Study of the Relationship Between New Polyurethane Composites for Biomedical Applications and Fungal Contamination

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Extracellular matrix biocomposites based on poly(ester urethane), and hydrolyzed collagen, elastin, chondroitin sulfate and hyaluronic acid have been prepared. Different surface morphologies for each of the test matrices were revealed by Environmental Scanning Electron Microscope (ESEM). The biological material used is a very wide spread aggressive fungal species in all media for life, Mucor mucedo. Evaluation of the biologically active potential of these biocomposites against Mucor mucedo has been done through inoculation onto Sabouraud –agar nutrient medium. Fungal growth was monitored over 60 days.Visual observations of the fungal growth show that Mucor mucedo was aggressive and caused rapid grow after 72 h of inoculation onto Sabouraud-agar nutrient medium, but the biopolyurethane composites samples relatively to the fungal attack particularly showed a different relationship.

Keywords: biomaterials; composites; polyurethanes; Mucor mucedo; extracellular matrix

The research dealing with synthetic polymers have evidenced the increasing need of new materials with properties adapted to functional applications in medicine, agriculture, industry, ecological reconstruction, etc. These research activities require the knowledge of the polymer characteristics and contamination/degradation action of microbiotics under various environmental factors.

Polyurethane materials have gained a considerable position as an important class of polymers suitable for extremely different practical applications as fibers, paints, foams, resins, elastomers, and many others [1-6]. Moreover, this class of polymers has gained a considerable position as useful biomaterials for implants or biomedical devices [7-10]. Application of polyurethanes in endoprothesis and as tissular adhesives are based on non-toxicity, chemical and biological inertia and physico-mechanical stability. Use of polyurethanes includes: endotracheal tubes, vascular prostheses, components of the artificial hearts, membranes for dialysis, adhesives for bone tissue and materials for dental recovering, due to their fairly good biocompatibility and antithrombogenicity characteristics [5, 11, 12]. The blends between biopolymers and synthetic polymers are of particular significance because they can combine biocompatibility with good processability and mechanical resistance and can be used as biomedical and biodegradable materials [13]. Currently, modern biotechnological methods such as recombinant products of biotechnology, bacterial fermentation, and advancing genetic engineering, nanotechnology provide that new specific required properties are given to the natural polymers and create the usage potential for uncommon biopolymers and their derivatives and blends.

In order to improve the biological characteristics of the materials used in contact with blood and tissues for long periods, natural polymers like collagen, fibrin and glycosaminoglycans were more often used than other natural products [14-17]. Recent studies showed that elastin represents a better contact surface than collagen due to its antithrombogenic properties [18].

The biodegradable component is mainly the natural polymer. A polymeric composite may be defined as a product formed by a continuous phase, the matrix which surrounds the biodegradable filler. The polymer scaffold in tissue engineering design represents the critical element, capable to mimic the role of the extracellular matrixes found in tissues. Extracellular matrices composed of proteins and glycosaminoglycans bring cells together and control the tissue structure, regulate the function of the cells, and allow the diffusion of nutrients, metabolites, and growth factors [19, 20]. Collagen is the most widely used tissue-derived natural polymer, and it is a main component of extracellular matrices of mammalian tissues including skin, bone, cartilage, tendon, and ligament. The combination with non-collageneous structural components results in modification of the ionic exchange function, as well the reactivity with cells and biodegradability. Collagen meets many of the biological design parameters, as it is composed of specific combinations of amino acid sequences that are recognized by cells and degraded by enzymes secreted from the cells (i.e., collagenase). Collagen has been used as a tissue culture scaffold for artificial skin due to the ready attachment of many different cell types and its cell-based degradation. The attachment of cells to collagen can be altered by chemical modification, including the incorporation of fibronectin, chondroitin sulfate, or low levels of hyaluronic acid into the collagen matrix [21]. Collagen gels have been utilized for reconstruction of liver [22], skin [23], blood vessel [24], and small intestine [25]. Collagen and elastin are two of the key structural proteins found in the extracellular matrices of many tissues [26, 27]. These proteins are important modulators for the physical properties of any engineered scaffold, affecting cellular attachment, growth and responses to mechanical stimuli [28-30].

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) that plays an important role in regulating the expression of the chondrocyte phenotype. It comprises alternating units of β-1,3-linked glucuronic acid and *N*-acetyl-galactosamine with sulfation at either the 4 or the 6 position of the *N*-acetyl-galactosamine residues. Chondroitin sulfate is also involved in intracellular signaling, cell recognition, and the

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connection of extracellular matrix components to cell-surface glycoproteins [19]. Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression [31].

