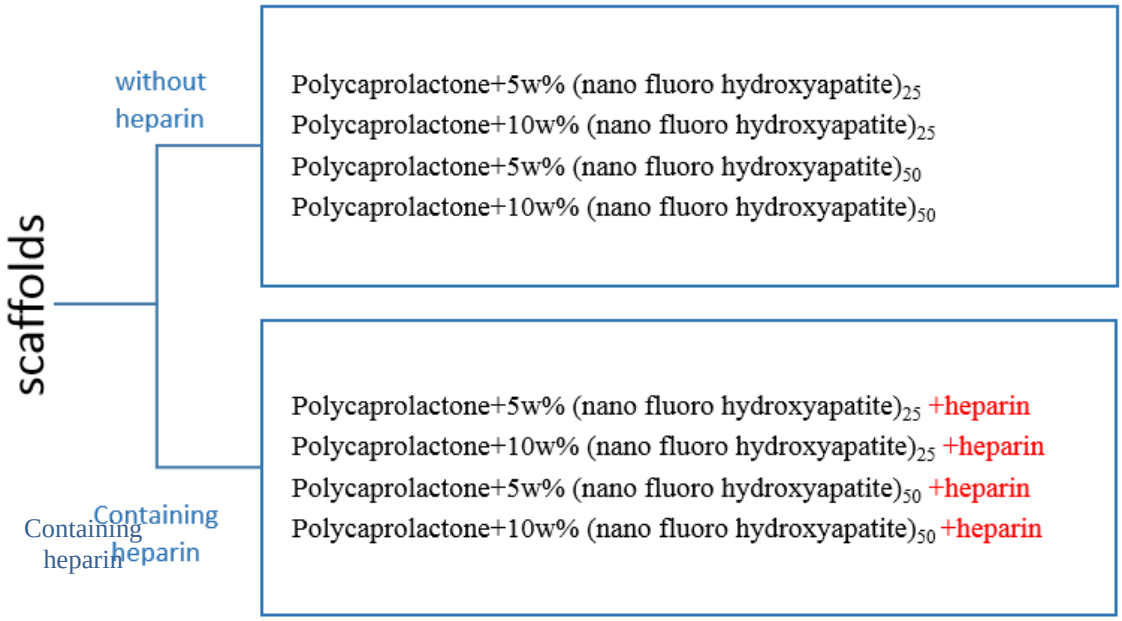


Figure 2. The composition of the produced scaffolds

*2.3 Water absorbance assay of the scaffolds*

Water absorbance assay of the samples was done according to ASTM D570 standard. The PCL5%(H-nFHA$_{25}$), PCL10%(H-nFHA$_{25}$), PCL5%(H-nFHA$_{50}$), PCL10%(H-nFHA5$_0$) Scaffolds, and polycaprolactone scaffold in a circle form with a diameter of 10 mm and a height of 3 mm, were produced. For each scaffold, three samples were constructed. First, the samples were weighed using a digital scale. Subsequently, they were immersed in distilled water and placed on an oven shaker at 37°C. After a certain time, the samples were brought out of water. Excess water was removed, and the samples were reweighted using a digital scale. The swelling of the samples during 144 hours of immersion in distilled water was evaluated. The percentage of water absorption was calculated via Equation 1 [21]:

$$\Delta M\% = \frac{M_1 - M_2}{M_2} \times 100 \qquad (1)$$

$M_1$ is the dry sample weight, $M_2$ is the wet sample weight and ΔM% is the sample swelling percentage in water. The amount of samples' swelling in distilled water was assessed after 144 hours.

