Cellulose Acetate / Hydroxyapatite / Nystatin Biocomposites with Antifugal Activity

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The aim of our study was to obtain cellulose acetate composites based on hydroxyapatite and nystatin with increased local therapeutic effect in the therapy of candidiasis. The porous composites were obtained by phase inversion method. Antifungal susceptibility of the samples was investigated by the Kirby Bauer Disk Diffusion test. The method has demonstrated an antifungal effect of the samples against Candida albicans as well as the fact that the polyene antibiotic nystatin might be an optimal choice as active agent incorporated in a composite. Also we have studied the swelling and the degradation of the membranes. These measurements help us better understand the water and PBS absorption capacity of the polymer matrix.

Keywords: Composite, cellulose acetate, hydroxyapatite, nystatin, antifungal activity

The idea to produce bioactive materials with antibacterial activity dates back at least to the early 50s when some authors have proposed the first dental cements and resin combinations with antibiotics [1, 2]. Calcium phosphates like hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, are known for their excellent bioactivity, biocompatibility and osteoconductivity [3-5]. As the major mineral constituent of the natural bone hydroxyapatite is a great material for use in bone regeneration. The hydroxyapatite has been used in the last decades for the manufacture of porous scaffolds, interconnected, with isotropic pore structure. In particular, the researches were directed to the use of hydroxyapatite as the transmission system of the biomaterials containing the stem cells in the diseased areas or lesions of the body to enable the bone regeneration. Although pure hydroxyapatite is bioactive, it is very difficult to incorporate therapeutic agents into the hydroxyapatite structure without destroying surface functionalities. To overcome this limitation several hydroxyapatite composites and polymers (such as chitosan, polyurethane, lactic acid, poly lactic-co-glycolic) have been developed [6].

Cellulose $(C_6H_{10}O_5)_n$, is the major component in the rigid cell walls in plants, a linear polysaccharide polymer with many glucose monosaccharide units. As an acetate ester of cellulose, cellulose acetate is a biodegradable polymer used in the construction of the medical devices or in the drugs release from matrix systems to enhance its action against local fungal infections [7-9]. Currently, polymer-ceramic composite materials are being developed with the aim of the obtaining of novel and controlled release drug delivery systems [10, 11].

In this work, hydroxyapatite and cellulose acetate are combined to form ceramic-polymer hybrid systems with applications in the biomedical field. The biologically active substance included in their matrix is nystatin (Nys), a polyene antifungal antibiotic, one of the oldest antifungal drugs, produced by *Streptomyces noursei* strains [12] commonly used for prophylaxis and treatment of candidiasis. The therapeutic role of the composites is demonstrated by the antimicrobial test against the yeast strain *Candida albicans*. In the present paper the Kirby Bauer Disk Diffusion test was applied.

Experimental part

Materials and methods

All chemicals were of analytical grade and used as received without further purification. Experiments were performed in triply distilled and deionized water. All chemicals were supplied by Sigma-Aldrich (Germany).

Synthesis of hydroxyapatite powder

Hydroxyapatite (HA) powder was synthesized by Rathje method [13] consisting in drop-wise addition of phosphoric acid (0.1M) to a suspension of calcium hydroxide (0.167M) under stirring according to the reaction bellow:

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O \quad (1)$$

The synthesis procedure is described entirely in a previous work [14-17]. After calcination the fine green powder obtained has been characterized by SEM technique.

Porous membrane preparation

The composites were obtained by adding calculated amounts of hydroxyapatite and/or nystatin in the cellulose acetate solution. The solution obtained was molded in a Petri dish and a dried membrane resulted after 2h. The preparation of the membranes is also described in previous works [18-21]. Finally, we have obtained four composites: cellulose acetate membrane (CA), cellulose acetate with nystatin (CA-Nys), cellulose acetate with hydroxyapatite (CA-HA) and cellulose acetate with hydroxyapatite and nystatin (CA-HA-Nys).

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Characterization. The morphology and chemical composition of sample surface were elucidated by scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX), with QUANTA 200 3D Dual Beam scanning electron microscope (FEI Co., USA). For the SEM-EDX investigations, gold sputtering was used to create a conductive coating surface.

