MATERIALE COMPOZITE ANTISEPTICE PENTRU APLICAȚII BIOMEDICALE
ANTISEPTIC COMPOSITE MATERIAL FOR BIOMEDICAL APPLICATIONS

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The scope of the present work was to study the release of methylene blue from a composite material based on collagen and hydroxyapatite.

The obtaining of the composite material was achieved by co-precipitation of hydroxyapatite in the collagen matrix (at a pH of 10.5). Later, methylene blue was added dropwise because the composite material is intended to act as support for releasing methylene blue; the release of methylene blue ensuring the antiseptic nature of the material.

The composites were then characterized by: Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), scanning electron microscopy (SEM) while the release process was monitored by UV-Vis spectroscopy.

The characterization of the collagen/hydroxyapatite composite material revealed a good mineralization of collagen matrix a high stability of the mineral phase, as well as the capacity to bind/absorb methylene blue.

From the point of view of the release process, the results showed a gradual release that was evidenced by UV-Vis spectroscopy, the release evolution being monitored at a wavelength of 663 nm, characteristic.

Therefore, studies suggest the use of collagen/hydroxyapatite composite material as support for tissular regeneration of bone tissue, methylene blue providing antiseptic properties.

În lucrarea de față s-a studiat eliberarea albastrului de metilen dintr-un material compozit de tip colagen-hidroxiapatită.

Obținerea materialului compozit s-a realizat prin coprecipitarea hidroxiapatitei în matricea colagenică (la un pH de 10,5). Ulterior a fost adăugat albastru de metilen prin picurare, materialul compozit dorindu-se a avea rolul de suport pentru eliberarea albastrului de metilen; eliberarea albastrului de metilen asigurând caracterul antiseptic al materialului obținut.

Materialele obținute au fost caracterizate prin: spectroscopie de IR cu transformată Fourier (FT-IR), difracție de raze X (XRD), microscopie electronică de baleiaj (SEM), în timp ce procesul de eliberare a fost monitorizat prin spectroscopie UV-Vis.

Caracterizarea materialului compozit colagen-hidroxiapatită a evidențiat o bună mineralizare a matricii colagenice, o stabilitate mare a fazei minerale, precum şi abilitatea de a lega/absorbi albastru de metilen. Din punct de vedere al procesului de eliberare, rezultatele au arătat o eliberare treptată care a fost pusă în evidență cu ajutorul spectroscopiei UV-Vis, evoluția eliberării fiind urmărită la lungimea de undă de 663nm, caracteristică.

Prin urmare, studiile realizate sugerează o posibilă utilizare a materialului compozit de tip colagen-hidroxiapatită ca suport pentru regenerarea tisulară a țesutului osos, albastru de metilen asigurând proprietăți antiseptice.

Keywords: *hydroxyapatite, collagen, drug release, methylene blue*

1. Introduction

Drug delivery systems (DDS) are new tools with applications both in the fields of industry and medicine.

Drug delivery is a domain of high importance for medicine and healthcare [1-6]. A wide range of improvements can occur by controlling drug delivery: bioavailability, by preventing premature degradation and enhancing uptake, the number of side effects will decrease by targeting exactly the diseased cells and sites, and a constant drug concentration by controlling the drug release rate.

Many synthetic biomaterials were tested as bone graft materials but the most promising of them seems to be the collagen (Coll)/hydroxyapatite (HA) (nano) composite materials because because of the similarity with natural bones [7-9].

Calcium phosphate-based bioceramics such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) are well-known for their excellent biocompatibility due to the resemblance in composition with apatite found in natural bones [10-12].

Research studies have been done using HA with the purpose to deliver stem cell-containing biomaterials to the sites of disease or injury to allow bone regeneration. Although pure HA is bioactive, it is very difficult to incorporate therapeutic agents within HA without destroying the biofunctionality of its surface. In order to overcome this limitation, several composites of HA and polymers have been developed [13-16].

Methylene blue (3,7-bis(dimethylamino)-phenothiazin-5-iumchloride) is an oxidation/reduction indicator with blue/gray color that is applied both in biology and chemistry. At room temperature, it appears as an odorless, dark green powder and dissolution in water produces a blue solution that is usable as redox indicator in analytical chemistry. Methylene blue (MB) was in

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the past just one of the many new synthetic chemicals used as dyes in the textile industry. Then, further researches in this domain proved that MB can be used in a wider range of experimental applications.

