MATERIAL NANOSTRUCTURAT BIOACTIV MAGNETITĂ - ACID USNIC CU ACTIVITATE ANTIMICROBIANĂ
MAGNETITE - USNIC ACID NANOSTRUCTURED BIOACTIVE MATERIAL WITH ANTIMICROBIAL ACTIVITY

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With the continuous increasing rate of antibiotic resistance, novel therapeutic approaches aiming to reduce resistance and bacterial tolerance to antibiotics are currently developing. Nanotechnology seems an efficient approach in dealing with resistant infections. One of the most challenging idea is to produce materials and surfaces exhibiting high antimicrobial effects but also a good biocompatibility with human cells. In this paper, we report a newly fabricated biocompatible, resorbable bioactive wound dressing based on polyvinyl alcohol and usnic acid functionalized magnetite nanoparticles (Fe_3O_4@UA). This material revealed a good anti-staphylococcal activity, mainly due to the bio-active natural usnic acid (UA) compound entrapped into Fe_3O_4@UA. This wound dressing produces an efficient and prolonged release of the bio-active compound. Furthermore, its absorbent structure recommends this material as a good candidate for treating infected wounds.

Odată cu creşterea continuă a ratelor de rezistență la antibiotice, noi abordări terapeutice cu scopul de a reduce rezistența şi toleranța bacteriană față de antibiotice sunt în prezent în curs de dezvoltare. Nanotehnologia pare a fi o abordare eficientă pentru combaterea infecțiilor cu bacterii rezistente, prin obținerea de materiale şi suprafețe cu pronunțată activitate antimicrobiană şi cu o biocompatibilitate ridicată. În aceast studiu a fost obținut un pansament biocompatibil, resorbabil pe bază de alcool polivinilic (PVA) şi magnetită funcționalizată cu acid usnic (Fe_3O_4@UA), ca substanță bioactivă. Acest material a evidențiat o bună activitate anti-stafilococică, în principal datorată acidului usnic încărcat în Fe_3O_4. Acest pansament realizează o eliberare eficientă şi prelungită a compusului bioactiv, iar structura sa absorbantă recomandă acest material pentru utilizarea în tratamentul infecțiilor de plagă.

Keywords: ***magnetite nanostructure, usnic acid, antimicrobial activity, functionalized nanoparticles***

1. Introduction

The increased evolution of antimicrobial-resistant bacterial species represents a major concern for clinicians worldwide. Scientists are aware that antibiotic resistance derives from a multitude of factors that includes the widespread and sometimes inappropriate use of antimicrobials, the extensive use of these agents as growth enhancers in animal food, and, with the increase in regional and international travel, the relative ease antimicrobial-resistant bacteria cross geographic barriers [1]. *Staphylococcus aureus* is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections, and its great capacity to survive and adapt to different environmental challenges [2]. It has been estimated that the mortality of *S. aureus* bacteremia is approximately 20–40% despite the availability of effective antimicrobials [1]. *S. aureus* has been recently proposed as the leading overall cause of nosocomial infections and, as more treated outside the hospital setting, is also an increasing concern in the community [3]. Because of its cosmopolitan abundance into the environment and prevalence on human skin, this Gram-positive bacterium is also the main microorganism incriminated for wound infections or burns-associated infections. Depending on their site, infected wounds can be difficult to treat without an invasive procedure or without using high amounts of wide usage antibiotics, that recently have been reported to become otiose [4].

Because of the fact that antibiotics use seems to become more and more inefficient in treating resistant infections, novel therapeutic approaches, using alternative antimicrobial compounds are needed.

In the last years, an impressive number of investigations involving several types of iron oxide nanomaterials have been carried out in order to evaluate their use in antimicrobial therapy [5].

Magnetic particles are usually made of Fe_3O_4, Fe_2O_3, Fe_2CoO_4, CrO_2 [6]. These magnetic particles have been widely used for cancer

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treatment [7-13], drug delivery and targeting [14,15], inhibition of microbial biofilm [16,17], stabilization of essential oils [18], antimicrobial therapy [19,20], magnetic resonance imaging [21-25] etc. Among them, magnetite is a well-known magnetic material, properly characterized in many aspects, whose toxicity has been demonstrated to be low, and well tolerated in the human body [26,27].

