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Biological Evaluation of a Novel Tissue Engineering Scaffold of Layered Double Hydroxides (LDHs)

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Abstract. Bone Tissue Engineering (BTE) composed of three main parts: scaffold, cells and signaling factors. Several materials and composites are suggested as a scaffold for BTE. Biocompatibility is one of the most important property of a BTE scaffold. In this work synthesis of a novel nanocomposite including layered double hydroxides (LDH) and gelatin is carried out and its biological properties were studied. The co-precipitation (pH=11) method was used to prepare the LDH powder, using calcium nitrate, Magesium nitrate and aluminum nitrate salts as starting materials. The resulted precipitates were dried. X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) analyses were used to characterize the synthesized powders. The results demonstrated the presence of nanocrystals of Ca-LDH and Mg-LDH as Hexagonal and Layered Morphology. The obtained powders were composed to gelatin via solvent casting method then freez dried. The scaffold was prepared via membrane lamination method from the resulted layers that linked together with gelatin as binder. In order to investigate the scaffold cytotoxicity MTT assay was done with a osteosarcoma cell line. No toxic response was observed in specimens. As a major result, it was demonstrated that the specimen showed a significant cellular response. Then osteosarcoma cells were cultured for 7-day and 14-day extract of powders. The composites osteoconductivity was investigate with cells alkaline phosphatase extraction. The results demonstrated that the Ca-LDH/gelatin composite scaffold has a good potential for bone tissue engineering applications and Mg-LDH specimen has a better osteconductivity.

Introduction

Layered double hydroxides (LDHs) are also known as anionic clays, from a family of layered materials which are used in a variety of applications such as medical applications, drug delivery, electrodes, anion exchangers, catalysts, and catalyst precursors [1-5] . The general formula for these compounds can be represented as $[M^{II}_{1-x}M^{III}_{x}(OH)_2]^{X+}(A^{n-})_{X/n}.mH_2O$, where M^{II} and M^{III} are di-and trivalent metals, such as Zn^{2+}, Ni^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+}, respectively, and A is an anion, such as Nitrate, Carbonate, organic components, biologic agents, proteins and anionic drugs [5, 6] The main structure of LDHs is composed of infinite sheets of positively charged brucite-like $Mg(OH)_2$, where Al^{3+} have replaced a fraction "X" of the divalent cations in octahedral coordination. Due to neutralizing the additional positive charge, anionic molecules were accommodated into the inter-layer distance. LDHs have attracted the interest due to their ability to intercalate a variety of anions in their inter-layer distance [1-3].

Tissue engineering (TE) is a promising field of regenerative medicine that prepares outstanding opportunities to repair damaged tissues and organs. The main concept underlying TE is combining a scaffold or matrix, living cells, bioreactors and signaling factors to form a 3-D tissue engineered construction to promote process of repair and regeneration of tissues. The scaffold is a 3-D structure that supports cell attachment, proliferation, growth and differentiation and also guides the development of the required tissue and acts as a drug delivery vehicle [7][6]. A scaffold with the ability of drug delivery can enhance the regeneration of the tissue. Ca-LDHs and Mg -LDHs because of the presence of calcium and magnesium in their primary structure (natural body elements) and also their strong biocompatibility have the potential for bone tissue engineering applications [3, 7].

Materials and methods

The powder of Ca-LDH was prepared via the precipitation method using calcium nitrate and aluminum nitrate salts as starting materials with the ratio of Ca/Al=2. The pH was adjusted at 11 and the suspension was aged for 22 hours in room temperature. Then the resulted precipitates were washed and undergone a hydrothermal post-treatment in 90°c for an hour and then dried in oven (90°C for 3 days). The process was exactly repeated to preparing Mg-LDH powder.

The X-ray diffraction patterns of powders were obtained using a Siemens D5000 diffractometer with CuKa radiation (k = 1.5418 A°). The size and morphology of the product particles were measured using Philips XL30 scanning electron microscope. Furthermore, the Fourier transform IR spectroscopy (FTIR), using IFS-48 device made by bruker company was utilized to confirm the resulted data.

The resulted powders were composed to gelatin solution in the temperature of 40° then stirred one hour to obtain the nanocomposite of LDH-gelatin via solvent casting method then freezed 24 hours to prepare for freez drying process. The scaffolds was prepared via membrane lamination method from the resulted layers thay cut into circular pieces. Afterwards, they linked together with gelatin solution as binder.

