Alendronate Sodium Intercalation in Layered Double Hydroxide/Poly (ε-caprolactone): Application in Osteoporosis Treatment

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Background: Osteoporosis is a bone disease alters the amount and variety of proteins in bone tissue and increases the potential of bone fracture. Antiresorptive therapy is one of the most popular treatment methods for osteoporosis. To reduce side effects and enhance the bioavailability of drug agents, the controlled delivery of drug is commonly utilized.
Objectives: We investigated the controlled release of Alendronate in different composites of layered double hydroxide (LDH) using poly (ε-caprolactone) (PCL) as a matrix.
Materials and Methods: We prepared different microsphere composites of ALD intercalated in various amounts of LDH, using PCL as a matrix. The controlled release of ALD from these composites is subsequently investigated. Samples are characterized and *in vitro* cell cytotoxicity, attachment, osteogenic activity including alkaline phosphatase activity and mineralization are examined using MG-63 human osteosarcoma cells.
Results: The results showed that the release of ALD is more desirable and controlled in the samples having a higher amount of LDH incorporated into the PCL matrix. MG63 cells show a significant increase in viability, attachment, and mineralization while alkaline phosphatase activity remains almost at a constant level after 3 weeks.
Conclusions: Overall, the findings showed that by incorporation of 15 wt% of LDH, the composite microsphere is capable of holding the antiresorptive drug longer and release it in a more controlled manner. This is an advantageous and promising characteristic for a carrier that could be used as a potential candidate for osteoporosis treatment.

Keywords: Alendronate, Layered double hydroxide, Osteoporosis, Poly (ε-caprolactone).

1. Background

Over the last century, the incidence of osteoporosis has continued to increase as a result of progressive increase of elderly population. The loss of bone strength associated with this disease can significantly enhance the risk of skeletal fractures, deteriorates the micro architectural bone tissue which leads to the pain and reducing the quality of the patient (1-3). The most frequently affected sites of the osteoporotic fracture are the femoral neck of hip, spine and wrist (4). In recent 30 years, biomaterial research outcome has reached to a multitude of materials for replacement of osteoporotic bone as well as treatments consist of testosterone replacement and osteoporotic medication (5). Bisphosphonates such as sodium alendronate (ALD) are

the pioneer drug classes for treatment of osteoporosis which could prevent osteoclast-mediated bone resorption, reduce bone turnover and maintain the bone tissue microstructure (6). However, passive absorption of ALD which depends on its concentration in the biological fluid is a remaining challenge (7-10). Hence, a key matter is increasing the bioavailability of these antiresorptive drugs which prolongs the drug access to bone sites without exceeding the toxic level (8). This can be achieved by drug delivery systems based on biomaterials that can deliver drugs continuously in a controlled manner. **Table 1** represents the delivery systems that have been used for enhanced delivery and efficiency of this drug.
To encapsulate ALD and enhance its bioavailibility,

Table 1. A summary of Alendronate delivery by various systems for osteoporosis treatment.

Drug carrier	Application	References
Self-dissolving microneedle arrays	Increase the bioavailability without skin irritation for undergoing treatment of osteoporosis	(11)
Polyethylene glycol-conjugated ALD	suitable for bone tissue	(12)
PEG–ALD	Increase the growth plate in an osteoporotic model	(13)
N-(2-hydroxypropyl) methacrylamide copolymer	Bone diseases treatment	(14)
nanocomposite plasticized PLGA film with alendronate–LDH clay	biocompatible with osteoblasts and after 5 week incubations and bone nodule formation	(15)
Gellan gum based hydrogels	An injectable system to inhibit osteoclastic differentiation for over 25 days	(16)
mesoporous silica based matrices	Increase total drug loading from functionalized materials for bone disease	(17)
Doxorubicin poly (ethylene glycol)- ALD	Tumor growth, reduce bone loss and bone metastastic tumor	(18)
Paclitaxel with conjugation of PEG and ALD	As a core- shell system for anti-cancer bone-target system	(19)

layered double hydroxides (LDHs) as a hydrotalcite-like compound was used. These anion clays have lamellar structure with positive charge that could easily intercalate anions such as vitamins, anti-inflammatory, anti-fibrinolytic, and anticancer agents (20-22). LDHs as a drug carrier provides many advantages including ease of preparation, feasibility of being modified with various organic anions, good biocompatibility, stabilization of the loaded drug, and controlled release of the drug (23-25). However, the exposure of LDH to physiological and chemical triggers could result in its deterioration leading to an undesirable dose of the released drugs. As a consequence, the use of polymeric matrices such as poly (ε-caprolactone) having sufficient mechanical integrity and biodegradability which is capable of hosting and protecting the drug carrier, is essential. Previous studies have indicated that polymer/LDH nanocomposites have mechanical, optical and thermal properties which rarely exist in neat polymer or micro scales composites (10, 26, 27). Still, while many reports are available on the PCL reinforced LDH for tissue engineering applications, there has not been much investigation yet on a convenient method to prepare potential delivery system for osteoporotic medication (23, 24, 28-30).

2. Objectives
In this study, a delivery system for controlling the release of ALD, an antiresorptive drug, using LDH as carrier and poly caprolactone as polymeric matrix, is developed. To obtain a slow drug release system, different concentrations of LDH/ALD were dispersed into poly caprolactone matrix using solvent evaporation method. The release behavior of ALD from developed delivery systems was investigated. Furthermore, the effect of different specimen on the cytotoxicity and morphology of osteoblast-like MG63 cells were investigated. Finally, osteogenic activity of the specimens was tested through alkaline phosphatase activity and Alizarin Red S staining assay.