Iron oxide nanoparticles functionalized with penicillin, streptomycin, erythromycin, kanamycin and cefotaxime have proved an inhibitory activity on *S. aureus* growth and the biofilm formation, superior to that exhibited by each antibiotic alone [28]. Iron oxide nanoparticles functionalized with amoxicillin and kanamicin revealed decreased MIC values against *Escherichia coli*, comparing with the MIC values of plain antibiotic solutions. Improved MIC values were also obtained in the case of iron oxide nanoparticles functionalized with cefotaxime against *Pseudomonas aeruginosa* [29].

Iron oxide nanoparticles functionalized with chitosan improved the antimicrobial activity of the cefazolin, cefaclor, cefuroxime against *E. coli* and also penicillins, beta-lactam and beta-lactamase inhibitors associations, and cefepime against *P. aeruginosa* [30]. Iron oxide nanoparticles functionalized with chitosan and polyvinyl alcohol has been reported to modify and improve antimicrobial activity of gentamicin, ciprofloxacin and cefotaxime against *S.aureus* and *P. aeruginosa* [31].

A recently reported study highlights the usefulness of iron oxide nanoparticles functionalized with usnic acid (UA) to improve the antimicrobial activity against Gram-positive and Gram-negative strains. The UA incorporated into the magnetic nanoparticles exhibited a very significant inhibitory effect on the biofilms formed by *S. aureus* and *Enterococcus faecalis,* on a wide range of concentrations, while in case of the Gram-negative microbial strains, the UA loaded nanoparticles inhibited *E. coli* biofilm development, only at high concentrations, and they proved no inhibitory effect for *P. aeruginosa* biofilms [32].

In the light of above mentioned this paper reports a novel resorbable nanostructured bioactive wound dressing with antimicrobial activity.

2. Materials and Methods

2.1. Materials

All chemicals were used as received. $FeCl_3$, $FeSO_4 \cdot 7H_2O$, NH_4OH (25%), usnic acid, and CH_3OH were purchased from Sigma-Aldrich ChemieGmbh (Munich, Germany). Polyvinyl alcohol, PVA (Fluka) with the average molecular weights 130,000 g/mol (hydrolysis degree 86.7 – 88.7 mol%) were used in the study.

2.2. Synthesis of usnic acid functionalized magnetite nanoparticles

In our previous work we report the successful fabrication and characterization by TEM, XRD, FT-IR and TGA of novel water dispersible 10 nm structures based on Fe_3O_4 and UA [10]. Briefly, UA and NH_4OH were added in deionized water under vigorous stirring. Then, Fe^{3+} and Fe^{2+} were dissolved in deionized water and Fe^{+3}/Fe^{2+} solution was dropped into the basic solution of UA. After precipitation, the prepared black precipitate (Fe_3O_4@UA) was repeatedly washed with methanol, separated with a strong NdFeB permanent magnet.

2.3. Preparation of wound dressing

Aqueous solutions containing 5 % PVA and 1% Fe_3O_4@UA dispersion were used. These solutions were mixed slowly and to achieve complete uniform dispersion of Fe_3O_4@UA into PVA gel. Then the prepared gel was poured into plastic moulds to be lyophilized. The freeze-drying parameters were: freezing at -55°C (2 h), followed by manual main drying at 0.1 mbar (12 h at -55°C followed by 8 h at 0°C and 16 h at 35°C).

2.4. Characterization

A Nicolet 6700 FT-IR spectrometer (Thermo Nicolet, Madison, WI) connected to software of the OMNIC operating system (Version 8.0 Thermo Nicolet) was used to obtain FT-IR spectra of PVA control and WD. The samples were placed in contact with attenuated total reflectance (ATR) on a multibounce plate of ZnSe crystal at controlled ambient temperature (25^{o}C). FT-IR spectra were collected in the frequency range of 4,000–650 cm^{-1} by co-adding 32 scans and at a resolution of 4 cm^{-1} with strong apodization. All spectra were ratioed against a background of an air spectrum.

SEM analysis was performed on a HITACHI S2600N electron microscope, in secondary electrons fascicle, on samples covered with a thin silver layer.

The transmission electron microscopy (TEM) images were obtained on finely powdered samples using a Tecnai™ G2 F30 S-TWIN high-resolution transmission electron microscopy from FEI (FEI Company, Hillsboro,OR, USA). The microscope was operated in transmission mode at 300 kV with TEM point resolution of 2 Å and line resolution of 1 Å. The finely micronutrient powders was dispersed into pure ethanol and ultrasonicated for 15 min. After that the diluted sample was put onto a holey carbon-coated copper grid and left to dry before it was analyzed through TEM.