*2.4 Assessment of Biodegradability*

For biodegradability analysis and investigation of the weight decrease, three samples from each of the PCL5%(H-nFHA$_{25}$), PCL10%(H-nFHA$_{25}$), PCL5%(H-nFHA$_{50}$), PCL10%(H-nFHA$_{50}$) and also PCL scaffolds were cut in equal sizes and their weight was measured up to 5 decimal places ($M_i$). the samples were placed in separate glass tubes and, the phosphate buffer saline solution was added 100 times the weight of the samples. The samples were suspended in the solution and kept in a shaker oven at 37 °C for 6 weeks. After that, the samples were brought out from the solution and dried at 40 ℃. Once completely dried, their weights were measured ($M_d$). These values were used for the calculation of the biodegradability and water absorption value of each scaffold sample. In addition,

the pH value of the solution was measured after one week to assess any changes compared to the control solution. The weight loss percentage is calculated via Equation 2 [21]:

$$weight\ loss\ = \frac{M_i - M_d}{M_d} \times 100 \quad (2)$$

*2.5 MG-63 Cell Culture*

In the study [20], cell culture was performed using the MG-63 cell line prepared from the Iran Pastor Institute cell bank.

*2.6 Assessment of alkaline phosphatase activity*

The alkaline phosphatase enzyme is one of the hydrolyser enzymes that is naturally found in all tissues of the body. In most cells, this enzyme is the cell membrane enzyme type. This enzyme has a major role in the mineralization of the bone matrix [22]. Therefore, when the osteoblasts are producing bone matrix, the activity of this enzyme increases drastically and is regarded as a characteristic agent for distinguishing the osteoblast's activity in the ossification process. ALP assay was carried out by the ALP assay kit (Pars Azmoon, Iran) on the 14th day. Because of the presence of ALP in the environment, the p-nitro phenyl phosphate is decomposed and transformed into the p-nitro phenyl. P-nitrophenyl makes a yellow color and eventually, this decomposition reaction terminated by adding 100µl of 1N solution of NaOH. In the last stage, the color changes were assessed in 405 nm wavelength using a miv reader.

## 3. Results and Discussion

*3.1 Assessment of the water absorption of the scaffolds*

The infiltration of water molecules into the polymer network continues until reaching an equilibrium level where the osmosis pressure of the liquid reaches the pressure of the water infiltrated into the polymer network and finally, the liquid infiltration equilibrates while the infiltration continues on the microscopic scale. Generally, when hydrophilicity increases, the water absorption increases. With an increase in immersion time, the crystal parts would hydrolyze, and amorphous decomposed chains exit the polymer matrix [23,24].

The PCL5%(H-nFHA$_{25}$), PCL10%(H-nFHA$_{25}$), PCL5%(H-nFHA$_{50}$), PCL10%(H-nFHA$_{50}$), and also PCL scaffold samples were placed in distilled water at 37℃ for 144 hours. After that, the samples were taken out and weighed up to 3 decimal places. The voids between polymer networks are suitable places for water absorption. Therefore, the more lateral couplings in the network, the less water absorption is observed. As can be seen in Figure 3, with an increase in H-nFHA content, the water absorption of the scaffolds increases which is due to the increase in hydrophilicity and the existence of nanofluor and heparin molecules in the scaffolds. The composites containing different amounts of PCL/H-nFHA absorbed high volumes of water, up to 500 percent of their primary weight. This may be regarded as the water molecules entrapped between the pores of the scaffolds. The water absorption in PCL was much lower in comparison to heparinized nano fluoro hydroxyapatite-containing samples. Among all of the prepared samples, the PCL/10%(H-nFHA$_{50}$) scaffold showed the highest water absorption value.