3. Materials and Methods

3.1. Materials
Na-Alendronate (Fosamax) was a gift from (Alborz Darou, Iran) under license of (Cipla Company, India). Poly (vinyl alcohol) (PVA $M_w \approx 30,000$), dichloromethane, Al $(NO_3)_3.9H_2O$, $Ca(NO_3)_2.4H_2O$, ethanol, sodium hydroxide, hydrochloric acid, Alizarin Red S solution and Glutaraldehyde solution 25% were purchased from (Merck, Germany). In addition, Poly (ε-caprolactone) (PCL Mw 70,000-90,000) Dulbecco's Modified Eagle's Medium (DMEM), sodium dodecyl sulfate (SDS), trypsin, Triton™ X-100, Phenylmethanesulfonyl fluoride solution (PMSF), formaldehyde solution, acetic acid and ammonium hydroxide solution were obtained from Sigma-Aldrich. MG63 (human osteoblast-like osteosarcoma) cells were obtained from Stem cell technology research center (BONYAKHTE Company, Iran). Alkaline phosphatase assay kit and Quick Start™ Bradford protein assay were purchased from Pars Azmun co, Iran and (Bio-Rad Laboratories Inc, USA), respectively. All other chemicals were of analytical grade and used without any purifications.
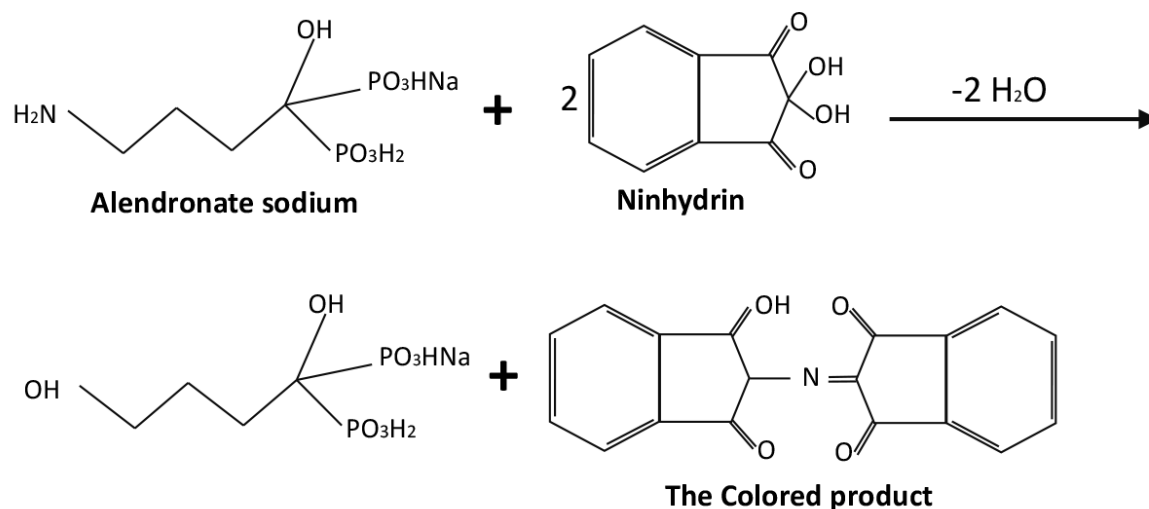
Figure 1. An schematic representation of ninhydrin binding to Alendronate.

3.2. Microsphere Sample Preparation

3.2.1 LDH Preparation

The LDH-NO$_3$ suspension was synthesized as previously described (31). In a typical synthesis, Al(NO$_3$)$_3$.9H$_2$O (12.5 mmoL) and Ca(NO$_3$)$_2$.4H$_2$O (25 mmoL) were added to 100 mL deionized water in a glassy reactor under nitrogen flow and stirred at 2.23 g (200 rpm) for 15 min. The pH was adjusted to 11 by dropwise addition of 0.4 M aqueous NaOH solution. The suspension was left in the reactor for 2 h at room temperature and washed several times by deionized water and centrifuged to remove the OH ions and decrease the pH to 7. Next, the suspension was poured into a hydrothermal reactor and heated at 120 ˚C for 48 h in a vacuum oven. Finally, the suspension was washed with deionized water, centrifuged at 1788 g (4000 rpm), and vacuum dried for 10 h at room temperature.

3.2.2 Intercalation of Alendronate into LDH

LDH-Alendronate (LDH-ALD) microhybrids were prepared using the ion-exchange method. Typically, 10 g.L^{-1} suspension of LDH was added to 1 mL aqueous ALD solution with the ratio of ALD: NO$_3^-$ =2:1. Moreover, the suspension was stirred at 150 rpm for about 24 h. Finally, the precipitates were collected by centrifuging at 1788 g (4000 rpm), washed 3 times with DI water and dried at room temperature overnight.

3.2.3 Preparation of Microspheres

In order to prepare microspheres of PCL/LDH-ALD, a water/oil/water double emulsion was formed using solvent evaporation-extraction. Initial concentration of 5, 10, 15 % (w/w) of LDH-ALD and ALD without LDH as a control were prepared. PCL solution was

prepared by dissolving the pre-determined amount of LDH-ALD into the 40 mL of 1% (w/v) PVA to obtain an aqueous solution. As for the control the ALD was added to 1% (w/v) PVA solution. The dissolved PCL in dichloromethane was slowly added to 1% (w/v) PVA and the resulting emulsion was stirred at 36000 rpm with homogenizer for 90 s. To ensure complete evaporation of dichloromethane, the emulsion was stirred gently for 24 h at room temperature. The microsphere samples were collected by centrifuging at 1788 g (4000 rpm) and dehydrated by freeze-dried for 10 h. The specimens were referred as L0, PL5, PL10, PL15, L100 which is based on the amount of the LDH-ALD in the microsphere samples.