2.5. In vitro wound dressing evaluation

S. aureus ATCC 25923 was purchased from American Type Culture Collection (ATCC) and glycerol stocks were obtained immediately. A

single colony, obtained after streaking a glycerol stock on LB agar, (Oxoid) was inoculated in 5mL Luria Broth and incubated overnight at 37°C, with 200 rpm shaking. Overnight cultures were diluted to a standard optical density of 0.5 McFarland and this inoculum was used for further experiments.

Antimicrobial activity of newly fabricated resorbable wound dressings was assessed by adapted disk diffusion. The *S. aureus* diluted cultures were inoculated onto Petri dishes containing Muller Hinton agar (Oxoid). After inoculation the plates were allowed to dry for 10 minutes at room temperature and resorbable wound dressings were added at three time points. First time point (T_0): wound dressings were added immediately after inoculation; second time point (T_1): wound dressings were added after 6h; and third time point (T_2): wound dressings were added after 12h of incubation at 37°C of the inoculated plates. After the addition of wound dressings, the plates were incubated for 24h at 37°C. When the incubation period expired, growth inhibition zones around wound dressings were analyzed.

1 x 10^5 HCT8 cells (human colon carcinoma cell lines) (ATCC CCL 244™) were seeded in each well of an 6 wells plate and maintained in RPMI 1640 (Gibco, NY, SUA) supplemented with 10% heat-inactivated bovine serum, penicillin/streptomycin at 37°C with 5% CO_2. After 24 hours, in each well one sponge was added (WD was cut into squares with sides of 0.5 cm). The effects were evaluated after 24 hours using fluorescein diacetate (FDA) and propidium iodide (PI) staining according to [33,34].

3. Results and Discussion

This paper reports a newly antibiotic-free wound dressing based PVA and Fe_3O_4@UA used as a prevention system after infection against *S. aureus*.

Characterization of magnetite nanoparticles was reported in a previous paper highlighting the purity, crystallinity and nanometric size of the Fe_3O_4@UA. The XRD pattern has characteristic peaks at 30.5°(220), 35.9°(311), 37°(222), 43.5°(400), 57.3°(511) and 63.1°(440), which match the standard pattern of Fe_3O_4 well. UA content was estimated by TGA as the difference between weight loss for the region at approximately 800°C for Fe_3O_4@UA and Fe_3O_4, and it is approximately 6.4% [32].

In this paper prepared Fe_3O_4@UA was characterized by TEM and the particles exhibit a rather spherical shape and are relatively monodispersed, with a mean diameter of 10 nm (Fig. 1). Using this type of particles, in the combination with PVA, a novel nanostructured wound dressing was prepared.

FTIR analysis was carried out on PVA polymer and WD (based on PVA and Fe_3O_4@UA) samples. PVA chains contain acetate groups which did not undergo hydrolysis in the production process of polyvinyl alcohol ((hydrolysis degree 86.7–88.7 mol%) from polyvinyl acetate [35]. The bands at 2937 cm^{-1} and 2913 cm^{-1} is assigned to CH_2 asymmetric and the symmetric stretching. The band at 1425 cm^{-1} is attributed to O-H and C-H bending. The absorption peak at 1088 cm^{-1} is assigned to C–O stretching. The band at 1732cm^{-1} is due to the stretching of C=O group from incomplete deacetylation [35, 36]. The assignments of the various bands are in good agreement with reported literature [35, 37].

The SEM micrographs of WD at different magnifications (Fig. 2) suggest that the PVA-Fe_3O_4@UA composite matrices are porous, with a three-dimensional interconnected microstructure. The uniformity in the pore structure is observed. It is also seen that PVA-Fe_3O_4@UA has a stratified structure interconnected with small fibrillar segments. These small segments form a highly interconnected network between layers (Fig. 3a).