Hyaluronate is one of the glycosaminoglycan components in natural extracellular matrices and plays a significant role in wound healing. Hyaluronate can be formed into hydrogels by covalent cross-linking. Hyaluronate is degraded by hyaluronidase, which exists in cells and serum [32]. Hyaluronate has shown excellent potential for tissue engineering applications such as artificial skin [33], facial intradermal implants [34], wound healing [35], and soft tissue augmentation [34]. However, hyaluronate may potentially transmit disease or act as an adjuvant in eliciting an immune response [36].

The introduction of biodegradable polymers into a synthetic polymer matrix restricts the action of a fungal, microbial or enzymatic attack [37-39]. Such limitations appear even when the biodegradable component occurs as a continuous phase in the composite material. It was found that mycelium of *Mucor mucedo* exhibits cell attractant and cell binding properties in fibroblast cultures, effects that could conceivably assist movement and attachment of fibroblasts in the healing wound. Fibroblasts are responsible for laying down collagen in the wound and hence rebuilding tissue [40]. In our previous work we demonstrated that the polyurethane mixed with extracellular matrix molecules (hydrolyzed collagen, elastin, chondroitin sulfate and hyaluronic acid) allowed cell attachment and growth over the culture period and did not interfere with morphological and functional characteristics of the cells evidencing a high biocompatibility [14, 15].

The aim of the present study is to investigate the relationship between *Mucor mucedo* and these new polyurethane composites because it is very important for the assessment of their potential application for biomedical devices.

Experimental part

Materials

Synthesis of polyurethane (PU)

The PU used in this study was synthesized in our laboratory and has the structure: $[MDI\text{-}PEA\text{-}(MDI\text{-}EG)_4]_n$ and composition determined from elemental analyses: 60.01 wt % C; 4.23 wt. % N; 6.16 wt % H; 29.60 wt % O.

Poly(ethylene glycol) adipate (PEA) is a commercial product purchased from Fibrex SA, Savinesti, Romania (M_n =2000 g/mol, purity of 97 %). Diphenylmethane-4,4'-diisocyanate (MDI), a commercial Merck product, was distilled prior to use. Ethylene glycol (EG) is a commercial product purchased from Fibrex SA, Savinesti, Romania, with a purity of 95 %.

The poly(ester urethane) was obtained from aromatic diisocyanate, MDI, with PEA and EG as chain extender, in a two-step polyaddition process, in N,N-dimethylformamide (DMF), according to procedure previously described [41-43]. In the first stage of the reaction in NCO-terminated prepolymer was formed. PEA was dehydrated for 3 h at 120° C, followed by addition of MDI. The reaction mixture between diisocyanate and macrodiol was maintained for 1 h under nitrogen atmosphere, at 90°C. The amounts of diisocyanate and PEA were controlled at an NCO: OH molar ratio of 5:1. The second step involved in the reaction of the free isocyanic group with the chain extender (EG). The reaction temperature was lowered to 60°C when the chain extender was added, and the reaction continued for 1.5 h. The polymer was precipitated in water and dried under vacuum, for several days. The spectrometric analyses FT-IR and NMR(DMSO-d_6) confirmed the structure of the synthesized polyurethane. The weight-average molecular weight of the PU is $M_w \approx 150\ 000$; the PU obtained is soluble in DMF, dimethyl sulfoxide and it is a film-forming polymer.

Hydrolyzed collagen (HC) was prepared in the laboratories of the National Institute of Research and Development for Biological Sciences (Bucharest); elastin (KEL), hyaluronic acid (HA) and chondroitin sulfate (CS) were purchased from Sigma.

Preparation of polyurethane composite matrices

Four polyurethane composite matrices were prepared as presented in table 1.

Table 1
POLYURETHANE COMPOSITE MATRICES

Polyurethane composite matrices	Weight ratio
PU/ HC	90.9/9.1
PU/HC/KEL	90.1/9/0.9
PU/HC/KEL/CS	90/9/0.9/0.1
PU/HC/KEL/HA	90/9/0.9/0.1

First, PU was dissolved in dimethylformamide (DMF) to obtain a 30 % (w/w) solution. Then, a suspension of 50% (w/w) HC in DMF was added into the PU solution at 60°C and stirred for 1 min (PU/HC). One quarter of this blend was moved in a capped test tube. The 50 % (w/w) KEL solution in DMF was added in the remaining blend, under vigorous stirring, at 45-50°C. After 5-10 min, the mixture became homogeneous and cleared (PU/HC/KEL). One third was separated in a capped test tube. The remaining composite was divided in two and in each part was added the powder of CS and HA respectively, according to table 1, under vigorous stirring, at 45-50°C. The four bio-composites were poured in glass moulds and dried for 15 h in an air-ventilated drying stove and then under vacuum for 55 h to obtain polymeric sheets.