3.3. Drug Release

Alendronate release from the samples were analysed by dissolving ninhydrin as a detecting reagent. The release kinetics of drug in a fixed volume of a physiological phosphate buffer saline (PBS 1x pH 7.4) was studied by ultraviolet spectrometric measurement (UV-2401 PC Shimadzu, Japan). A predetermined amount of each sample was added to 3 mL of phosphate buffer saline, placed in dialyze bag, placed in 200 mL PBS solution and incubated at 37 ºC while shaking at 2.70 g (110 rpm). After each time point, 200 μL of the above mentioned solution was withdrawn and replaced with the same amount of fresh medium. Since Alendronate does not have any absorbance within UV-visible region, the complex of ninhydrin and alendronate was used to monitor the alendronate release by measuring the absorbance at 568 nm. The mechanism of ninhydrin binding to Alendronate is depicted in **Figure 1.** The ninhydrin and NaHCO$_3$ mixture with the ratio of 5:1

were added to each sample and heated up to 90 °C followed by cooling down to the room temperature to obtain the final purple product (32).

3.4. Cytotoxicity Assay

To measure the viability of MG63 cells as an indicator for cellular metabolic activity and cytotoxicity, MTT assay was performed. In 24 well plates, cells were cultured at a density of 10^4 cells.mL^{-1}, containing different microsphere samples. After 1, 3, and 7 days, cells contain oxidoreductase enzymes reduced MTT solution (500μg.mL^{-1}) added previously and incubated for 4 h. the insoluble formazan crystals were dissolved in DMSO as a solubilization solution and the intensity of resulting MTT solution was quantified at 540 nm using a multi-well spectrophotometer (Biochrom, Canada). At last, the absorbance of cells was divided by that of the control to obtain the percentage of cell viable.

3.5. Alkaline Phosphatase Activity

To evaluate the osteogenic activity of MG63 in the presence of different microsphere samples alkaline phosphatase (ALP) activity was performed after 7, 14, and 21 days of incubation. This colorimetric assay was carried out using alkaline phosphatase assay kit (Abcam, USA, ab83369) based on the manufacturer's protocol. In a concise manner, In 6 well plates, cells with microsphere samples were cultured at a density of $2x10^4$ cells.mL^{-1}. After washing cells with cold PBS, they were scrapped by lysis buffer. Cells were prepared for ALP activity analysis and total protein content after homogenizing and centrifuging for 15 min at 4 °C. In this kit, dephosphorylated p-nitrophenyl phosphate turns yellow regarding the level of ALP in the samples. First, in order to stop ALP activity of samples, stop solution was added to the solution of cell lysis. After 60 min in the dark room, 50 μL of p-nitrophenylphosphate was added to each sample, the qualified amount of p-nitrophenol is measured at 405 nm. To normalize the ALP activity, the standard curve of total protein content using the BCA protein assay kit (EMD Millipore Company, Darmstadt, Germany) is obtained. The result was determined at 562 nm by Thermo Scientific™ Multiskan™ GO Microplate spectrophotometer (Thermo Scientific™, USA).

3.6. Morphological Study

A similar amount of each microsphere sample was used to prepare a disk like sample for evaluation of the osteoblasts morphology. The adherent cells on the disk like samples were examined after 3 days of incubation using SEM. The cells were fixed by 1% (v.v^{-1}) glutaraldehyde for 1 h followed 3 days in 3% (v.v^{-1}) glutaraldehyde and then washed with deionized water. Finally, the specimens were dehydrated using a graded series of ethanol in PBS including 30%, 50%, 70%, 90% and 100% for 10 min each which was followed by SEM examination.

3.7. Mineralization Study

Alizarin red S staining assay as a colorimetric sensitive assay is determined by the extraction of the calcified mineral of MG63 cells at low pH in the presence of different microsphere samples to the culture media after 15 days. Briefly, cells at a density of $2x10^4$ cells. well^{-1} were cultured in 6 well plates. Monolayers were washed twice with distilled water and they were fixed by adding 4 % paraformaldehyde at room temperature for 20 min. To prepare the calcified depositions samples, first, alizarin red S solution was added, stained for 10 min and the wells were washed several times in order to remove excess dye. The qualified amount of calcified depositions in bright red were investigated by light microscopy. Regarding quantification of staining, cells should be attached loosely to scrape from the plate. Therefore, to each well, 1600 μL acetic acid 10% v.v^{-1} was added and the plates were incubated for 30 min with gentle shaking at room temperature. Monolayer was transferred to a micro centrifuge tube and after vigorous vortexing for 30 s, heated to 85 °C for 10 min and transferred to ice for another 5 min. Finally, the tubes were centrifuged for 15 min at 16099 g (12,000 rpm) and in order to ensure that pH of each sample was 4.1, 100 μL ammonium hydroxide 10% v.v^{-1} was added. The absorbance of supernatants in aliquot of 200 μL was measured at 405 nm in triplicate.

3.8. Statistical Analysis

Experimental data were presented as means ± standard deviation. Significant differences among the groups was determined using analysis of a one way (ANOVA) and values of $p \leq 0.05$ were determined as statistically significance.