The MTT assay was utilized to evaluate the biological properties of specimens. The extraction process was done according to the ISO 10993-5 in which 1 mL of culture medium was added to each specimen with 0.1 gr weight and reserved in incubator for 14 days. A specified amount of culture medium was kept in the same condition as control to subsequent comparisons.

The human primary osteogenic sarcoma cell line MG63 (Pasteur Ins, NCBI, Iran) was cultured in RPMI (GIBCO, Scotland) supplemented with 10% fetal bovine serum (FBS, Seromed, Germany), 100 U/ml penicillin and 100 μg/ml, streptomycin (Sigma, USA). Also, the 14-day extract of scaffolds were obtained by similar processing.

Proliferation rate of cells on the specimens were measured by using dimethylthiazol diphenyltetrazolium bromide (known as MTT) assay. At the first day human osteosarcoma cells were seeded into a 96-well microtiter plate at 1×10^4 cells per well. After 24 hours, the culture medium of each well was removed and replaced with 90 μL extract plus 10 μL FBS. In the next 24 hours, the medium eliminated and 100 μL of a 0.5 mg/mL solution of MTT (Sigma, USA) was added to each well followed by incubation for 5 h at 37 °C. The purple formazan crystals (formed in the mitochondria of the cells) were detected and later dissolved by addition of 100 μL isopropanol (Sigma, USA) per well. The plates were then incubated at 37 °C for 15 min prior to absorbance measurements. The optical density (OD) was recorded on a multiwall microplate reader (ICN, Switzerland) at 545 nm and normalized to the control OD. The absorbance is directly a measure of cell viability and toxicity.

To investigate the osteocoductivity of powders and scaffolds, the extraction of Alkaline phosphatase (ALP) enzyme should be measured as a indicator of osteoblasts activity. The 14-day extracts of powders and scaffolds were exposed to osteosarcoma cells for 7 days and 14 days. Then the obtained culture media of each specimen was seperated. The concentration of ALP was measured via Autoanalyzer-902, Hitachi company, (Germany) using ParsAzmoon Kit (Iran).

Results and Discussion

The XRD patterns of specimens are showd in figure 1. In general, there are three major part in XRD pattern of LDH specimens: 1- A complex of sharp with acute angles indicate the (00l) atomic planes. Indeed these peaks represent the interlayer distance and the thickness of brucite-like layers. 2- A peak for the atomic plane of (110) in relatively larger angles (almost 60 for CuKα radition). This peak intensity is equal to the half of two neighbor cations (Mg^{2+}/ Ca^{2+} and Al^{3+} in this work) 3- The 3rd part of XRD patten of LDHs, is a group of peaks related to (01l) atomic planes. Mostly, these peaks are applied to determine the arrangement of atomic layers [7].

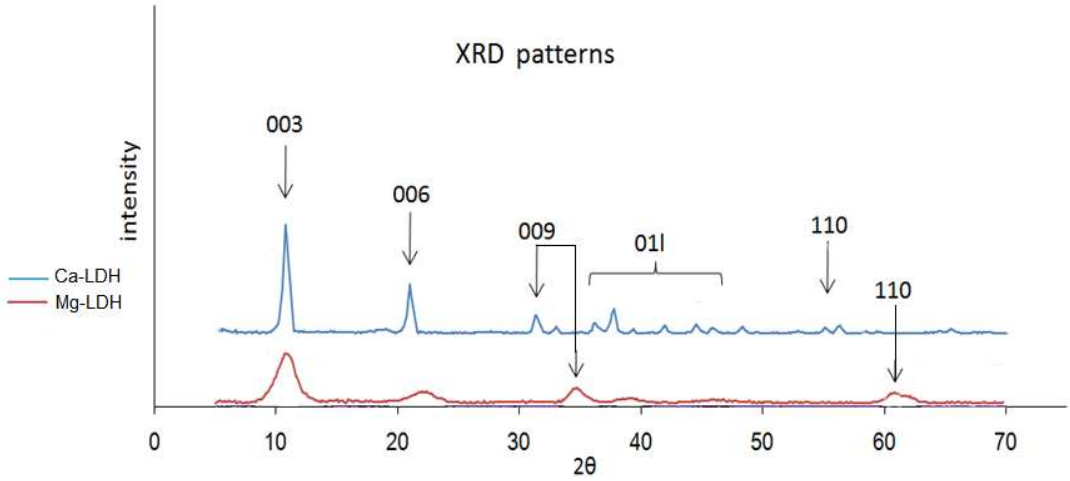

Fig. 1. XRD patterns of specimens.