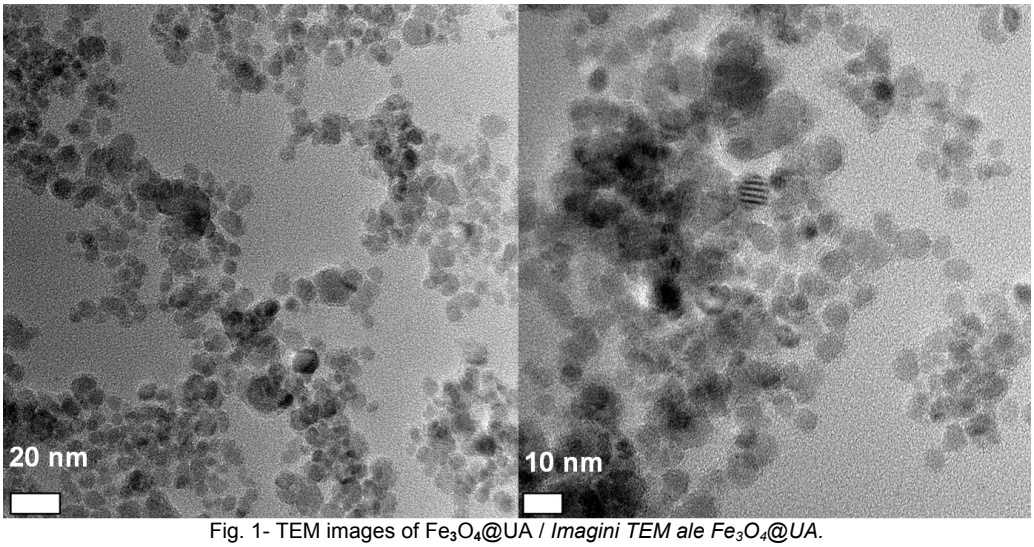

Fig. 1- TEM images of Fe_3O_4@UA / *Imagini TEM ale Fe_3O_4@UA*.