4. Results

4.1. FTIR Analysis

The Fourier transform infrared spectra of PCL, LDH, LDH-ALD and PCL-ALD are depicted in **Figure 2**. For LDH-ALD, the broad absorption bound at 3450 cm^{-1} is related to the O–H vibration mode of the hydroxyl group and water molecules. The absorption at 1617 cm^{-1} is assigned to the bending vibration of O–H band in LDH.
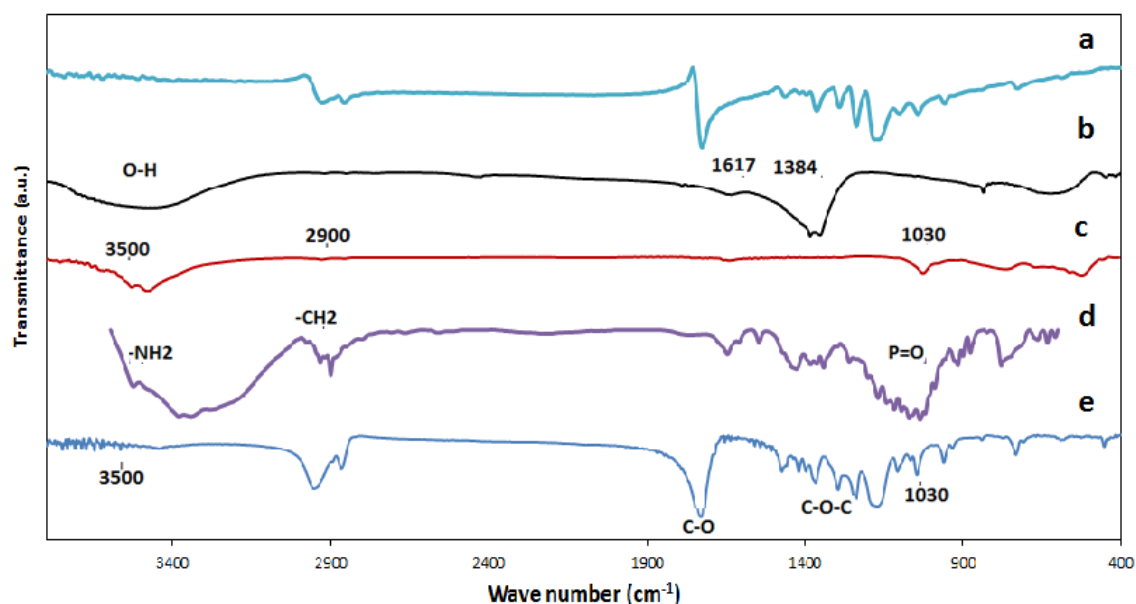
Figure 2. Fourier transform infrared spectroscopy of (a) PCL, (b) LDH, (c) LDH-ALD, (d) ALD and (e) PCL-ALD.

Furthermore, the absorbance bands in the range of 400-800 cm^{-1} represent lattice vibration bands of Ca–O and O–Al–O. The absence of 1384 cm^{-1} band indicated the elimination of the NO$_3$ ions from LDH interlayer and their replacement with Alendronate anions. The weak absorption band at ~2900 cm^{-1} is attributed to stretching mode of CH$_2$ in LDH-ALD (33). The primary vibration mode of amine bands as well as stretching mode of phosphate and carbonate bands in ALD, PCL-ALD and LDH-ALD were appeared at 3500, 1030, and 850-890 cm^{-1}, respectively. This is an indication for no characteristic changes in LDH and PCL bands after ALD incorporation. The main characteristic peaks of neat PCL as indicated in PCL-LDH spectra are 730 cm^{-1} for CH$_2$ long chain rocking motion vibrations, 1275–1050 cm^{-1} for C–O–C aliphatic ether stretching vibrations, 1450 and 1380 cm^{-1} for CH$_2$ and CH bending vibrations, 1735 cm^{-1} for C–O stretching vibrations, and 3000–2800 cm^{-1} for CH$_2$ stretching vibrations (25).

4.2. Morphology of Microsphere Samples
Figure 3 shows the SEM images of different microsphere samples. As can be seen in **Figure 3a** LDH showed uniform hexagonal flake-like particles with high aspect ratio with particle size in the range of 2.5-7.5 mm. The SEM images of PL5, PL10 and PL15 revealed that Ca.Al-ALD lamellar structures have completely lost their stacking orders and were inhomogeneously distributed on the surface of polymer spheres.

