Synthesis and Biomedical Application of Hydroxyapatite as Carrier for Loading and Controlled Delivery of Antibiotics

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The hydroxyapatite (HA) nano and microparticles were synthesized by wet-chemical precipitation in order to use them as drug carriers for biomedical applications. Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS) and Fourier Transform Infrared Spectroscopy (FTIR) were performed to assess their size, external morphology and chemical composition. The properties of HA particles as drug carriers for antibiotics delivery were evaluated with doxycycline and chloramphenicol. The amount of drug loading and release was determined by UV-Visible spectrophotometry. The antibacterial properties of loaded HA particles were evaluated using gram-positive Bacillus subtilis bacteria and gram-negative Pseudomonas aeruginosa bacteria. The synthesized particles of HA exhibit a high adsorption capacity (around 99%) and good controlled release properties for doxycycline. The adsorption of chloramphenicol on HA was extremely low (about 2%). According to the results, the compatibility between the drug and substrate is an important factor in the absorption process, and the hydroxyapatite is a very promising carrier for controlled release of antibiotics.

Keywords: hydroxyapatite synthesis, drug carrier, drug release, antibacterial activity, doxycycline, chloramphenicol

The study of micro and nanoparticles in biomedicine has an important role in the development of new generation of drug delivery systems. Micro and nano-sized particles are especially desirable over conventional drug carrier due to their high specific surface areas which allow an excellent drug loading capacity and a controlled drug release. Additionally, these drug delivery systems have the advantages of improving the therapeutic efficacy by providing a constant drug concentration in blood, concomitant with a reduction in the drug intake and its toxic effect.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) is a natural inorganic compound which is found in the hard tissues of bones and teeth of vertebrates. Due to its biocompatibility, bioactivity and nontoxicity, nano and micro-size HA particles have been successfully used in medical and pharmaceutical fields as material in bone tissue engineering [1] and drug delivery systems for proteins [2], genes [3], antibiotics [4-6], and other drugs [7].

Various techniques have been utilized to prepare nano and micro-size HA particles, including solid-state reaction process [8], hydrothermal precipitation method [9], microemulsion process [10], spray drying [11], and wet-chemical precipitation [12-17]. Among these methods, wet chemical process is generally preferred because is a simple technique, offers a rapid synthesis and has a low cost for industrial production. In addition, this method has the advantage that the reaction media is devoid of extraneous elements and water is the only by-product. In these conditions, it is of great importance in the process of HA synthesis to focus on the precise control of particle size, morphology and chemical composition.

In the present work, the wet-chemical precipitation method has been used to obtain nano and micro-sized HA particles. Their structural and morphological properties were investigated by Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS) and Fourier Transform Infrared Spectroscopy (FTIR). The potential of the synthesized HA as drug carrier was evaluated from adsorption and release experiments using doxycycline and chloramphenicol antibiotics. The amount of drug loaded and delivered was detected by ultraviolet–visible (UV-vis) spectrophotometry. The antimicrobial activity of loaded antibiotic was evaluated using *Bacillus subtilis* and *Pseudomonas aeruginosa* bacteria.

Experimental part

Materials

High purity calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$), ammonium phosphate dibasic (($(NH_4)_2HPO_4$), and ammonia solution were used for the preparation of HA (Sigma Aldrich Chemicals). Two antibiotics, doxycycline hyclate (Sigma Aldrich, figure 6) and chloramphenicol (ACROS ORGANICS Chemicals, fig. 7), were used for adsorption and desorption studies. The chemicals, sodium hydroxide (NaOH) and potassium phosphate monobasic (KH_2PO_4) were purchased from Scharlau Chemicals. Phosphate-buffered saline solution (PBS) of pH = 7.4 (0.2 M NaOH/0.2 M KH_2PO_4 K) were prepared in double distilled water. All chemicals were of analytical grades and used as received, without further purification. The bacterial strains of *Bacillus subtilis* and *Pseudomonas aeruginosa* were provided by the Microorganism Collection of our Microbiology Laboratory (of National R&D Institute for Chemistry and Petrochemistry -ICECHIM Bucharest).