Swelling and degradation measurements

Swelling and degradation tests were performed by immersing all membranes into deionized water and into phosphate-buffered saline (PBS) solution. The swollen sample weights were measured after removing the surface water in excess or PBS by gently tapping the surface with filter paper. Swelling ratio (*SR%*) was estimated from the swollen state and the final dried weight by means of the following equation:

$$SR\% = \frac{m_f - m_0}{m_0} \cdot 100 \quad (2)$$

where m_0 denotes the initial weight of the dried sample (g) and m_f is the weight of the swollen sample (g) at time t (min). The kinetic study of the process involves determination of the material swelling degree at the various times by means of the *SR%* versus t (immersion time) plot.

The weight loss (*WL%*) was calculated from the initial dried weight m_0 and the final wet weight m_f using the following equation:

$$WL\% = \frac{m_0 - m_f}{m_0} \cdot 100 \quad (3)$$

The kinetic study of the degradation process involves the determining of the degradation degree of the material at different times by Weight loss % versus immersion time plot.

Antifungal test

The present assay was performed to evaluate the antifungal activity of the composites against the strain *Candida albicans* isolated from the tegumentary tissue and it has the following composition: glucose – 10g, peptic digest of animal tissue – 5g, yeast extract – 3g, malt extract – 3g, aniline blue – 1g, agar – 20g, distilled water – 1000mL, with *p*H = 6.2 ± 0.2.

Yeast strain was kept refrigerated (4°C), periodically passed on the Sabouraud medium with the following formula: glucose – 20g, peptone – 10g, agar – 15g, substances dissolved in 1000mL of distilled water at *p*H = 7 of the culture medium at a temperature of 25 ± 0.2°C.

The test microorganism was inoculated (10^4 cfu/mL) in the Sabouraud liquid medium at 37°C.

In this study we applied the diffusion method (Kirby Bauer Disk Diffusion test) [21]. Kirby Bauer test provides qualitative information on the sensitivity of bacterial species on antimicrobial therapeutic agents (such as antibiotics, silver, zinc, etc.).

This method does not determine accurately the minimum inhibitory concentration (MIC, minimum quantity of an antimicrobial agent that inhibits cultivation of a strain of bacteria) or minimum bactericidal concentration (MBC, the least amount of antimicrobial agent that kills 99.9% of bacteria strains tested), but it offers preliminary information on the sensitivity of microbial species tested against antimicrobial use.

The accuracy and reproducibility of this test are dependent on the maintaining a standard set of procedures recommended by the NCCLS (Manual on Antimicrobial Susceptibility Testing) [22].

In our research we used a candida inoculum aged 20h. It was obtained by seeding of the yeast strain on the liquid media Sabouraud (with no agar). The medium was distributed in Petri dishes (about 20mL of Sabouraud medium) and inoculated with yeast inoculum (10^8 bacteria/mL), using a sterile cotton wool swab in order to obtain a uniform dispersion of the cells on the surface of the medium yeasts.

The composites were placed in the center of each Petri dish disc-shaped (thickness 4 mm). In the same time a blank test strain (*Candida albicans*) containing only culture medium and inoculum yeast was prepared with the role of positive control development. Immediately, the plate was incubated for 18h at a temperature of 35 ± 0.2°C, under atmospheric (ambient) pressure.

Results and discussions

The present paper relates the obtaining of the composites based on cellulose acetate and hydroxyapatite including an active drug substance (i.e. nystatin) in their structure. Antimicrobial activity of the obtained composites was tested.

The hydroxyapatite powders were prepared by wet chemical process and their characterization was presented elsewhere [17]. The SEM image (fig. 1) shows the morphology of the hydroxyapatite samples. It is seen that the hydroxyapatite powder is composed of nanosized primary particles which tends to form agglomerates with intergranular micropores.