4.3. Release Behaviour
To investigate the release of the drug from PCL and microsphere samples, it is necessary to use ninhydrin in order to be able to detect the free ammonia band in the samples containing ALD at 568 nm. **Figure 4** presents the time-dependent release profile of drug from different microsphere samples. As clearly could be seen (**Fig. 4**), all the samples showed three stages of drug release. The first stage displays an initial burst release which is an indication for the loosely deposited drug on the surface of LDH. It should be noted that, the fast release stage could enhance the amount of released drug leading to undesirable toxicity which depending on the application needs to be controlled (34). The second stage depends on the diffusion behaviour of anions and the final stage would occur during the release of the drug molecules from microspheres (29). As the release of the drug takes place through the above mentioned stages, it would be very helpful for controlled release application for many days and suitable for osteoporosis treatment. The PL15 sample showed the longest period of time in stage 3 and therefore a better efficiency in controlled drug release. Many investigations have been carried out on the controlled release of Alendronate using different substrates such as chitosan to enhance Alendronate encapsulation around 70% by their opposite charges (35), polyethylene glycol (PEG) for treatment of bone metastasis (36), poly lactic-co-glycolic acid (PLGA) for long-term delivery of Alendronate (37), high molecular
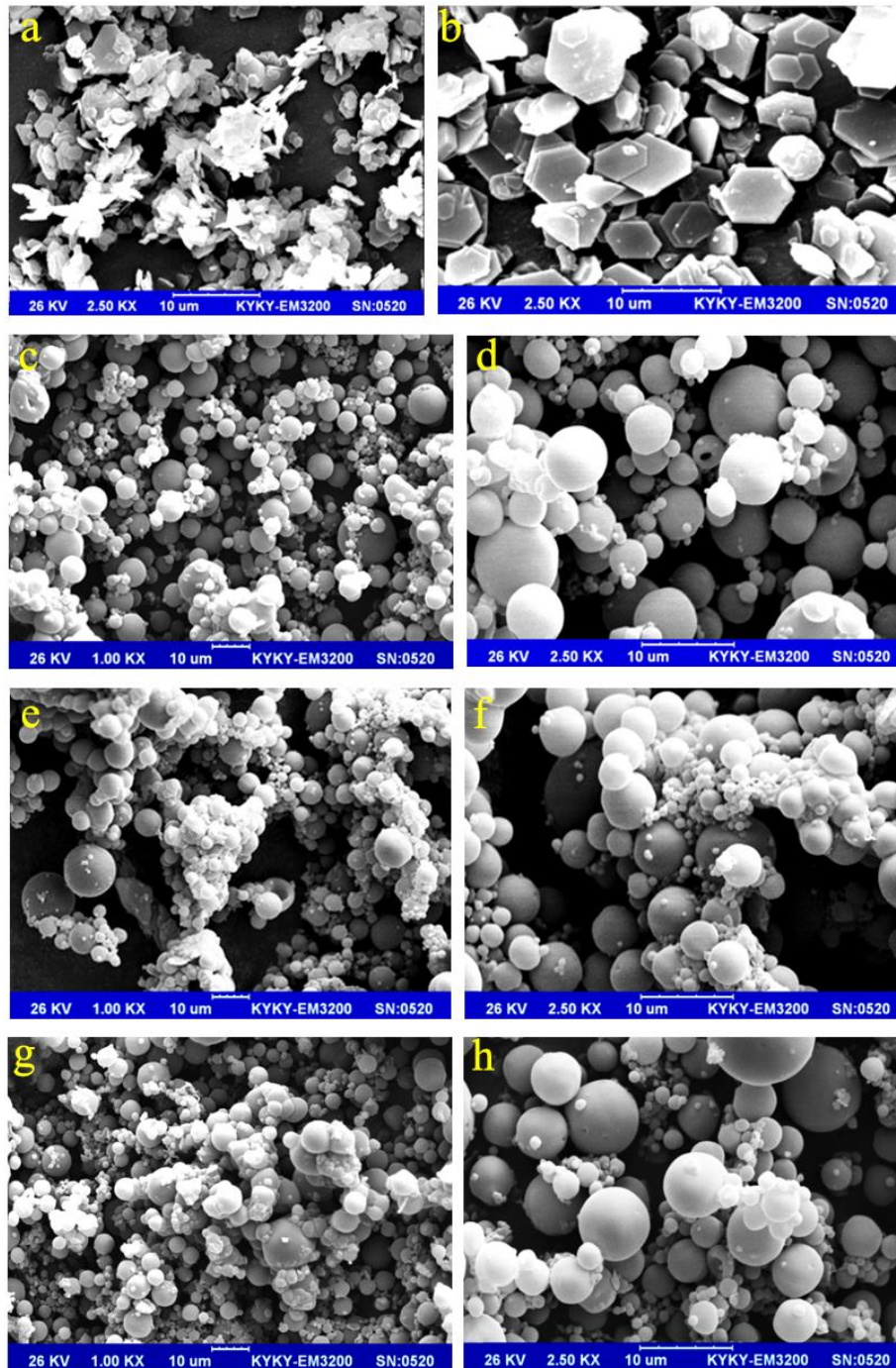
Figure 3. Morphological characterization samples by scanning electron microscopy; (a,b) L100, (c,d) PL5, (e,f) PL10, (g,h) PL15.

weight poly caprolactone to increase particle size and encapsulation efficiency (34) and LDH as a suitable drug carrier for co loading of Alendronate and Tetracycline (36). The current study revealed that different composites of LDH in PCL could provide various rates of controlled release during 60 days. Despite of the significance burst release, PL15 indicates the best rate for the controlled release among the samples.