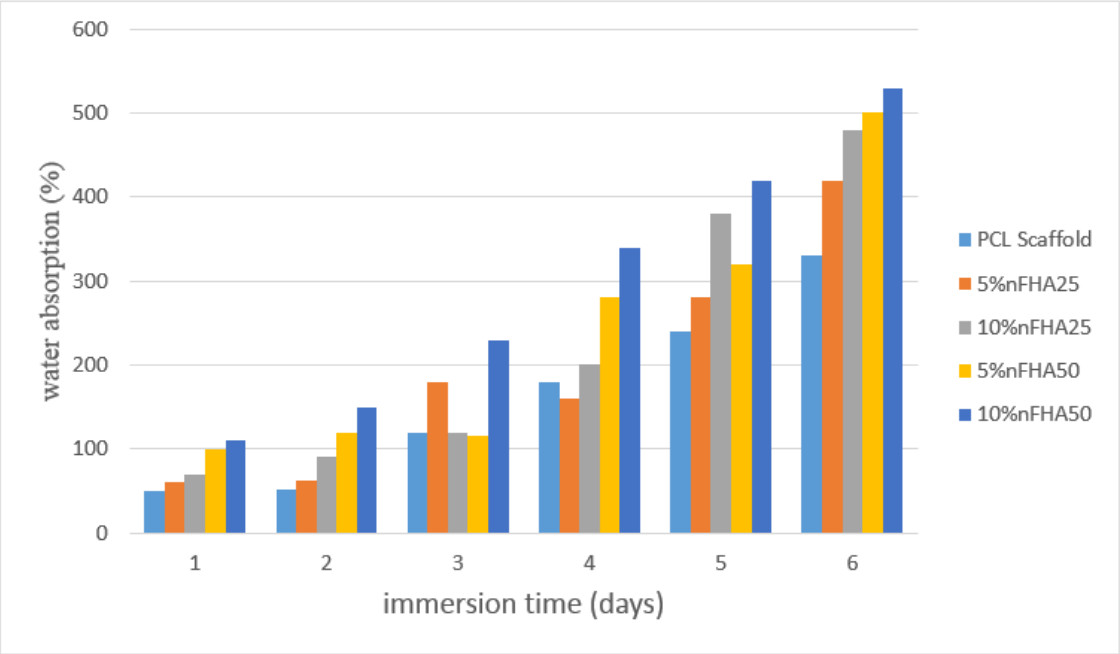


Figure 3. Diagram of the water absorption value of the PCL5% (H-nFHA$_{25}$), PCL10% (H-nFHA$_{25}$), PCL5% (H-nFHA$_{50}$), PCL10% (H-nFHA$_{50}$), and PCL composite scaffolds during 6 days of immersion

*3.2 Assessment of biodegradability of the scaffolds*

The hydrolytic degradation of aliphatic polyesters is a complicated process that consists of several reactions and diffusion stages, such as water absorption, ester hydrolysis, dispersion, and dissolving the pieces.

Polycaprolactone is a semi-crystalline linear aliphatic polyester that is biodegradable due to the hydrolysis of its aliphatic ester bonds [15]. To investigate the amount of sample weight loss, after 6 weeks of sample immersion in PBS solution, they were taken out of the solution and their weight was measured after complete desiccation. The results are demonstrated in Figure 4. The weight loss investigation process was carried out at 37˚C and pH of 7.42. As can be seen the longer the immersion time of the samples, the more weight loss percentage occurs. The weight loss for PCL and PCL5% (H-nFHA$_{25}$), PCL10% (H-nFHA$_{25}$), PCL5% (H-nFHA$_{50}$), PCL10% (H-nFHA50) scaffolds were 2.3%, 4.5%, 5.2%, 5.6% and 6.1%, respectively. PCL/10%w (H-nFHA$_{50}$) scaffolds had the highest rate of biodegradability. All scaffolds hold their shape until the end of 6 weeks which show that the samples' degradation is in the primitive stage, and their outer layers have not been degraded yet.