MB is usually prescribed for urinary infections as analgesic/anti-infective/anti-spasmodic that is a combination of drugs which also contains phenyl salicylate, benzoic acid, hyoscyamine sulfate and methenamine. MB is also used for endoscopic polypectomy as an adjunct to saline, epinephrine and for injection into the submucosa around the polyp to be removed. This allows the identification of submucosal tissue plane after removal of the polyp and is useful to determine if more tissue needs to be removed or if it is a high risk for perforation [17-18].

Collagen/hydroxyapatite composite materials are widely used for hard tissue repairs; adding methylene blue (MB) to the composites is intended to allow avoiding infections.

2. Experimental

In this study it was used a composite material based on collagen and hydroxyapatite which was obtained by co-precipitation technique. The antiseptic characteristics are assured by the use of methylene blue.

The mineralization process was carried out directly on the collagen matrix. The mineralization was made by mineralization by using $Ca(OH)_2$ suspension and NaH_2PO_4 solution (both purchased from Fluka). Between the two immersions of collagen matrix in $Ca(OH)_2$ suspension and phosphate solution after draining the excess of water, a volume of methylene blue was pippeted on the collagen matrix surface. The entire process is shown in Figure 3. The concentration and volume of the $Ca(OH)_2$ suspension, NaH_2PO_4 solution are presented in Table 1 and the procentual composition of the composite material are presented in Table 2.

The synthesized samples were characterized by:

a) X-ray diffraction (XRD) to identify the phases of the material and to determine their concentration. X-ray diffraction analysis was performed on a Shimadzu XRD 6000 diffractometer at room temperature. It was used the Cu Kα radiation from a Cu X-ray tube. The samples were scanned in the Bragg angle, 2θ range of 10–87^o at a scan rate of $2^{o}\cdot min^{-1}$.

b) Fourier transform infrared spectroscopy (FTIR) - IR spectroscopic measurements were performed using a Shimadzu FTIR 8400 spectrophotometer. The spectra were recorded over the wavenumber range of 400– 4600cm^{-1} with a resolution of 4cm^{-1}.

c) Scanning electron microscopy (SEM) was used to examine the microstructure of the samples. SEM images were obtained with a HITACHI S2600N with an EDAX probe. All samples were covered with a silver layer prior to imaging.

d) The release of methylene blue was monitored by UV-Vis spectroscopy, at 663nm corresponding to the maximum of absorption of methylene blue. The UV-Vis spectroscopy analyses were performed on a Thermo Scientific Evolution 300 Spectrophotometer.

Figure 1 presents the UV-Vis absorption spectrum recorded for the 100 ppm methylene blue solution. The maximum absorption points of methylene blue are found at 246, 291, 612 and 633nm.

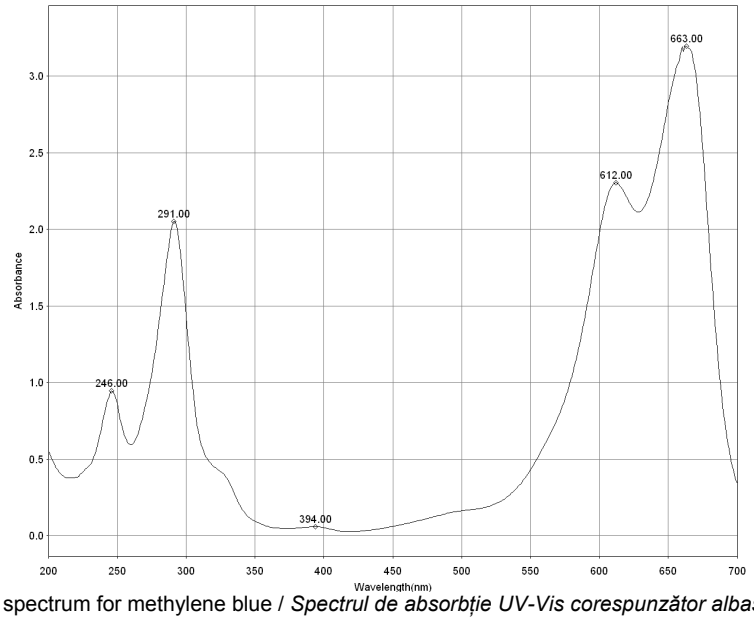

Fig. 1 - UV-Vis absorption spectrum for methylene blue / *Spectrul de absorbție UV-Vis corespunzător albastrului de metilen.*

Based on the calibration curve in Figure 2, the quantification is done at 663 nm because the absorbance is maximum at this value and linearity on the desired range (8 -1000 ppm) is very good. The linearity coefficient was 0.9996.