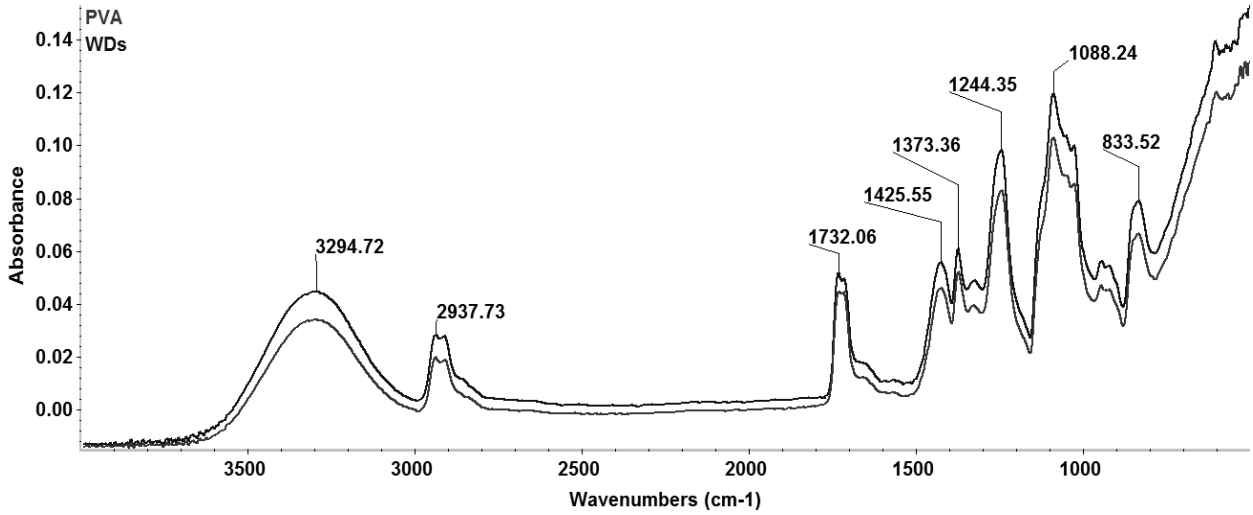

Fig. 2 - FT-IR spectra of PVA and WD / *Spectrele FT-IR ale APV şi WD.*

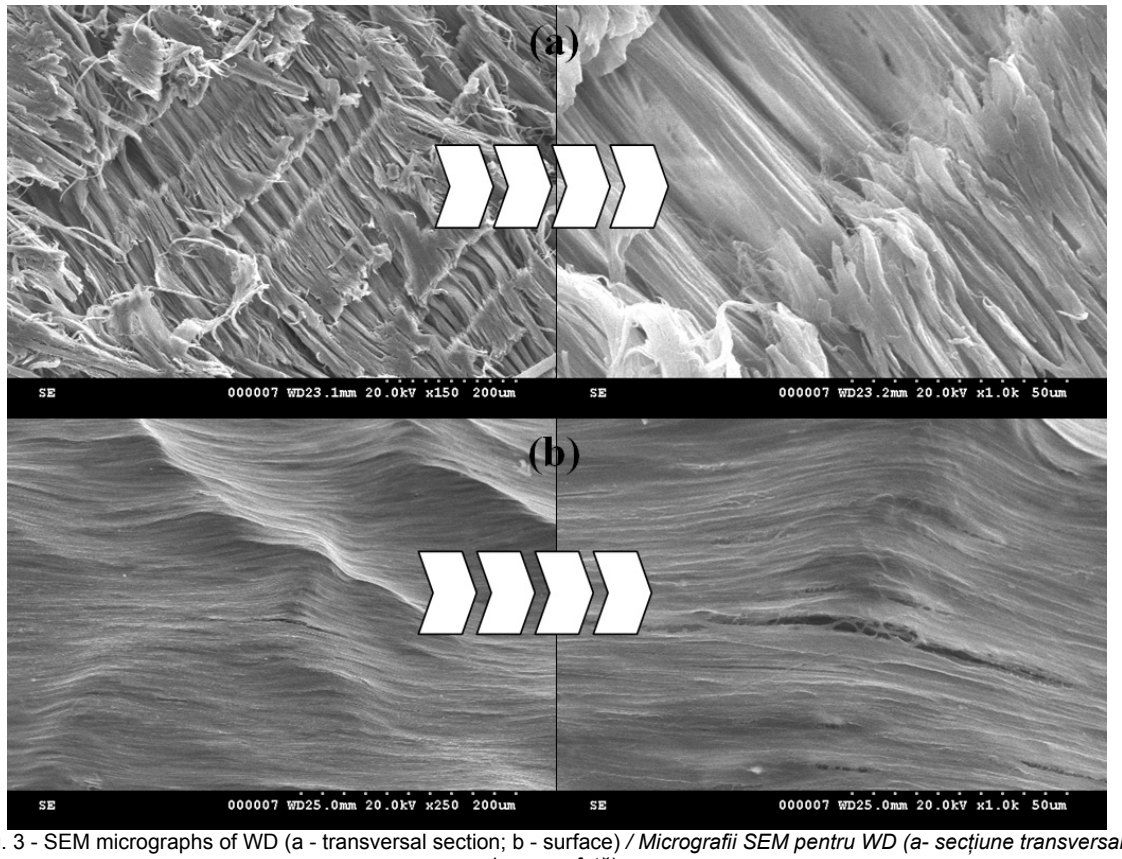

Fig. 3 - SEM micrographs of WD (a - transversal section; b - surface) */ Micrografii SEM pentru WD (a- secțiune transversală; b - suprafață).*

The morphological examination of the PVA-Fe_3O_4@UA surface (Fig. 3b) highlights a smooth surface with few cavities.

Biocompatibility assay demonstrated that newly developed WD has no effect against the eukaryotic cells growth. Tested tissue culture seems to grow normaly on the WD, maintaining also their typical morphology (Fig. 4).

Antimicrobial testing revealed that the newly developed nanostructured wound dressing is inhibiting *S. aureus* growth, especially if they are used immediately after infection. When the modified materials are added immediately after inoculation or after 6h post inoculation, growth inhibition area is similar with the one exhibited by Fe_3O_4@UA alone (Figure 5). This result reveals the efficiency of AU against staphylococcal development and the fact that the fabricated WD has the ability to release this active phyto-compound in efficient doses to inhibit bacteria growth.

Even if growth diameters are reduced indicating the low diffusion rate of the released compound into the culture medium, it is still