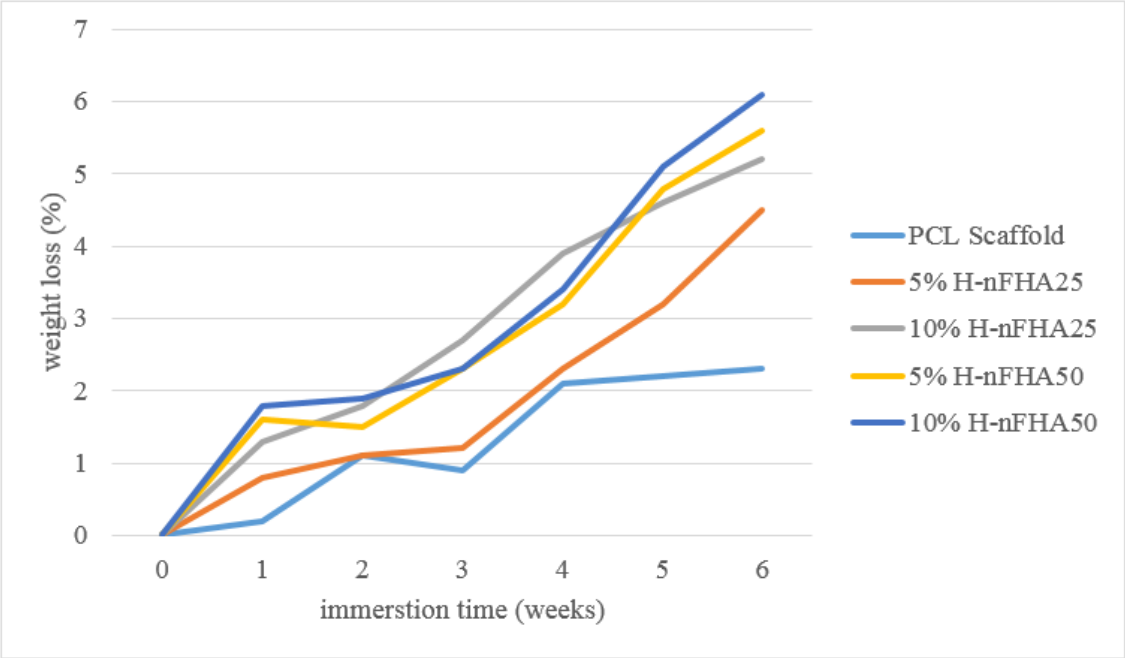


Figure 4. Diagram of the weight loss of PCL5% (H-nFHA25), PCL10% (H-nFHA25), PCL5% (H-nFHA50), PCL10% (H -nFHA50), and PCL composite scaffolds during 6 weeks of immersion in PBS.

pH variation around each of the samples immersed in PBS was evaluated every week, and the results are illustrated in Figure 5. For the polycaprolactone scaffold, the pH variation decreased slowly in comparison to the primary pH value which was 7.42. The pH decrease may occur due to the increase in carboxyl and hydroxyl groups in the polymer structure which causes higher hydrophilicity and eventually higher degradation. pH variations had a declining trend for composite samples.