Synthesis of HA particles

HA powder samples were prepared by wet chemical precipitation as previously described [17]. Briefly, 250 mL of calcium nitrate tetrahydrate solution (1 M) was added drop-wise to 250 mL of ammonium phosphate dibasic solution (0.6 M) while stirring for one hour (1h) at room temperature. The pH of the system was maintained at 11 throughout the stirring process by using 25% ammonia solution. The obtained precipitate solution was stirred 1h for homogenization, refluxed at 100°C for another hour, and then was left to rest overnight. The precipitate was washed and filtered on filter paper under vacuum. The white precipitate formed was dried in an oven at 80°C for 24h. The powder was crushed in a mortar and subsequently subjected to calcinations in platinum crucibles at 800°C

for 1h, at 1000°C for 2h and at 1200°C for 4h. The prepared powders were mixed and used for further characterization. To see the reproduction of this approach, we made another batch of HA powders, under the same conditions. HA1 and HA2 labels were utilized to differentiate between the experiments.

Antibiotic loading on HA

Doxycycline hyclate and chloramphenicol solutions were used as water soluble models. In order to estimate the adsorptive amount of antibiotic on HA, 0.5 g of HA powder was immersed into 75 mL of antibiotics at different concentrations (1 and 2 mg/mL). The resulted suspension was stirred (250 rpm) for various time periods (6, 24, 30, 54, 78, 102, 126, 150, 174 h) at room temperature. The suspension was then centrifuged at 5000 rpm for 30 min. The supernatant was measured by UV-Vis spectrometer at a wavelength of 350 nm for doxycycline and 278 nm for chloramphenicol. The amount of loaded antibiotic was calculated by the difference in the drug concentration before and after adsorption using the equation:

$$Drug\ loading\ \% = \frac{C_i - C_f}{C_i} \times 100$$

where C_i and C_f are the initial and the final drug concentration of the aqueous antibiotic solution, mg/mL.

The sediment of the antibiotic-loaded hydroxyapatite was lyophilized for 24h and then used in further investigations.

In vitro drug release

The release study was carried out by dispersing 0.1 g of antibiotics loaded HA particles into a test tube with a stopper containing 50 mL of phosphate buffer medium at 37°C and *p*H 7.4. The experiments were performed under stirring at 200 rpm for a period of 242h. At the pre-determined sampling times, the tube was removed and centrifuged at 5000 rpm for 30 min. About 5 mL of supernatant was withdrawn for antibiotics determination and replaced with 5 mL fresh PBS. The amount of antibiotic from the supernatant was determined by UV-Vis spectrophotometric method.

In vitro antibacterial studies

The antibacterial activity of doxycycline was performed against the Bacillus subtilis and Pseudomonas aeruginosa. The bacterial strains were inoculated on Luria-Bertani (LB) medium and were incubated for 48h at 37°C.

In order to standardize the bacterial inoculum, the *growth* rates of *bacteria* were monitored by determining the optical density (OD) with a spectrophotometer BIOM at a wavelength of 590 nm. Petri plates were seeded with a suspension with an OD of 0.140, which corresponds to a concentration of 1.95 x 108 CFU/mL. (CFU = colony
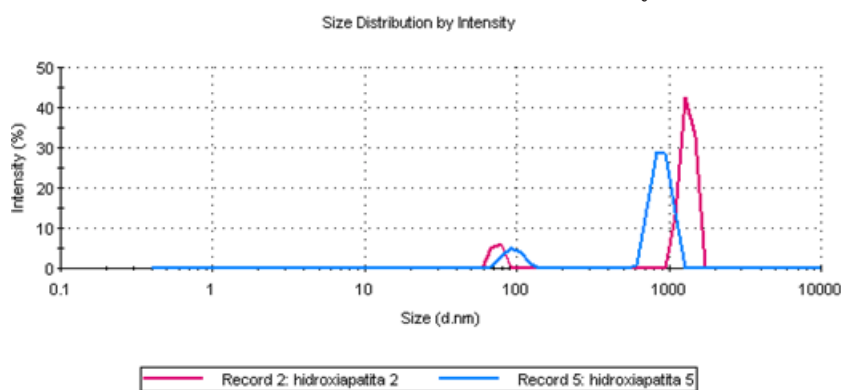
forming units; the number of viable germs of the material examined).