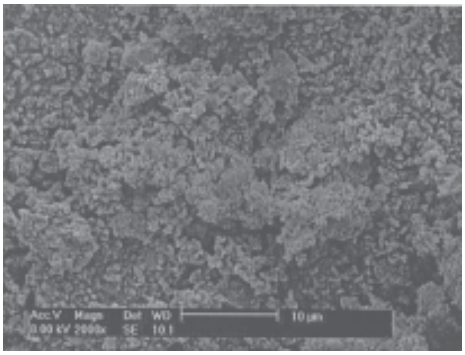

Fig. 1. SEM image of the hydroxyapatite powder

The cellulose acetate porous matrices filled with hydroxyapatite and/or nystatin were obtained by phase inversion method. Four types of porous scaffolds were obtained. In the SEM pictures of the cellulose acetate samples filled with hydroxyapatite and/or nystatin (fig. 2) the hydroxyapatite and/or nystatin deposits were observed. Hydroxyapatite crystals and/or nystatin particles can be seen spread over the internal surface of the scaffold as white spots. As shown in the SEM images, the macroporosity of the composite samples decreases with the addition of the hydroxyapatite and/or nystatin. The cross-section confirms the good incorporation of the hydroxyapatite and / or nystatin in the polymeric matrix.

A material that fulfills the requirements of biocompatibility can start to lose these qualities not only because of the processes of wear, fatigue, degradation, but also because the surrounding tissues, initially healthy, get sick or just get old. Therefore it is necessary to determine how the biomaterial behaves in contact with body fluids. The tests are aimed to study the processes of this swelling and degradation of the biomaterial processes under physiological conditions *in vitro*.

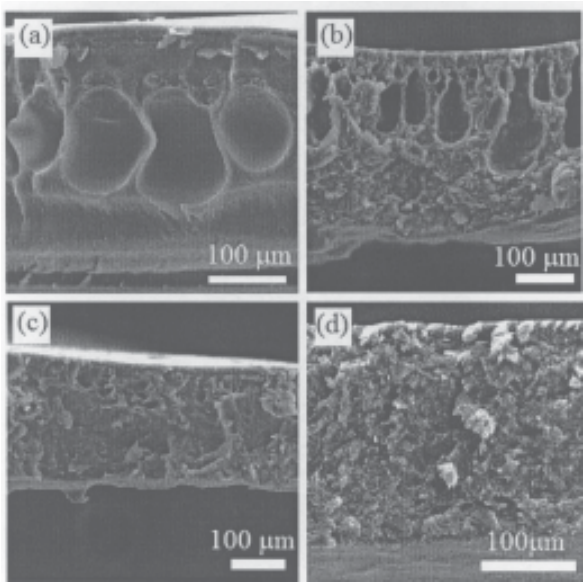

Fig. 2. The SEM images of the scaffolds in the cross-section: a. CA, b. CA-HA, CA-Nys, d. CA-HA-Nys

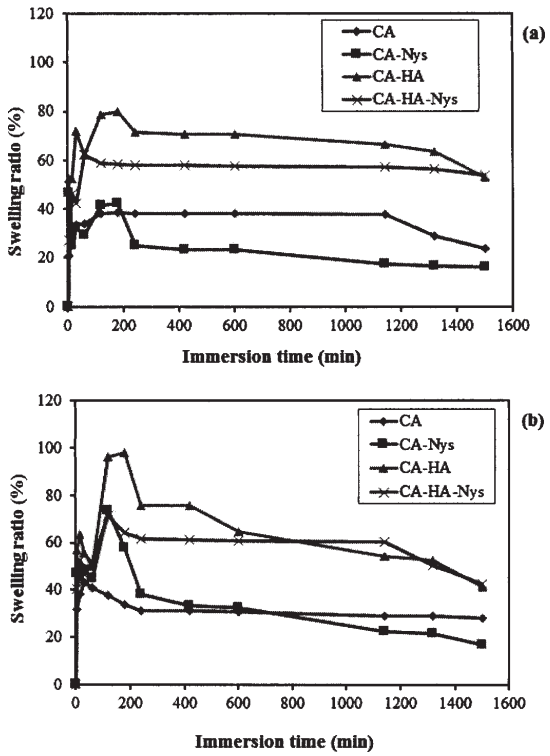

Fig. 3. Swelling ratio of composites at different immersion time in deionized water (a) and in PBS solution (b)

Thus, all membranes were incubated in deionized water and in PBS solution at ambient temperature and *p*H = 7.4 for various times. The figure 3 displays the swelling ratio in water (fig. 3a) and in PBS solution (fig. 3b) and the figure 4 presents the degradation profiles of the membranes in water (fig. 4a), respectively in PBS solution (fig. 4b) during the study period.