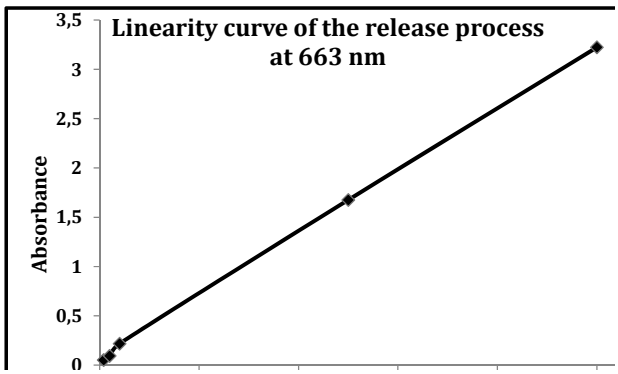

Fig. 2 - Linearity curve of the release process at 663 nm / *Curba de liniaritate a eliberării la 663 nm*

Table 1

Mineralization conditions / *Condițiile de mineralizare*

	CM*
Concentration of $Ca(OH)_2$ suspension, g/L *Concentratia suspensiei de Ca(OH), g/L*	0.739
Volume of $Ca(OH)_2$ suspension, L *Volumul suspensiei de Ca(OH), L*	0.5
Concentration of NaH_2PO_4 solution, g/L *Concentratia solutiei de NaH_2PO_4, g/L*	0.9318
Volume of NaH_2PO_4 solution, L *Volumul soluției de NaH_2PO_4, L*	0.5

* Composite material/*Material compozit*

Table 2

Procentual composition of Coll/HA-MB composite material

Compoziția procentuală a materialului compozit Coll/HA-AM

CM*	Coll(%)	HA(%)	MB** (%)
Coll/HA	19.8	79.2	1

** *Methylene blue/Albastru de metilen*

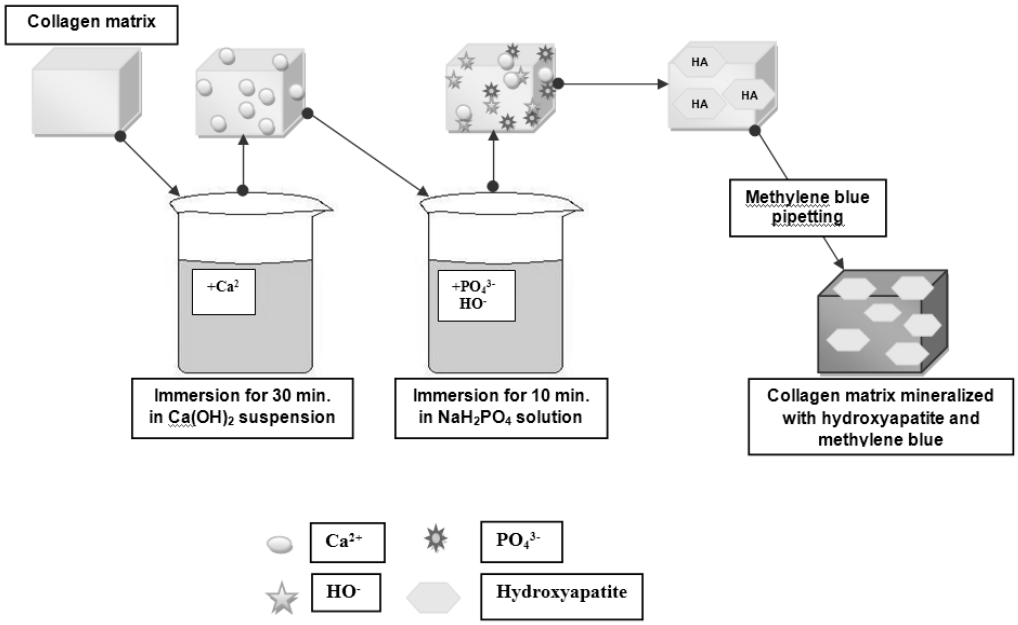

Fig. 3 - Schematic representation of the mineralization process / *Reprezentarea schematică a procesului de mineralizare*

3. Results and discussion

3.1. Scanning electron microscopy

Figure 4 presents SEM images for the collagen matrix used for obtaining antiseptic collagen/hydroxyapatite composite material. SEM pictures show the porous structure of the material, where both fibers and fibrils can be seen and are useful to make a comparison with the material that was previously analyzed. The collagen matrix is a layered porous structure with pore size of approximately 50 – 200μm and the distance between layers of about 100μm.