For the determination of antibacterial activity of the hydroxyapatite powders, two biochemical compounds containing 1 and 2mg/mL doxycycline loaded hydroxyapatite were used. Then from each biochemical compound, samples of 200 mg, 500 mg and 2300 mg were suspended in 1 mL of bidistilled water into Eppendorf test tubes. Paper disks with a diameter of 6 mm were placed on the surface of the solid culture medium in the Petri dish and impregnated with a 25 mL solution of compound withdrawn from the suspension in the Eppendorf test tubes. The Petri dishes were incubated at 37°C for 72 h. The bacterial cultures were microscopically observed and the antimicrobial activity was evaluated by measuring the inhibition zone of the tested microorganism on two perpendicular axes at 24, 48 and 72 h. Experiments were performed in triplicate.

Characterization

The chemical nature and the molecular bond structure of the samples were determined by using a FT-IR spectrometer (Perkin Elmerm Spectrum GX). The measuring resolution was 4 cm^{-1} and the spectral range was 400-4000 cm^{-1}.

The particle size analyses of the pure HA were investigated by dynamic light scattering (DLS) technique using a Nano ZS (Red badge) instrument. Prior to the DLS measurements, 0.01 g of the sample was suspended in 25 mL distilled water and sonicated for 5 min.

The morphology of the HA particles was observed by scanning electron microscopy using Environmental Scanning Electron Microscope (ESEM) FEI Quanta *200.*

The amount of drug in the adsorption and desorption studies was determined by using a UV-VIS spectro-photometer CINTRA 202.

Results and discussions
Dynamic Light Scattering measurement

The DLS image (fig. 1) shows small particles with a mean diameter of about 70-90 nm and particles with an average size of 900-1200 nm.

Scanning Electron Microscopy

The SEM images of the synthetized HA powders revealed a spherical morphology of the agglomerated particles with sizes around 1μm (fig. 2 a, b).
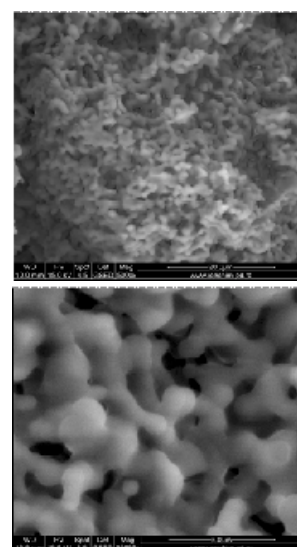

a

b

Fig. 2. SEM images of synthetized HA

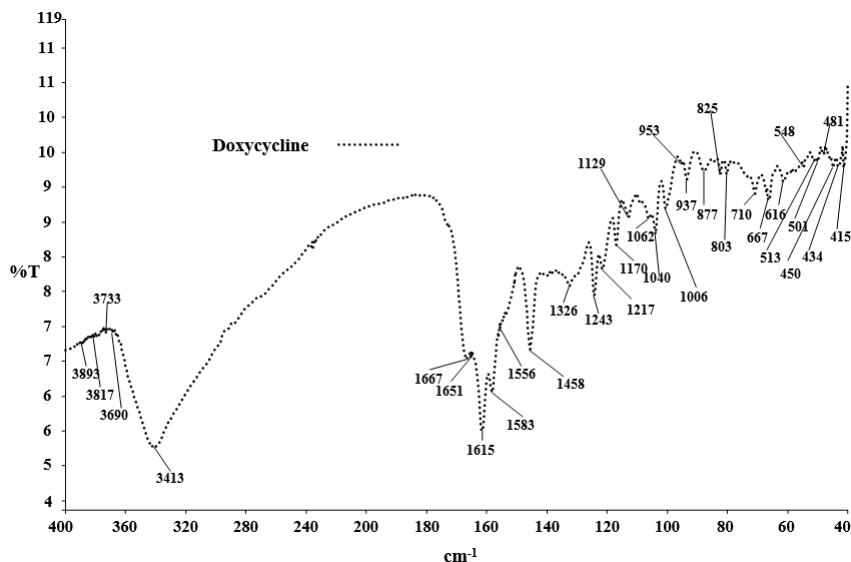Fig. 1. DLS image of synthetized HA
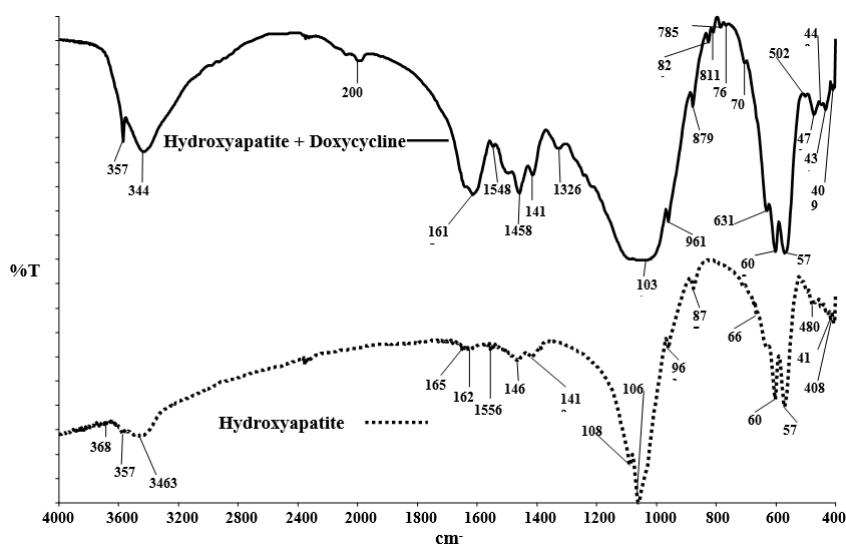
Fig. 3. FTIR spectrum of doxycycline