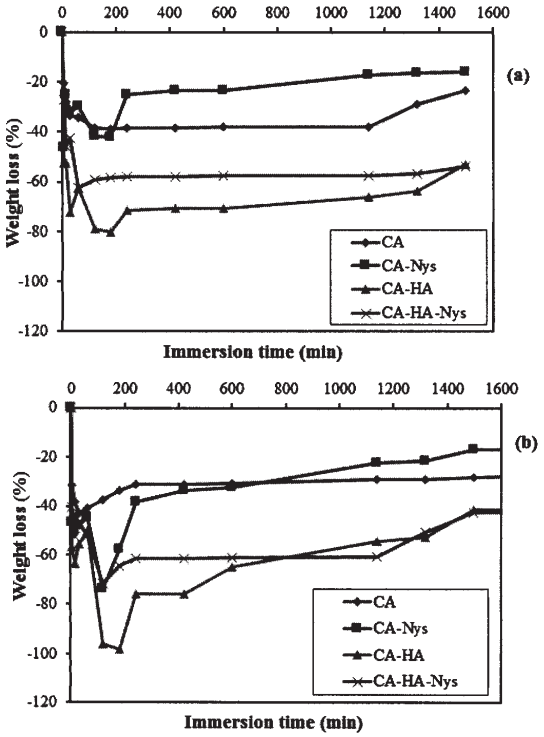

Fig. 4. Weight loss of composites at different immersion time in deionized water (a) and in PBS solution (b)

From analysis of the swelling data presented in figure 3, it was possible to conclude that the composites under investigation accept water and PBS at different rates. The highest degree of swelling in deionized water and in PBS solution reached in the shortest time was observed for CA-HA sample, followed by the CA-HA-Nys, CA-Nys and CA composites. The ability of cellulose acetate/hydroxyapatite and nystatin matrix to absorb enough water is an important factor in the formation of the gel layer which controls the drug release. The results obtained are in accordance with the previous paper dates [17] when the nystatin drug release is shown to be faster than in case of the cellulose acetate/nystatin composite. This result is probably a consequence of the increased number of hydrophilic groups in the polymer system due to the addition of hydroxyapatite.

The degradation of the developed membranes was also investigated. An analysis of the degradation profiles (fig. 4a and 4b) indicate that the membranes with hydroxyapatite in their structure show less weight loss compared with CA-Nys and CA composites. The addition of the hydroxyapatite in a polymer composite reduces its degradation rate. So, in the polymer matrix the hydroxyapatite acts as network stabilizer.

The microbiological activity of composites on yeast strain *Candida albicans* was studied. The susceptibility of nystatin loaded composites was tested using diffusion method Kirby Bauer. This is based on the properties of solid media to broadcast different culture at concentrations that decrease gradually at the deposition place to diffusion zone. After certain time the antifungal activity of tested materials was estimated by measuring the diameter of inhibition zones. The results presented in table 1 are expressed as: Susceptible (S), intermediate (I) or resistant (R) [22].

Interpretative criteria	Zone diameter (mm)
Susceptible (S)	≥ 15
Intermediate (I)	10 – 14
Resistant (R)	No zone

Table 1
INTERPRETATIVE CRITERIA FOR NYSTATIN

Table 2
INHIBITION ZONE AND INTERPRETATIVE CRITERIA OF THE SAMPLES

Nr.	Sample	Mass (g)	Inhibition zone (mm)	Interpretation criteria	Microbian inhibition (%)
1	Control sample	0	90	-	0
2	CA	0.1	0	R	0
3	CA-Nys	0.1	27.6	S	30.66
4	CA –HA	0.1	0	R	0
5	CA –HA-Nys	0.1	28	S	31.11

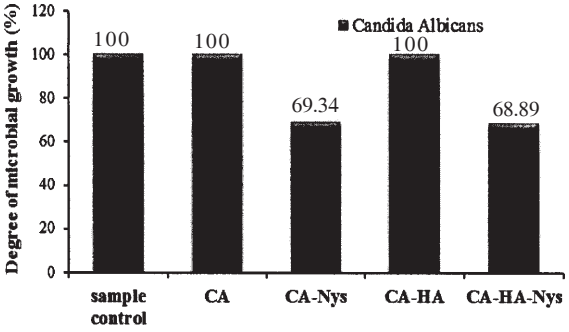

Fig.5. Degree of microbial growth of the composites

It could be seen that after 18 h the cellulose acetate membrane and cellulose acetate – hydroxyapatite composite had no zones of inhibition against the yeast strain *Candida albicans* and the samples containing nystatin had a very good inhibition zone (table 2).