4.4. MTT Assay

The results of MTT assay is presented in **Figure 5a**. Viability of the MG63 cells was the highest in L0 and L100 which were used as control followed by PL10 and PL15 samples. The cell viability in PL5 was the lowest compared to neat PCL or LDH-ALD. It is believed that the burst release of Alendronate (more than 65%) in less than 10 hrs resulted in the increased
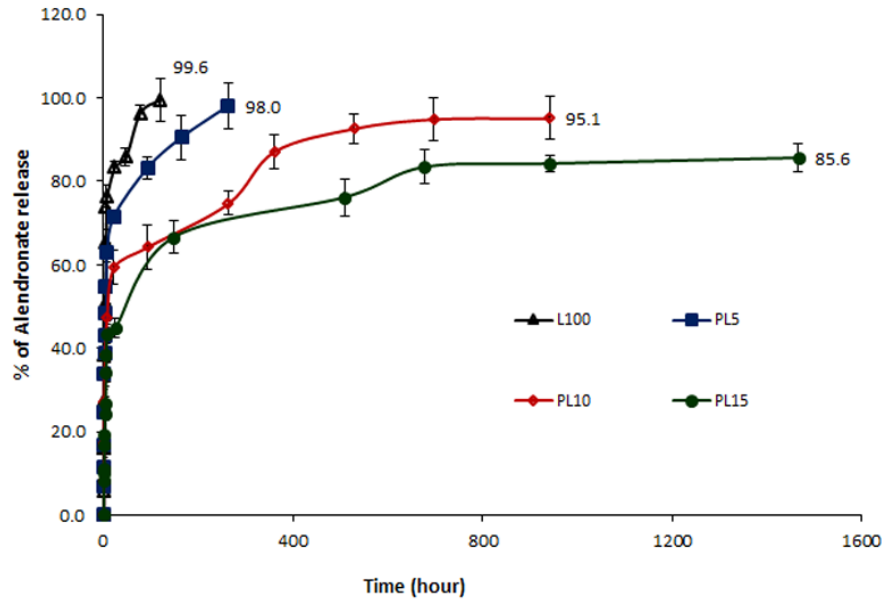
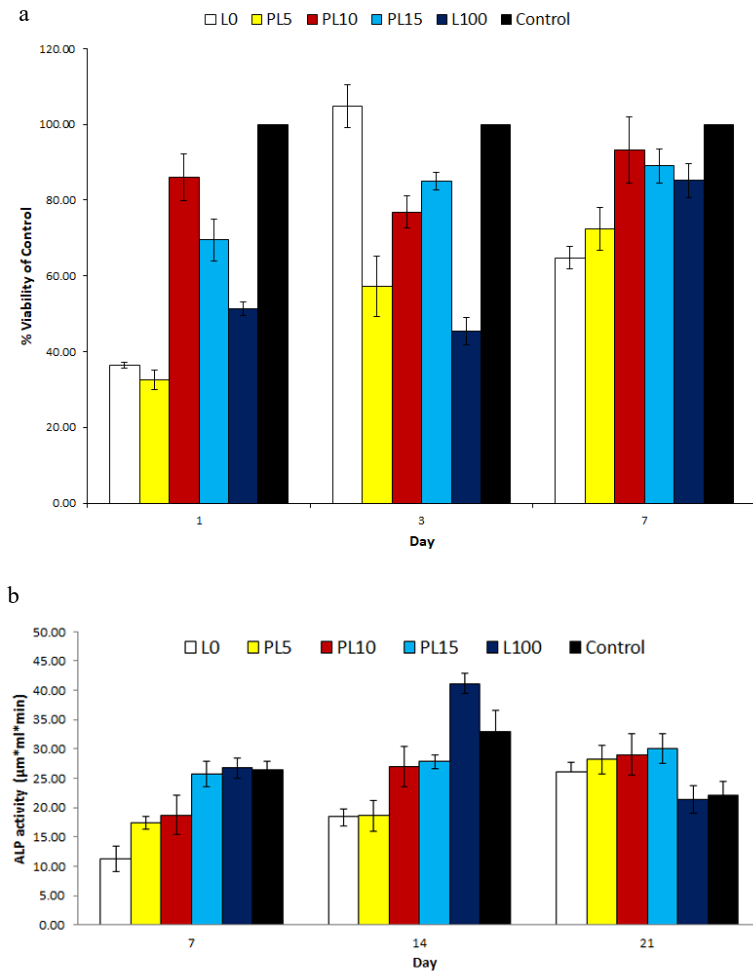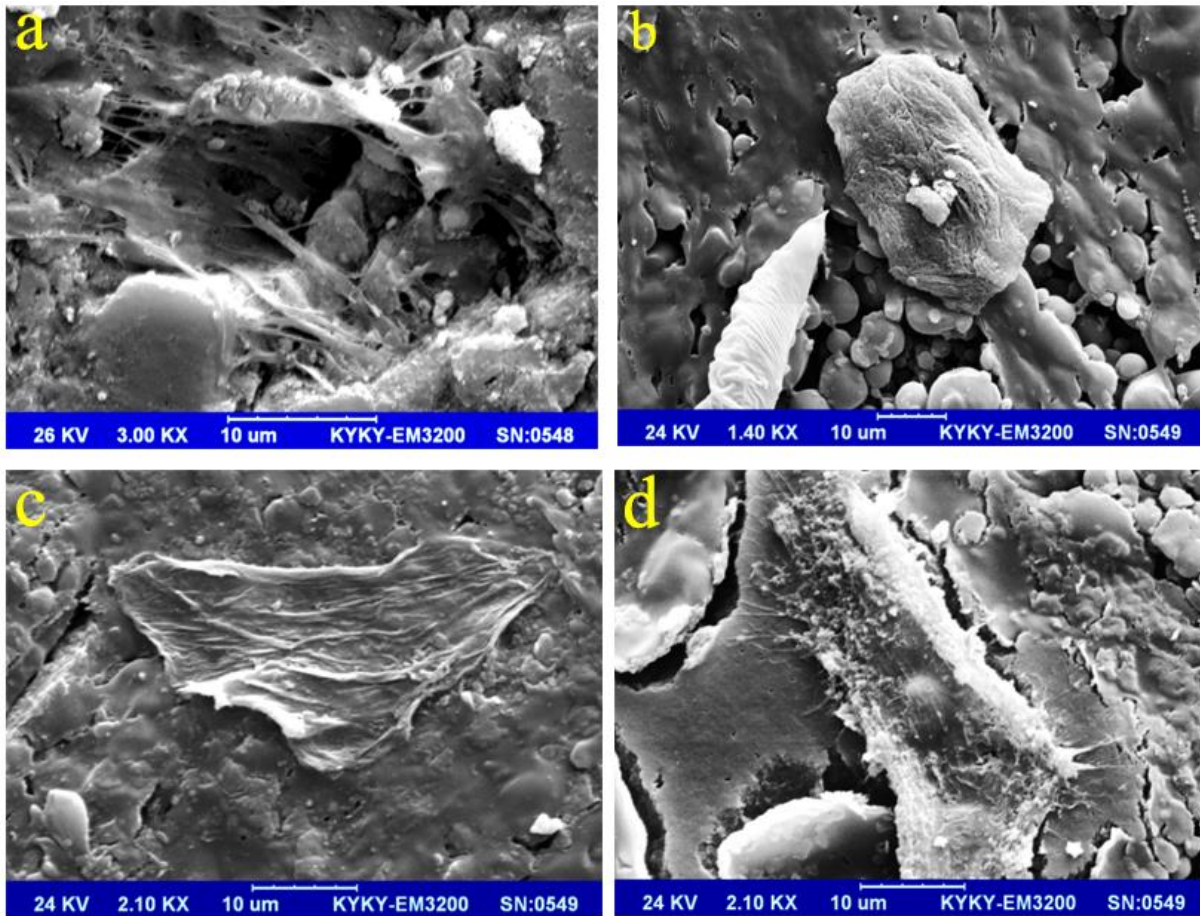**Figure 4.** Release behaviour of Alendronate in different samples.