Fig. 4. FTIR spectrum of microstructurated hydroxyapatite and doxycycline doped hydroxyapatite

FTIR spectroscopy

The FTIR spectra of pure doxycycline, microstructurated hydroxyapatite, and doxycycline loaded hydroxyapatite are given in the figures 3 and 4.

As seen in figure 3, the main characteristic absorption bands of doxycycline are at 3690 cm⁻¹ and at 3413 cm⁻¹ for -OH groups, at 1667 cm⁻¹ and 1651 cm⁻¹ for C=O of Amide I group. The band at 1615 cm⁻¹ is specific for -COOH group, and the bands at 1583 cm⁻¹ and 1556 cm⁻¹ are specific to NH from Amide II group. The bands at 1458 cm⁻¹ and 1326 cm⁻¹ are characteristic to -CH_2 group. In the spectrum of doxycycline, the vibration transmittance band at 1243 cm⁻¹ is ascribed to NH from Amide III group. The C-O group transmittance band, attached to hydrocarbons ring, at 1170 cm⁻¹, 1062 cm⁻¹, 1040 cm⁻¹ and 1006 cm⁻¹, are also specific to doxycycline.

The figure 4 displays the FTIR spectrum of doxycycline doped hydroxyapatite compared with the FTIR spectrum of hydroxyapatite For the spectrum of hydroxyapatite, transmittance bands at 3575 cm⁻¹ and at 3463 cm⁻¹ emphasize the presence of -OH, at 1089 cm⁻¹, 1061 cm⁻¹, 961 cm⁻¹, 603 cm⁻¹, and 573 cm⁻¹ the bands characteristic to PO_4^{3-}, at 668 cm⁻¹ for HPO_4^{3-}, and the transmittance bands at 1419 cm⁻¹ and 877 cm⁻¹ correspond to CO_3^{2-} anion due to the CO_2 adsorption from the atmosphere into HA particles. The characteristic transmittance bands associated with doxycycline at 1615 cm⁻¹, at 1548 cm⁻¹, at 1458 cm⁻¹ and at 1326 cm⁻¹ in the Hydroxyapatite + Doxycycline spectrum

indicate the adsorption of doxycycline on the HA. This is a physical process and the properties of doxycycline are preserved during the adsorption phenomenon, an important behaviour for a controlled drug release application.

Drug loading and release studies

The data of the drug loading study are shown in figure 5. The drug loading efficiency of hydroxyapatite has been found to depend on the adsorption time and the chemical structure of antibiotics. It was observed that doxycycline has an excellent adsorption capacity on hydroxyapatite particles. The adsorption equilibrium was attained after 100 h (and before 126 h, our measurements). After 174 h, a maximum of 1.979 mg/mL doxycycline (99% from the initial solution of 2 mg/mL of doxycycline) and 0.998 mg/ml doxycycline (99% of the 1 mg/mL solution) was loaded on the HA particles.