The index peaks of Ca-LDH specimen are relatively sharper than Mg-LDH specimen and have less width. Therefore, the Ca-LDH specimen has a better crystalinity and its crystal structure is more completed than the Mg-LDH specimen. This is because of the special arrangement of Ca-LDH. The bivalent cation (Ca^{2+}) is being arranged regularly in the octahedral sites because of its smaller size. Therefore, it results that the more crystalinity is observed in Ca-LDH (figure 2) [8]. Furthermore, the (110) atomic plane peak is observed in more acute angles in Ca-LDH specimen that indicates a raise in lattice parameter of a. The other noteworthy point is the disappearance of the group of (01l) peaks in Mg-LDH specimen that decreases the regulation of layers arrangement.

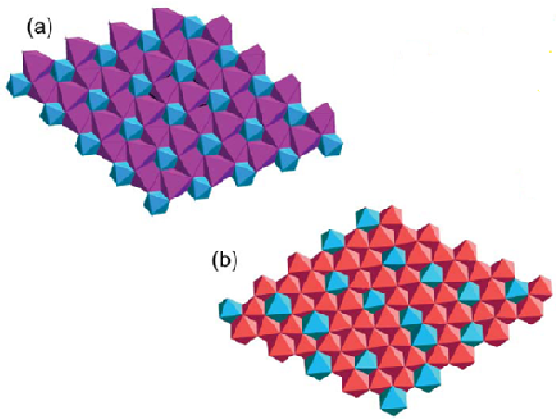

Fig. 2.The arrangement of cations in a: Ca-LDH and b: Mg-LDH [8].

The lattice parameters was calculated as ($c = d_{003}+2d_{006}+3d_{009}$, $d_{average} = c/3$, $a = 2d_{110}$); the values are listed in Table 1 together. The lattice parameter c depends on size of the interlayer anions, as well as on the strength of the electrostatic interactions between the electrostatic interactions between the interlayer spaces and the layers, wich depend on the degree of crystallinity. Ca-LDH showed a higher degree of crystallinity and also because of smaller size it can placed more

regularely on a monolayer of octahederals. It leads to create a stronger electrostatic attraction between Ca^{2+} ions and the other anions there, but this stronge attraction may result a weaker electrostatic attraction between layers and also increases the interlayer distance or lattice parameter c.

Table. 1.Lattice parameters of specimens (Angstrom)

specimen	a	$d_{average}$	c	d_{110}	d_{009}	d_{006}	d_{003}
Ca-LDH	3.316	8.645	25.935	1.658	2.877	4.312	8.68
Mg-LDH	3.038	7.86	23.58	1.519	2.56	3.98	7.94

Obviously, the quantitative results of the lattice parameters calculation confirm the previous analysis close to 25.935 angstrom. The FTIR spectra of specimens are showed on figure 3. The presence of carbonate was banished because no band is detected around 1364 cm^{-1},the position where the most intense carbonate band due to the antisymmetric stretching mod was expected. Four main regions can be detected for the overall FTIR spectra of LDHs. A group of sharp peaks indicate the OH bond stretching and vibrational modes around 3500 cm^{-1}. The peaks are around 1380 cm^{-1} indicates the nitrate group. A peak indicated the water absorption around wavenumber of 1600 cm^{-1}. A group of peaks under wavenumber of 1000 cm^{-1} indicate the stretching mode of OH-Mg and OH-Ca. In Ca-LDH specimen the peaks become more sharp and also have a greater intensity that illustrate the more complete crystal structure and also confirm the XRD pattern results.