According to the interpretative criteria of NCCLS [22], the CA-Nys and CA-HA-Nys composites with a diameter zone inhibition >15mm show a great susceptibility against fungal yeast. This proves that the antifungal activity of nystatin is not diminished or canceled by the presence of hydroxyapatite and cellulose acetate in the sample.

In the figures 5 and 6 we observed that the degree of microbial growth is 100% in the case of CA and CA-HA sample. This can be explaining by the fact that cellulose acetate minimizes the dispersion of calcium hydroxide into the medium containing the fungal yeast. Several studies have demonstrated that calcium hydroxide exerts lethal effects on bacterial cells [23, 24]. The antimicrobial property of calcium hydroxide is related to the release of hydroxyl ions in an aqueous environment. The viscous solution of cellulose acetate does not allow for dispersion of calcium hydroxide in the Sabouraud liquid medium and for this reason the samples CA and CA-HA have not the zone of inhibition.

The higher antifungal activity of the CA-Nys and the CA-HA-Nys membranes could be a result of the content of the nystatin in the membrane which increases its inhibitory potencies.

In conclusion, the composites based on hydroxyapatite and cellulose acetate with nystatin might be successfully used as controlled release drug systems with a very good therapeutic role.

Conclusions

In the present paper four composites including cellulose acetate, hydroxyapatite and nystatin were successfully prepared by phase inversion method. The SEM images show

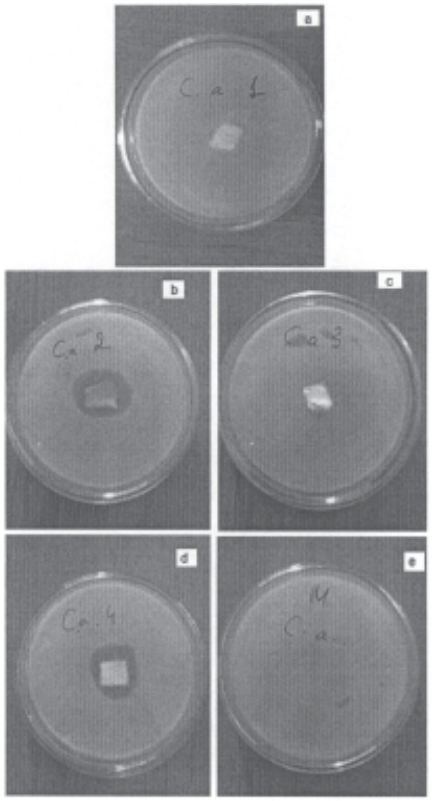

Fig. 6. Antimicrobial activity of composites on *Candida albicans*: a. CA, b. CA-Nys, c. CA-HA, d. CA-HA-Nys, e. control sample

that the macroporosity of the composite samples decreases by adding hydroxyapatite and/or nystatin. The cross-section confirms the good incorporation of the hydroxyapatite and nystatin in the polymeric matrix. The highest degree of swelling in deionized water and in PBS solution reached in the shortest time was observed for CA-HA sample, followed by the CA-HA-Nys, CA-Nys and CA composites. This result is probably a consequence of the increased number of the hydrophilic groups in the polymer system due to the addition of hydroxyapatite. We can conclude that the CA-HA membrane might be successfully used as polymer matrix in the controlled drug release by its ability to absorb water and PBS solution. The antimicrobial tests demonstrated the antifungal activity of the CA-Nys and CA-HA-Nys composites with a diameter zone >15mm. The membranes loaded with nystatin exhibited a clear inhibition effect on the *Candida albicans* growth suggesting thus their clinical potential use with a great appeal for the treatment of localized infections.

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