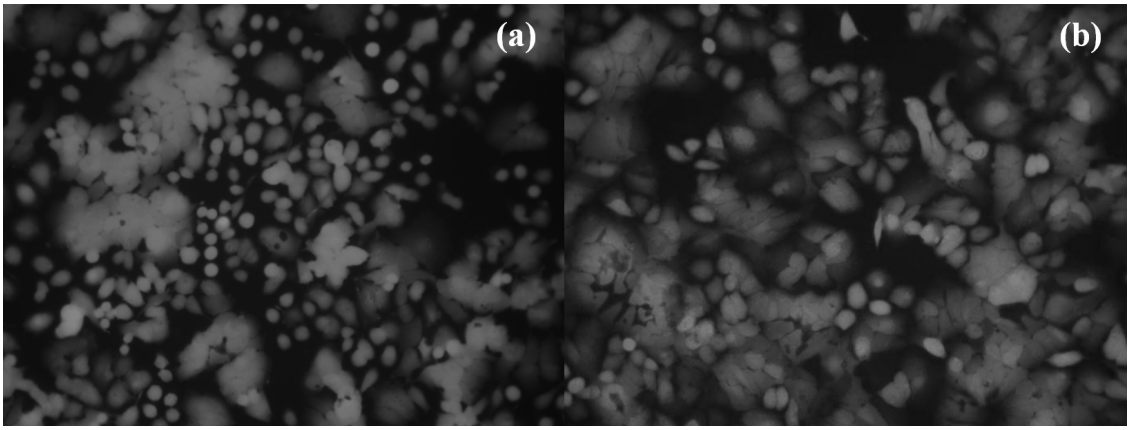

Fig. 4 - HCT8 cultures obtained after 24h incubation on the WD (a) and control (b), 200x, OI (FDA and PI staining) / *Culturi HCT8 obținute după 24h incubare în prezența WD (a) şi control (b), 200x, OI (colorație cu FDA şi PI).*

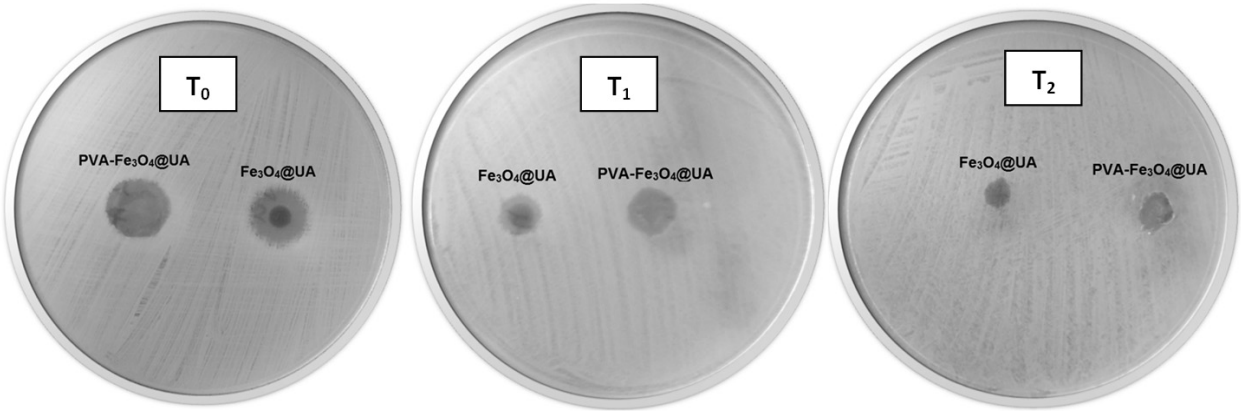

Fig. 5 - *S. aureus* growth inhibition on MH agar, after 24h incubation at 37°C, when WD was added at different time points. T_0, WD was added immediately after bacteria inoculation; T_1, WD was added at 6h after bacteria inoculation; T_2, WD was added at 12h after bacteria inoculation / *Inhibiția creşterii culturii de S. aureus pe mediu MH solid, după 24h incubare la 37°C, la nivelul ariei de depunere a materialelor adăugate la anumite perioade de timp după însămânțarea plăcii; T_0, WD a fost adăugat după inocularea bacteriană; T_1, WD a fost adăugat la 6 ore după inocularea bacteriană; T_2, WD a fost adăugat la 12 ore după inocularea bacteriană.*

obvious that the modified wound dressing exhibits a good antimicrobial activity, even in the presence of high bacterial densities. This capacity is due to the bioactive UA natural compound, which is released in active form and acts locally, but also to the absorbent properties of the material.

4. Conclusions

This paper reports the successful fabrication of a novel nanostructured resorbable material with good anti-staphyloccal properties, due to the release of UA in active forms. WD based on PVA and Fe_3O_4@UA has also proved a good biocompatibility, allowing the normal development of HCT8 eukaryotic cells *in vitro*. Because of its absorbent properties the efficiency of this novel WD is maintained also in the presence of high bacterial densities, exhibiting an evident antibacterial effect for at least 12 h.

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