The capacity of chloramphenicol loading on hydroxyapatite was very low. After 174 h, a maximum of 2% chloramphenicol was adsorbed on HA.

 The greater adsorption affinity of doxycycline on hydroxyapatite could be explained due to the chemical structure of doxycycline (fig. 6) and its compatibility with the HA particles, with various possibilities to form electrostatic interactions: hydrogen bonding between hydroxyl groups of doxycycline and hydroxyapatite, or
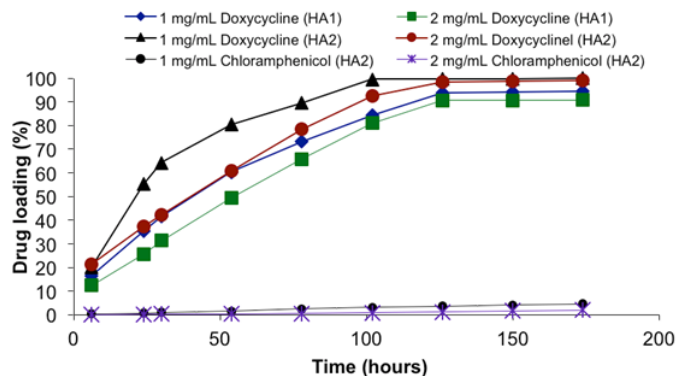
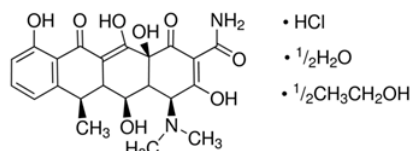Fig. 5. Percentage of drug loaded onto hydroxyapatite as a function of time

Fig. 6. Chemical structure of Dox.HCl

between hydroxyl groups of the hydroxyapatite and the unshared electrons of the oxygen (of the carbonyl group) and the nitrogen of doxycycline.

Chloramphenicol (fig. 7) has almost no affinity for HA particles. This could be explained due to a low solubility in
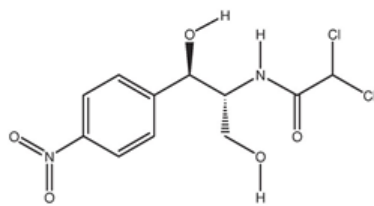

Fig. 7. Chemical structure of Chloramphenicol

water (2.5 g/L) and less potential to form electrostatic interactions, with hydrogen bonding with the OH groups of hydroxyapatite.

The loaded hydroxyapatite exhibits a sustained and slow release of the drug, which prevents the burst release of doxycycline and prolongs the drug effect (fig. 8). This behavior could be attributed to the fact that a high amount of doxycycline was adsorbed in the channels of the porous HA and a small amount of doxycycline was attached to the surface of the HA particles. The more drug in the channels of HA, the slower the desorption process. The doxycycline release after 242 h was 36% from the 0.998 mg/mL antibiotic solution and 55% from the 1.979 mg/mL drug-loaded sample. The results demonstrate that HA
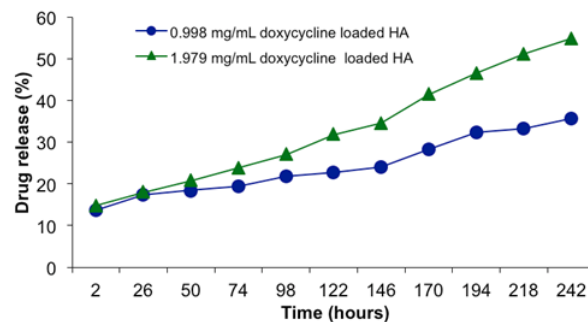

Fig. 8. The release profile of doxycycline from hydroxyapatite

could be used as a carrier for a compatible drug when a slow rate of release over a long period is desired.

Antibacterial activity

The results of the antibacterial activity of doxycycline-loaded hydroxyapatite are given in table 1. The zone of inhibition around the pure hydroxyapatite and doxycycline-loaded hydroxyapatite against *Bacillus subtilis* and *Pseudomonas aeruginosa* is shown in the figures 9, 10 and 11.