Figure 5 presents the SEM image of the collagen/hydroxyapatite composite material for different magnifications. Regardless of the selected magnification, there is a very good mineralization of collagen matrix, the collagen/hydroxyapatite composite being homogeneous. At lower magnification, SEM images allow visualization of the microstructure of the composite, being able to identify pore sizes of 50-150 μm within the layered material. These size are optimal for bone integration as reported by different researchers [19].

Due to the instability of the collagenous structure in the electron beam, the capture of images was possible at a maximum magnification of 3500 x which allowed just the visualization of hydroxyapatite agglomerates and not for the mineral phase particles, the size of the agglomerates being smaller than 500nm.

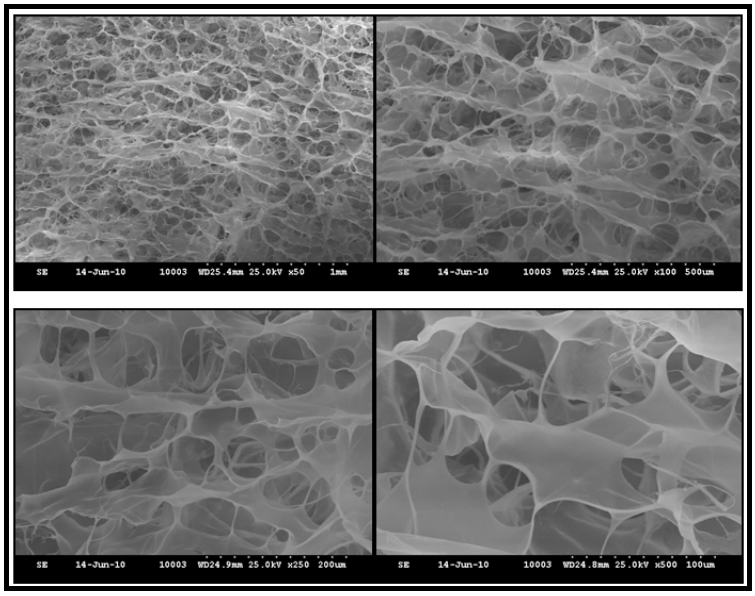

Fig. 4 - SEM images recorded on a collagen matrix at magnifications of 50, 100, 250 and 500x / *Imaginile SEM înregistrate pe o matrice colagenică la măriri de 50, 100, 250 şi 500x.*