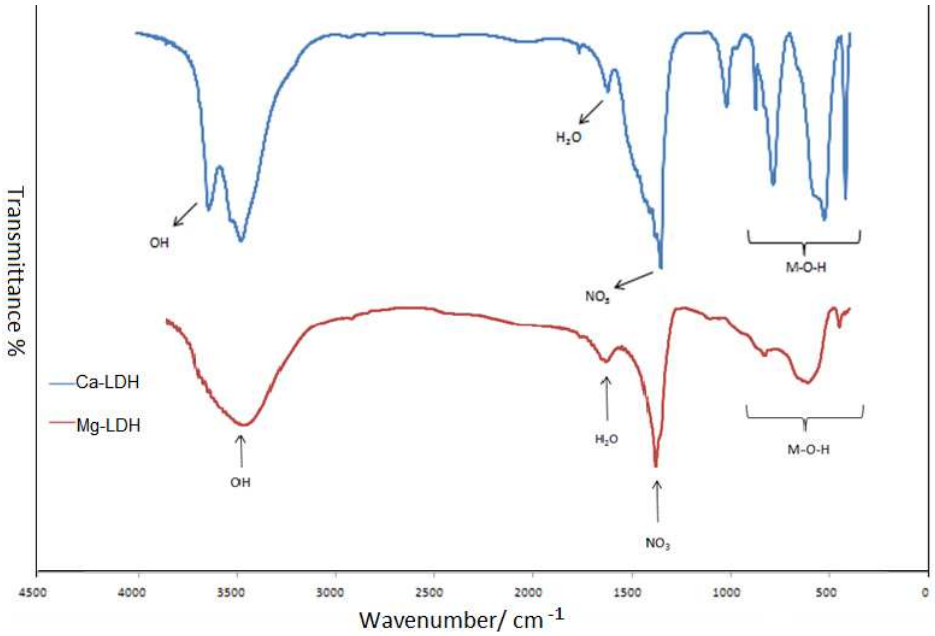

Fig. 3. FTIR spectra of specimens

The SEM images of specimens showed in figure 4. Both specimens are in layer structure and also nano-scale. The particle size is varies from 150nm to 1.5µm. Also, it was illustrated that the Mg-LDH layer structure irregularly agglomerated and the particles were adhered to each other. Therefore, the layer structure was barely visible. Also because of the lack of cationic arrangement the crystal structure had been has a semi-hexagonal morphology. In contrast, in the Ca-LDH specimen, the layer structure arrangement because of the particles not adhered and less agglomeration was well arranged and showed a complete hexagonal morphology. The cations were exactly arranged on octahederal sites. Also the placement of some particles in the direction of perpendicular showed that the leyer thickness is about 100 nm.

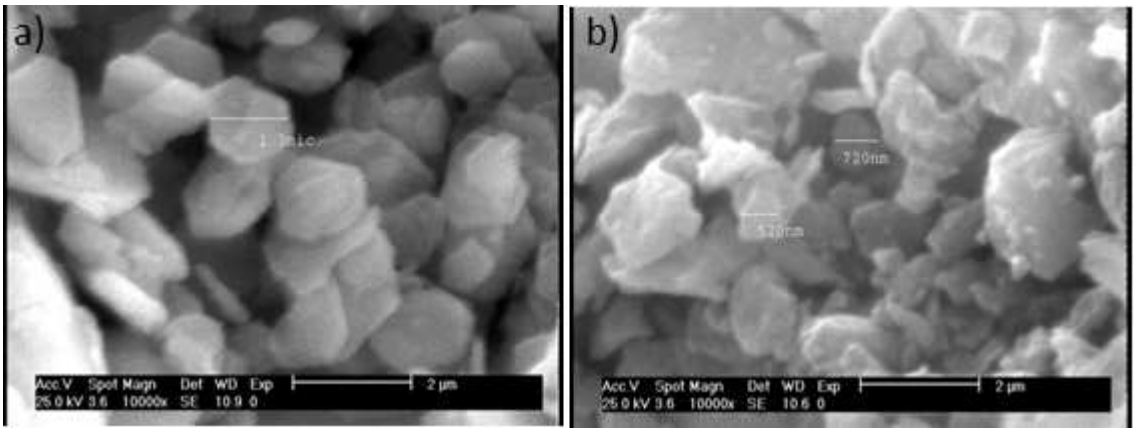

Fig. 4.SEM images of a) Ca-LDH, b) Mg-LDH

The MTT assay was carried out to evaluate cell viability and cytotoxicity and the results are shown in figure 5. As indicated in MTT results, there is no significant difference between specimens over the time except and both of them showed a higher degree of biocompatibility in comparisson to the control specimen. The OD for the Ca-LDH specimen was larger than other specimen after 3 days. It maybe a reasult of presence of Ca^{2+} in culture media (the main element of hydroyapatite, natural bone inorganic phase[]). Similar results obtained in longer periods. Consequently, this specimen seems to be more biocompatible in comparison with the Mg-LDH specimen, but in longer periods the difference of OD values decreased. So, both specimens provide a stable condition for cell viability and metabolism over a 14- day period. There is a 20% decrease during the first week, and then stable condition is tend to be. These results suggested biocompatibility of two specimens in a long period of time. This reduction can be a result of reactivity function of LDH. Obviously, in the early hours the rate of reaction and cell-media interaction has its maximum value and during the reaction progress the rate of reaction is decreasing.