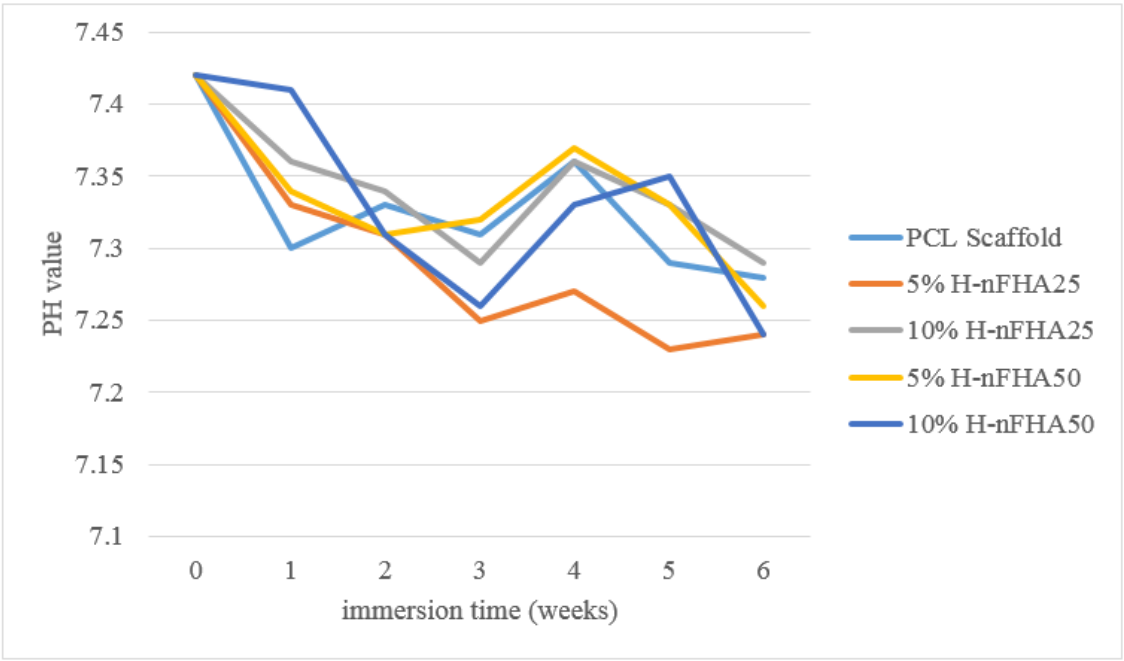


Figure 5. The diagram of PH of PBS solution for PCL5% (H-nFHA25), PCL10% (H-nFHA25), PCL5% (H-nFHA50), PCL10% (H-nFHA50), and PCL composite scaffolds during 6 weeks of immersion in PBS.

*3.2 ALP Activity assessment*

Alkaline phosphatase activity is considered a marker that reveals the activity of osteoblast cells during new bone formation. ALP secretion is one of the biological tests that is used for investigating the osteoblast cell activity value [23]. The amount of ALP secretion of MG-63 cells cultured on the heparinized scaffolds PCL5%(10%H-nFHA$_{50}$), PCL5%(5%H-nFHA$_{50}$), PCL5%(5%H-nFHA$_{25}$), PCL5%(10%H-nFHA$_{25}$), and heparin-free scaffolds PCL5%(10%nFHA$_{50}$), PCL5%(5%nFHA$_{50}$), PCL5%(5%nFHA$_{25}$), PCL5%(10%nFHA$_{25}$) were evaluated on 14th day after cell culture. On day 14 of cell culture, higher ALP activity was observed in heparinized samples in comparison to the samples having no heparin which may be attributed to the concurrent presence of heparin and nano fluoro hydroxyapatite in the scaffold structure [20].

## 4. Conclusion

In this study, water absorption and assessment of biodegradability of scaffolds constructed from polycaprolactone/ heparinized nano fluoro hydroxyapatite with 5 and 10 weight percent were investigated. To achieve this, we employed the phase separation (solid-liquid) method accompanied by freeze-drying, utilizing 1.4- dioxane as the solvent. The water absorption rate increased as the value of heparinized nano fluoro hydroxyapatite increased until the 6th day of immersion. The results showed that with the increase of H-nFHA$_{25}$ and H-nFHA$_{50}$ in scaffold structure, water absorption increased, and the PCL/10%w (H-nFHA$_{50}$) had the highest water absorption rate. For biodegradability assessment of the scaffolds, the samples were immersed in a PBS solution for six weeks. The results showed that among all scaffolds, PCL/10%w (H-nFHA$_{50}$) had the highest rate of biodegradability. For the cell study of the scaffolds, we utilized MG-63 cells, as stated in our recent study [20]. The results showed that the presence of heparin resulted in higher ALP activity on the 14th day after cell culture compared to the non-heparinized samples. This increase may be related to the synergy between heparin and hydroxyapatite nanoparticles. The results of ALP activity were consistent with the MTT assay and cell culture findings from our recent study [20]. Overall, this research demonstrates that PCL/H-nFHA scaffolds comprising heparinized hydroxyapatite nanoparticles are suitable for applications in bone tissue engineering.

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