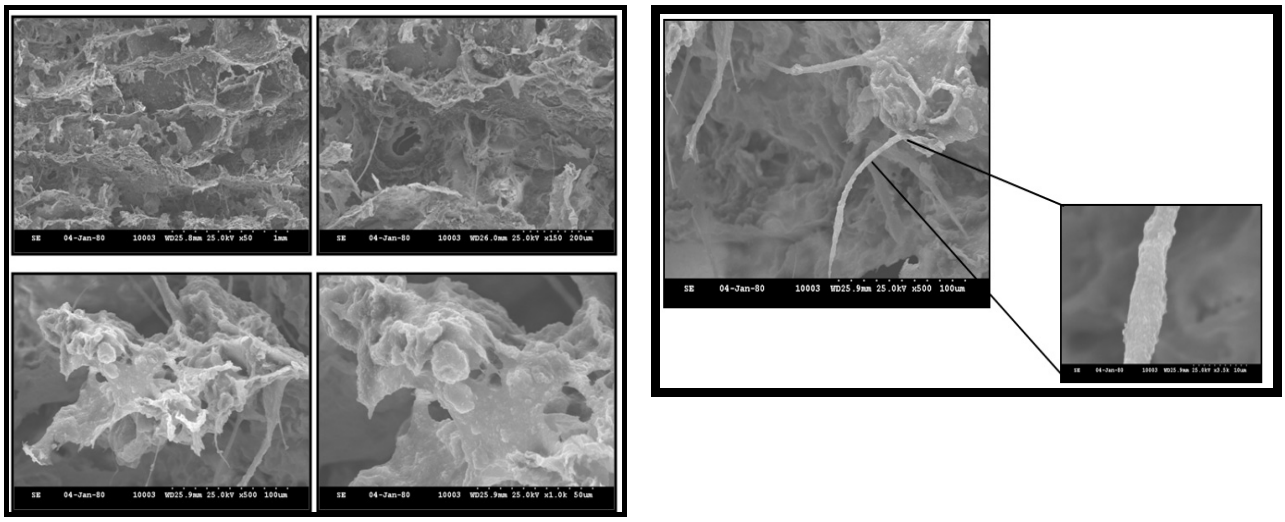

Figure 5 - SEM images recorded for the collagen/hydroxyapatite composite material at magnifications of 50, 100, 1000 and 3500x
Imaginile SEM înregistrate pe materialul compozit pe bază de colagen şi hidroxiapatită, la măriri de 50, 150, 500, 1000 şi 3500x

3.2. Fourier transform infrared spectroscopy – FTIR

IR spectroscopy is used to highlight the presence of hydroxyapatite deposit on the collagen matrix and chemical stability of the collagen matrix in the mineralization process.

Figure 6 presents IR spectra of Coll/HA composite material. The main absorption bands of collagen are present at 1510, 1541, 1652cm^{-1}, and those of HA are 565, 604, 1032cm^{-1}. The large absorption band in the range of 3000 – 3600cm $^{-1}$ is related to the associated hydroxyl groups from water, collagen and hydroxyapatite [20].

Figure 7 - shows the FTIR spectra recorded on a collagen matrix. The reason for recording this spectrum is to compare it with the collagen/hydroxyapatite composite material (Figure 6) in which mineral phase can be observed by the appearance of characteristic absorption bands at 1032 cm^{-1} and those from 565 cm^{-1} and 604 cm^{-1}.

As it can be noticed from Figure 8, the presence of methylene blue as antiseptic does not influence the structure and composition of the composite material maybe because the latter is present in very small amount. However, it can be noticed the wider bands in the spectrum of the composite material with methylene blue compared to the bands of the composite material without drug.

3.3. X-Ray diffraction

The XRD spectrum (Figure 9) proves the formation of hydroxyapatite on the collagen fibers as the main mineral phase. This could be done based on the diffraction peaks determination.

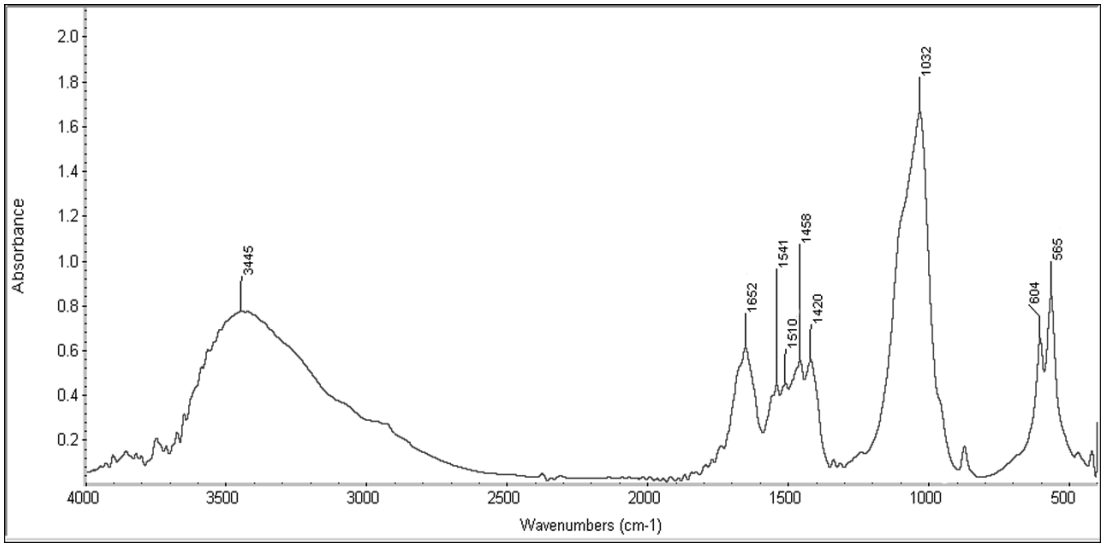

Fig. 6 - FTIR spectra of the collagen/hydroxyapatite composite material used for drug controlled release / *Spectrele FTIR ale materialului compozit colagen/hidroxiapatită, utilizat pentru eliberarea controlată de medicament.*