Decrease in release rate of some ions such as Ca^{+} can lead to a reduction in cell proliferation [9]. Recrystallization and Ca layer formation on LDH plate seems to have a role in cell proliferation and viability. Although MG63 is a established osteosarcoma cell line, but its function differs in comparison with other osteoblast cell lines or primary human osteoblast cells in biological functions and proliferation [10].

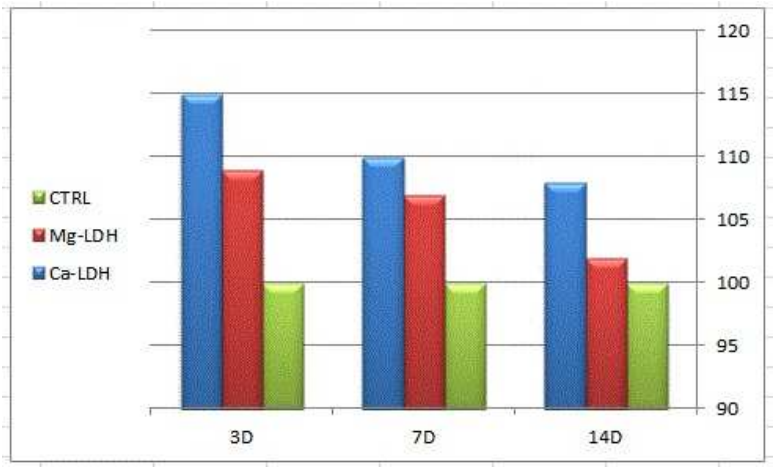

Fig. 5.OD in the MTT assay for MG63 osteosarcoma cell line cultured at a density of 10^4 cells/well.

Alkaline phosphatase activity as an indicator of osteoblastic phenotype is showed in figure 6. The Mg-LDH specimen showed a greater value of alkaline phosphatase extraction in first week and second week, but in second week the difference value of the amounts of extracted alkaline phosphatase between Mg-LDH and Ca-LDH became less. It may because of presence of Mg^{2+} in

culture media. The Mg^{2+} acts as a messenger agent of osteoconductivity [11]. similarly, over passing time it was reported that the rate of reaction and also alkalin phosphatase extraction was decreased.

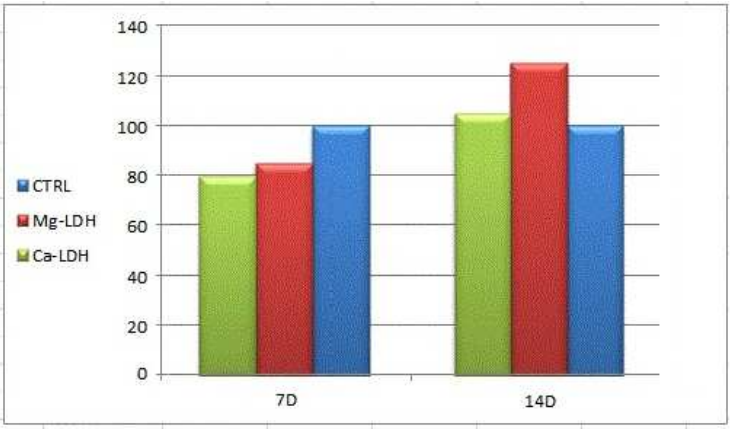

Fig. 6. The value of alkaline phosphatase extraction in LDH powder-extracted culture media

Conclusion

The LDHs have recently entered to the biomaterials field but the major capacity such as high degree of bioactivity, easy forming processing and ability to controled drug release; make it a suitable choice for bone tissue engineering insitu drug delivery applications.
In this work, Ca-LDH and Mg-LDH were synthesized, characterized and also biologically evaluated. Ca-LDH had a greater crystallinity and larger particle size. the lattice parameter a of this specimen is taller than the Mg-LDH's. Both specimens are biocompatible, but the Ca-LDH showed a better biocompatibility and the osteoconductivity of Mg-LDH is more than Ca-LDH whereas both of them showed better osteoconductivity than Control specimen.

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