As seen in figure 9, the compound A, loaded with about 1 mg/ mL doxycycline, has an antibacterial effect against *Bacillus subtilis*, even at the lowest amount of biochemical compound (200 mg). This effect is seen after an incubation of 48 h increases in intensity towards 72 h. The strain of *Pseudomonas aeruginosa* is not sensitive to the action of Compound A.
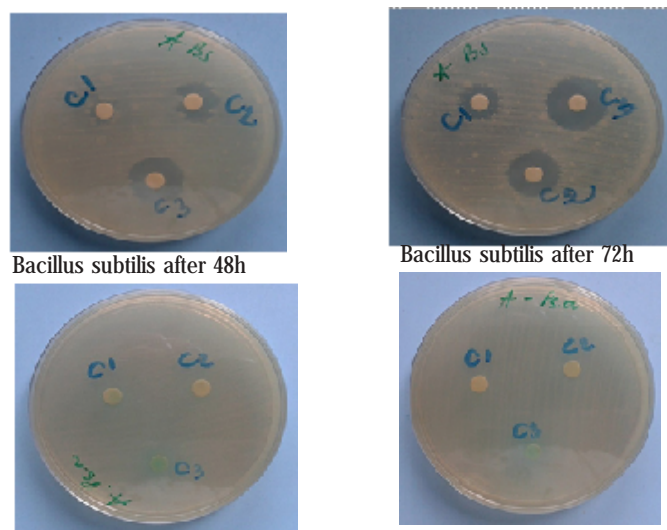

Bacillus subtilis after 48h

Bacillus subtilis after 72h

Pseudomonas aeruginosa after 48h

Pseudomonas aeruginosa after 72h

Fig. 9. The inhibitory effect of compound A (HA loaded with about 1 mg/ mL doxycycline) against the growth of the bacterial strains

Table1
THE INHIBITION ZONE OF THE HYDROXYAPATITE COMPOUNDS AGAINST MICROBIAL STRAINS

Compound A (hydroxyapatite loaded with about 1 mg/ mL doxycycline)				
Microbial strains	Sample of compound	Zone of inhibition (mm)		
		The period of incubation (hours)		
		24	48	72
Bacillus subtillis	C1 (200 mg)	10	11	11
	C2 (500 mg)	14	15.5	15.5
	C3 (2300 mg)	19	20	20
Pseudomonas aeruginosa	C1 (200 mg)	0	0	0
	C2 (500 mg)	0	0	0
	C3 (2300 mg)	0	0	0
Compound B (hydroxyapatite loaded with about 2 mg/ mL doxycycline)				
Bacillus subtillis	C1 (200 mg)	13	14.5	14.5
	C2 (500 mg)	15.5	16	16
	C3 (2300 mg)	24	25	25
Pseudomonas aeruginosa	C1 (200 mg)	0	0	0
	C2 (500 mg)	0	0	0
	C3 (2300 mg)	0	0	0

Bacillus subtilis after 48 h

Bacillus subtilis after 72 h
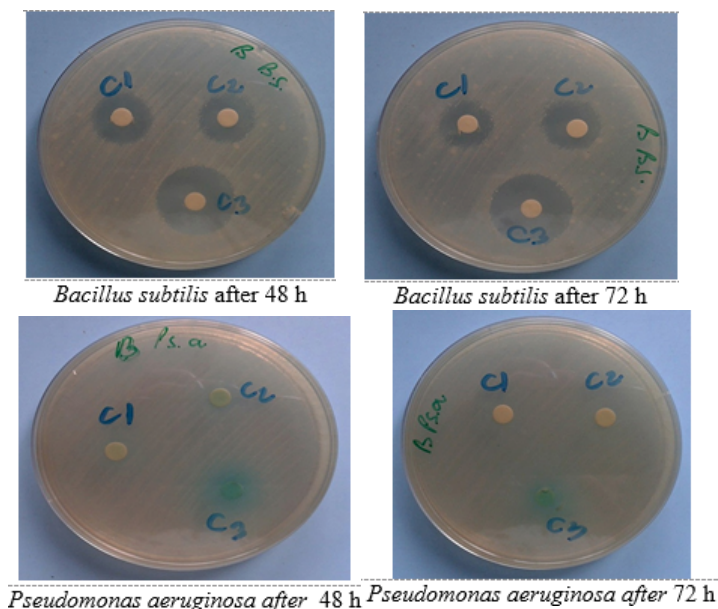
Pseudomonas aeruginosa after 48 h