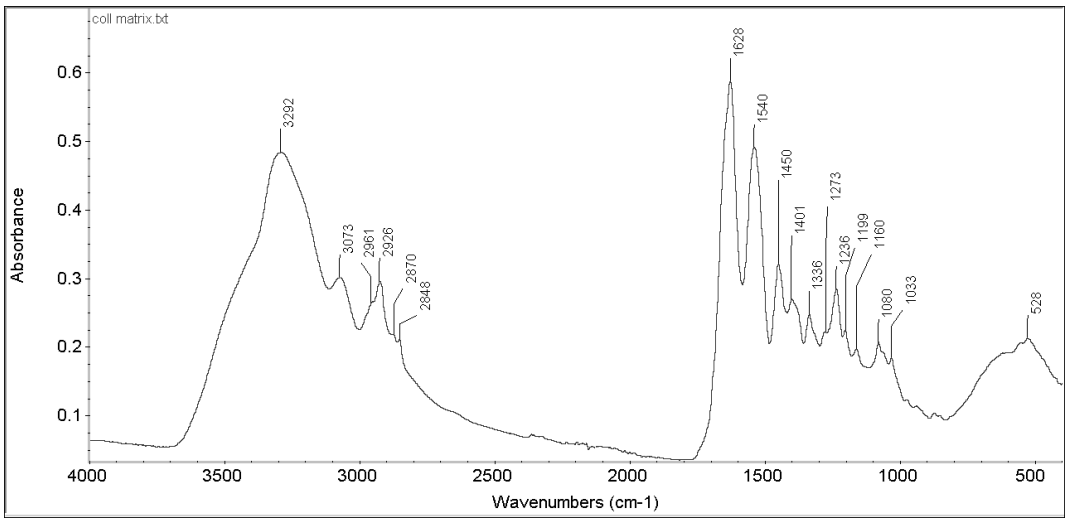

Fig. 7 - FTIR spectra of the collagen matrix used for drug controlled release / *Spectrele FTIR ale matricii colagenice utilizate pentru eliberarea controlată de medicament.*

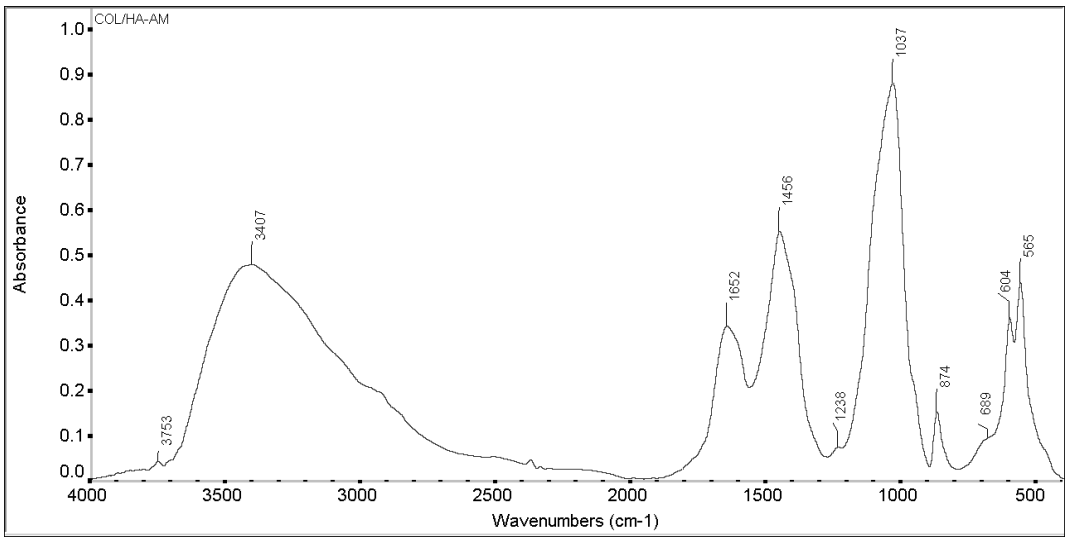

Fig. 8 - FTIR spectra of Coll/HAp composite and methylene blue used for controlled release / *Spectrele FTIR ale materialului compozit Coll/HAp și albastru de metilen utilizat pentru eliberarea controlată.*