Figure 5. (a) MTT assay on different microsphere samples and controls including PL5, PL10, PL15, L0 and L100 after 1, 3, and 7 days of culture of MG-63 cells. (b) ALP activity of MG-63 cells cultured on samples after 7, 14 and 21 days in triplicate. Data are the mean ±SD of five samples.

Figure 6. SEM observation of MG63 cells after 3 days of culture on (a) L100, (b) PL5, (c) PL10 and (d) PL15.

cell toxicity and decrease in cell proliferation of PL5. In addition, after 7 days of culture the PL5 and L0 specimens were shown increased cytotoxicity. The PL15 and PL10 samples demonstrated better cell viability and cell compatibility evidenced from an increased number of viable cells.

4.5. ALP Activity

ALP activity is an early indication for osteoblastic differentiation and their oseteogenic activity (34). **Figure 5b** shows that ALP activity of MG63 cells in the presence of different specimens after 1, 2 and 3 weeks of incubation. While the ALP activity of all samples has increased by time, it was not statistically significant in all the time points. After 7 and 14 days, the PL15 showed higher ALP activity compared to L0 and L5 specimens. The finding of this study is similar to the previous reports measuring the ALP activity of MG-63 cell treated with ALD (35,36,38). The ALP activity of PL10 and PL15 were approximately similar and did not show any significant increase after 14 days.

4.6. SEM Analysis

SEM images of MG63 cells after 3 days of incubation with different samples are shown in **Figure 6**. **(Fig. 6a)** shows that the osteoblast cells were covered the pores of LDH structure in L100 samples which was used as control. However, the spindle-like shapes of cells could not be observed in PL5 and PL10 samples having lower amount of LDH. **Figures 6b, c** exhibit that the cells were flat in shape but unable to fully cover the surfaces of PL5 and PL10 samples with cellular extension which is due to the lack of porous structure of these sample. However, higher number of cells that were attached on the surface with many filopodia penetrating into the pores of PL15 sample was detected (**Fig. 6d**). On PL15 sample, cells were completely flattened with an irregular pattern in a way that fully covered the surface that could be improved that the bioactivity of the samples was increased.

4.7. Alizarin Red Staining

Alizarin red which preferentially stains calcium

a

b
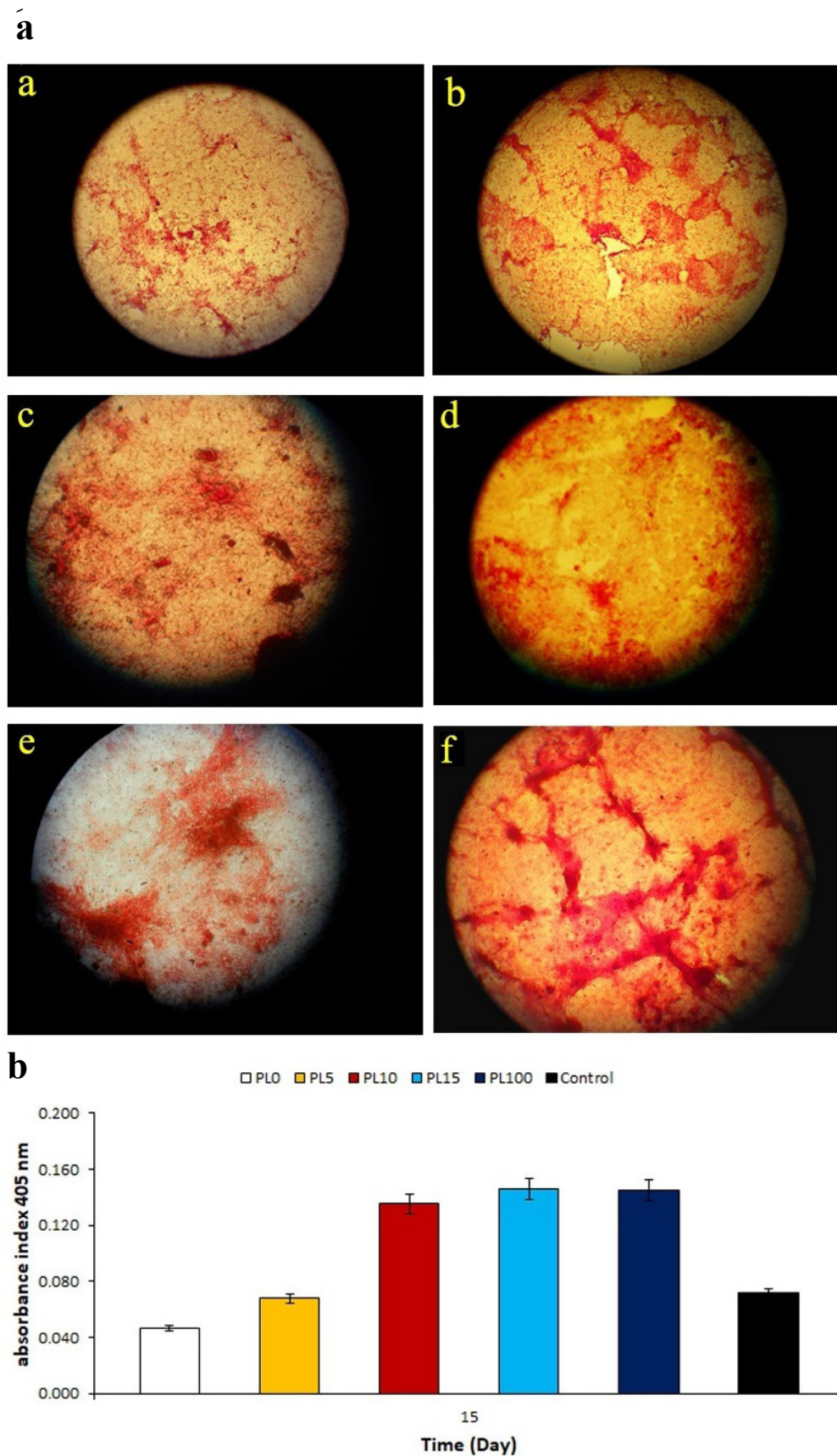
Figure 7. (a) Alizarin red S staining representing calcium containing matrix and mineral deposition on a) control b) L0 c) L100 d) PL5 e) PL10 f) PL15 after 15 days of incubation. (b) The osteogenic activity of MG63 cells was qualified in 2D culture plate at 405 nm wavelength in triplicate at the day 15.