Pseudomonas aeruginosa after 72 h

Fig. 10. The inhibitory effect of compound B (HA loaded with about 2 mg/ mL doxycycline) against the growth of the bacterial strains
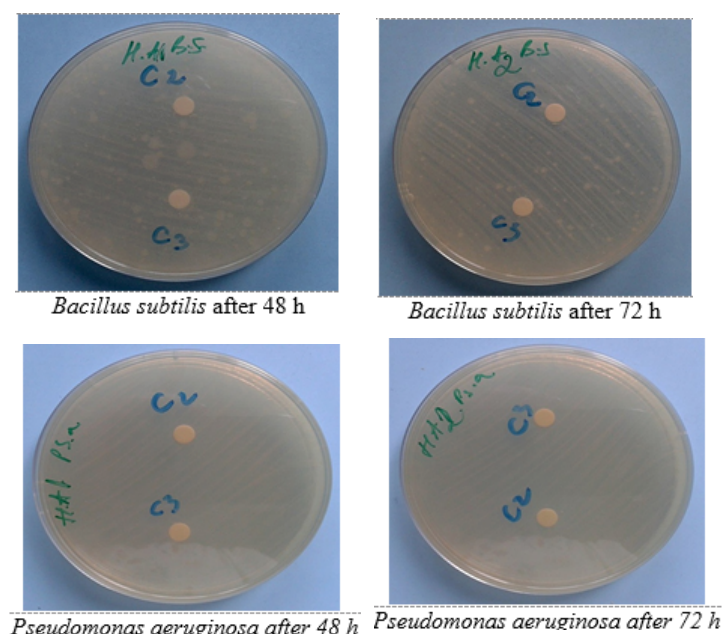

Bacillus subtilis after 48 h

Bacillus subtilis after 72 h

Pseudomonas aeruginosa after 48 h

Pseudomonas aeruginosa after 72 h

Fig. 11. The inhibitory effect of pure hydroxyapatite (HA1 and HA2) against the growth of the bacterial strains

Table 2
THE INTERPRETATION CRITERIA FOR INHIBITION ZONE DIAMETER ON THE BACTERIAL GROWTH

	MIC* (μg/mL)	Zone diameter (mm)
susceptible	≤ 4	≥ 20
intermediate	8-16	15-19
resistant	≥ 32	≤ 14

*MIC - minimum inhibitory concentration

A similar behavior was observed for compound B (fig. 10), where the inhibitory effect against Bacillus subtilis is clearly seen after 48 h. The diameter of the zone of inhibition for the compound B was higher for C1 and C3, compared with the values for compound A.

The results obtained in figure 11 show the absence of inhibitory effect on the bacterial growth of pure hydroxyapatite.

The interpretation criteria for the inhibition zone diameter (table 2) are made in accordance to the *Performance standards for antimicrobial disk susceptibility tests*; approved standard- ed. 11th; January 2012 M02-A11, vol. 32, no. 1 (Replaces M02-A10 Vol. 29 No. 1).

Conclusions

The hydroxyapatite nano and microparticles were prepared by wet-chemical precipitation method using $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$. The size, morphology and structure of the HA samples were characterized by Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS) and Fourier Transform Infrared Spectroscopy (FTIR). The HA particles with average diameters of 1 ìm were obtained. The drug loading and release behavior of the HA were also investigated. The drug-loaded HA exhibits a high adsorption capacity and a good drug controlled release property. The antibacterial activity of doxycycline, pure hydroxyapatite and doxycycline-loaded hydroxyapatite was performed against the *Bacillus subtilis* and *Pseudomonas aeruginosa*. The study demonstrates that hydroxyapatite particles loaded with doxycycline have an antibacterial effect against *Bacillus subtilis*, even at the lowest amount of biochemical compound. The strain of *Pseudomonas aeruginosa* was not sensitive to the doxycycline-loaded hydroxyapatite. The pure hydroxyapatite didn't show any inhibitory effect on the bacterial growth. The doxycycline-loaded HA particles have the potential for application in medicine as promising carriers for the release of various water-soluble drugs.

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