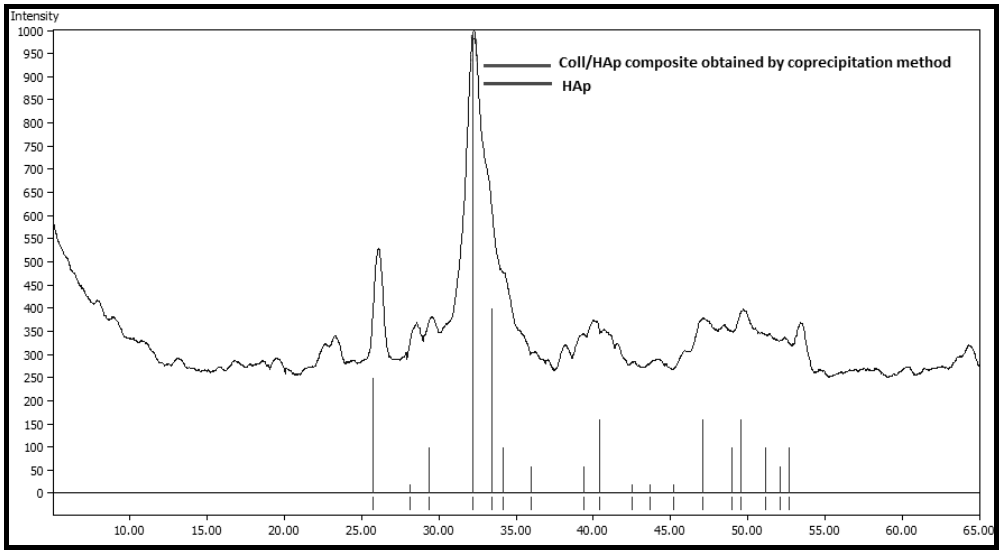

Fig. 9 - Diffractogram recorded on the collagen/hydroxyapatite composite material/*Spectrul de difracție înregistrat pe materialul compozit colagen/hidroxiapatită.*

3.4. Methylene blue controlled release process - UV-Vis spectrophotometry

Figure 10 presents the drug release process evolution of methylene blue from collagen/hydroxyapatite composite as measured by the increase of the concentration of liquid related to time, at a wavelength of 663nm. As it can be seen in the figure, the release of the drug occurs gradually. Also, the liquid concentration increases over time and after 26 hours, it starts to become approximately constant. However, at higher time values, there are some small increases of concentration rates, which indicates that the drug is still released from the composite material.

Figure 11 shows the evolution of methylene blue release from the collagen matrix at 663 nm. As It can be seen, concentration increases rather quickly in the first few minutes.

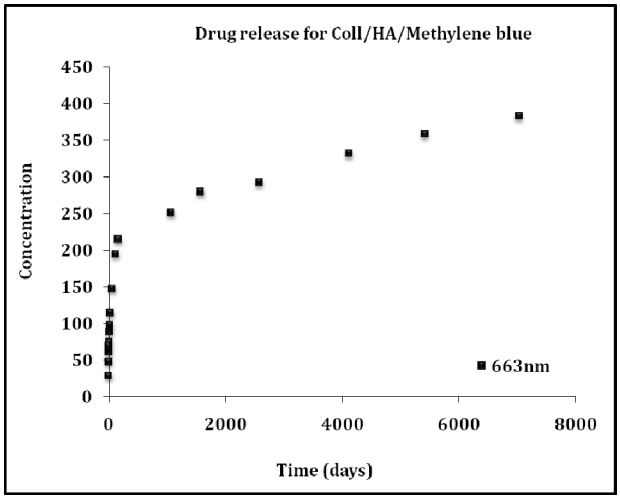

Fig. 10 - Methylene blue release curve from Coll/HA composite, recorded with UV-Vis spectrophotometer / *Curba procesului de eliberare controlată a albastrului de metilen din compozitul Coll/HA, înregistrat cu spectrofotometrul UV Vis.*

After approximately 1250 minutes, the curve tends to flatten, which indicates that the drug release concentration from the matrix is decreasing.