containing matrix has been widely used to identify the mineral deposition. The osteogenic activity of MG63 cells was determined by the Alizarin Red S staining after 15 days of incubation. As expected, L0 and PL5 showed no considerable mineralization after 15 days of culture. Furthermore, areas with nodular cell formation were significantly increased with increasing the LDH content and samples were clearly red as depicted in **Figure 7a**. The quantification of staining demonstrated that PL15 and L100 have higher number of osteogenic cells rather than other samples (**Fig.7b**). Therefore, the PL15 sample having higher amount of LDH presented the highest number of osteogenic cells with higher activity and matrix formation capability (39).

5. Discussion

In the present study microsphere samples of ALD intercalated in five different amounts of LDH in a matrix of PCL were prepared namely L0, PL5, PL10, PL15, L100. FT-IR did not show any characteristic changes in LDH and PCL bands after ALD incorporation and SEM techniques represent that lamellar structures of Ca. Al-ALD were distributed on the surface of polymer spheres. According to the results, PL5 composite showed a very fragile structure, since the pellet including this composite may break away during the pellet preparation or after sterilization. This could be attributed to the mechanical properties of this sample and could be due to the increase in the amount of LDH in the samples which resulted in enhanced flexibility of composite samples and improved mechanical properties of PL15. A slow drug release can be observed from microsphere samples compared to L100 containing 100% LDH during 60 days. This might be correlated to the structure and polymer chain relaxation of PCL which could control the release of the drug via diffusion process. The results also showed that by increasing the amount of LDH in microsphere samples the duration of drug release period was enhanced. It can be seen that the burst release in PL15 sample was the lowest among the samples whereas the other samples having lower amount of LDH showed a lower controlled drug release. The released ALD was quantified with UV spectroscopy which revealed that PL15 could release 85% of the drug during the 60 days of measurement while the other samples released almost 99% of the drug. This could be related to the intercalation of higher amount of the drug into LDH structure which inhibits the rapid burst release of the drug. In MTT assay study, we obtained better results in PL15 and PL10 samples. This result could be correlated to the higher amount of LDH which increased surface area and biocompatibility

of the samples leading to increased number of viable cells. Furthermore, the controlled drug releases of these samples could be another explanation for lower cell toxicity and higher cell proliferation (36). In addition, after 14 days, the ALP activity of PL10 and PL15 did not show any significant increase. It is assumed that the higher contact area provided by enhanced quantity of LDH could probably be the reason for the higher values of differential expression of cells after 3 weeks. On the other hand, several studies have demonstrated that the higher number of mature osteoblasts could cause the expression of several osteogenic marker genes which could result in ALP activity decrease after 21 days (37,40,41). Moreover, the morphological and alizarin red staining studies of samples indicated that firstly, on PL15, cells covered the sample in an irregular pattern which confirmed the increase of bioavailability of this sample and secondly, this sample seemed to be more capable for more osteogenic activity.

6. Conclusion

In the current study, different concentrations of LDH-Alendronate were adopted to synthesize microsphere-PCL composites. The *in-vitro* release study showed an initial burst release for all the samples which was directly proportional to the amount of LDH in the samples. The PL15 showed an acceptable controlled drug release and provided better biocompatibility, cell attachment, and mineral deposition of osteoblast-like MG63 cells. It is concluded that PCL-enriched LDH composite, PL15, has better osteogenic characteristics in comparison to the pure PCL, promising a bio-resorbable candidate for treatment of damaged osteoporotic bones. However, further studies should be directed toward implantation of the samples to evaluate bone formation *in vivo.*

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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