The degree of drug release from the matrix reaches about 57% of the total amount of methylene blue.

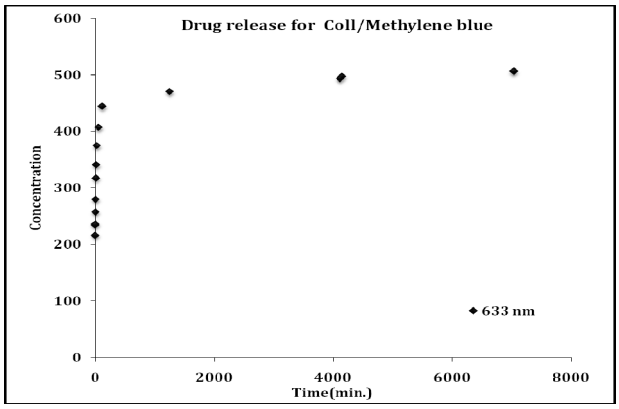

Fig. 11 - Drug controlled release curves from the collagen matrix, recorded with UV-Vis spectrophotometer / *Curbele procesului de eliberare controlată a medicametului din matricea colagenică, înregistrat cu spectrofotometrul UV Vis.*

The experimental study was made on Coll/HA composite material and on the collagen matrix in order to observe the release of methylene blue and to do a comparison between the two materials in terms of application as a carrier material for the controlled release process.

As could be seen from the two figures, the composite collagen/hydroxyapatite managed to retain much better the drug within its matrix and to release it slowly as compared to the simple collagen matrix where the release process was more quickly. This can be explained on the basis

of different interactions that occur between methylene blue and collagen or hydroxyapatite. Between methylene blue and hydroxyapatite there are stronger interactions due to the slower release rate of methylene blue in case of the composite materials towards the collagen matrix. Also, the differences related to the speed of releasing methylene blue from the collagen matrix and the composite are determined by the higher porosity of the collagen matrix, the advanced porosity showing better diffusion.

The low degree of recovery is a proof that strong interactions occur between methylene blue and the composite.

3.5. Absorbtion of methylene blue drug

Figure 12 presents the absorbtion process of methylene blue on the collagen matrix at 663 nm. The absorbtion process was made from 100 ppm methylene blue solution in order to maintain the theoretical conditions of the release process. As time goes on, it can be observed a gradual decrease of liquid's concentration. This is determined by the penetration of the organic drug in the collagen matrix. As time passes, even at higher values of time, the concentration continues to decrease.

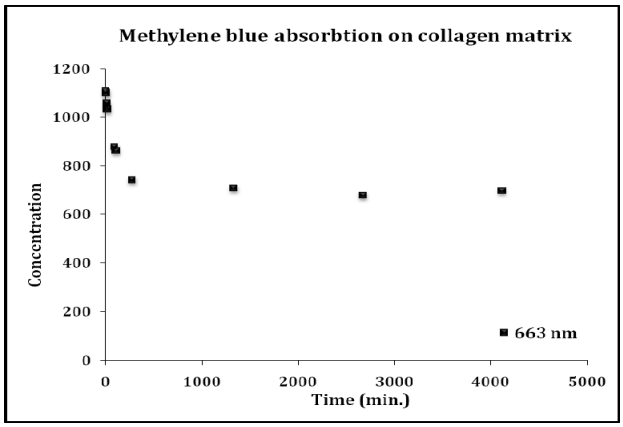

Fig. 12 - Drug adsorbtion curves on the collagen matrix, recorded with UV-Vis spectrophotometer / *Curbele procesului de adsorbtie a medicametului pe matricea colagenică, înregistrat cu spectrofotometrul UV-Vis.*

4. Conclusions

It was found that the release of methylene blue is slow and controlled.

The same was also observed in the adsorption process of the drug on the Coll/HA composite, namely a slow decrease in the concentration of the liquid. This was due to both material structure and the presence of mineral phase.

The release process of MB was quantified by UV-Vis spectroscopy at 663nm. By analyzing the release curve, two characteristic zones can be observed: a rapid delivery of the MB in the first 4h followed by a very slow release process, the recovery of MB being of ≈40% after 5 days.

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