The biological material is represented by *Mucor mucedo* [44], which was isolated from air, by placing sterile Petri dishes (15x100 mm) with added Sabouraud-agar medium (glucose-10 g; peptone-10 g; agar-15 g; distilled water-1000 mL) , for 30 min. Pure culture (100 %) of *Mucor mucedo* was obtained by repeated isolations in sterile box and it belongs to the fungal collection of Laboratory of Mycology-Faculty of Biology, Iasi. Inoculum was seeded by puncturing with a spore-laden inoculation needle into Sabouraud-agar medium, uniformly distributed in sterile Petri dishes with polymer fragments, (10x10 mm), placed in the middle. Control samples for each experiment are polymer fragments with dimensions 10x10 mm, which were seeded by puncturing with *Mucor mucedo* inoculum and placed into sterile Petri dishes, without added Sabouraud-agar medium. Two replicates of Petri dishes were incubated at 25°C, in the absence of light for 14 days, and afterwards the action of the fungus onto the selected polymer composites was monitored during 60 days. Fungal growth was measured after 72, 96, 120, 168 h and 60 days. The literature [45] shows that the temperature for optimal growth of *Mucor mucedo* is 25°C. The photographs were collected with a photo camera Canon Power Shot A530. The photomicrographs for detail visualisations were collected with a Trinocular Stereo Microscope Nikon SMZ800.

Environmental scanning electron microscopy (ESEM)
The ESEM studies were performed on samples fixed by means of colloidal silver on copper supports. The samples were covered with a thin layer of gold, by sputtering (EMITECH K550X). The coated surface was examined by using Environmental Scanning Electron Microscope (ESEM), type Quanta 200, operating at 15 kV with secondary electrons.

Results and discussions

The environmental scanning electron microscope (ESEM) overcomes many limitations of the conventional scanning electron microscope. The ESEM permits high resolution imaging of samples at relatively high pressures.

In figure 1 are presented the ESEM micrographs showing the surface morphological features of the polyurethane biocomposites.

Morphology of multiphase systems characteristic for composite materials plays an important role in determining the final properties, in particular the biomaterial qualities. Morphology of segmented polyurethanes is very complicated not only because of their two phase structure, but also because of other physical phenomena such as crystallization and hydrogen bonding in both segments [41].

The incompatibility between the polar hard segment and less polar soft segment in the polyurethane causes the heat of mixing to be positive and drives the two segments to phase separate. This phase separation influences the biomaterial qualities of polyurethanes [12]. By adding the natural extracellular matrix components into polyurethane continuous phase the morphology is found changed. Each of the components makes a different surface morphology due to its own organizational characteristics but also they interact to each other and finally the result is a unique structural biological matrix. It appears that the simple combination of these components (PU, HC, KEL, CS and HA) can be used in tailoring biomaterials for tissue engineering. The organization of collagen from fibril diameter and length to the overall orientation of the fibril in a network provides unique morphological and mechanical properties to individual tissues. Elastin is a fibrous protein found within arterial walls, ligaments, and other connective tissues where its principal function is to provide elasticity and resilience. Glycosaminoglycans (CS and HA) affect also collagen organization and tissue properties. The hyaluronic acid (HA) has the high capacity of lubrication, water sorption and water retention. Recently, HA has been widely used in biomedical applications such as scaffolds for wound healing and tissue engineering, ophthalmic surgery, arthritis treatment, and utilization as a component in implant materials. HA aids tissue formation and repairs and provides a protective matrix for reproductive cells and also serves as a regulator in the lymphatic system, and acts as a lubricating fluid in joints. Since hyaluronic acid plays an important role in many developmental and regulatory processes of the body, it has been used principally in biomedical applications. Presently, its major uses are in eye surgery, treatment of arthritis, and wound-healing preparations.

The tested biological material is a very wide spread fungal species in all media for life, which is known as ordinary fungus, [46]. From literature [47] it is specified that *Mucor mucedo* is a putrefying agent which can be isolated both from air and soil, from vegetal wastes, and animal material. The fungi are heterotrophic organisms with the majority of fungal species growing as multicellular

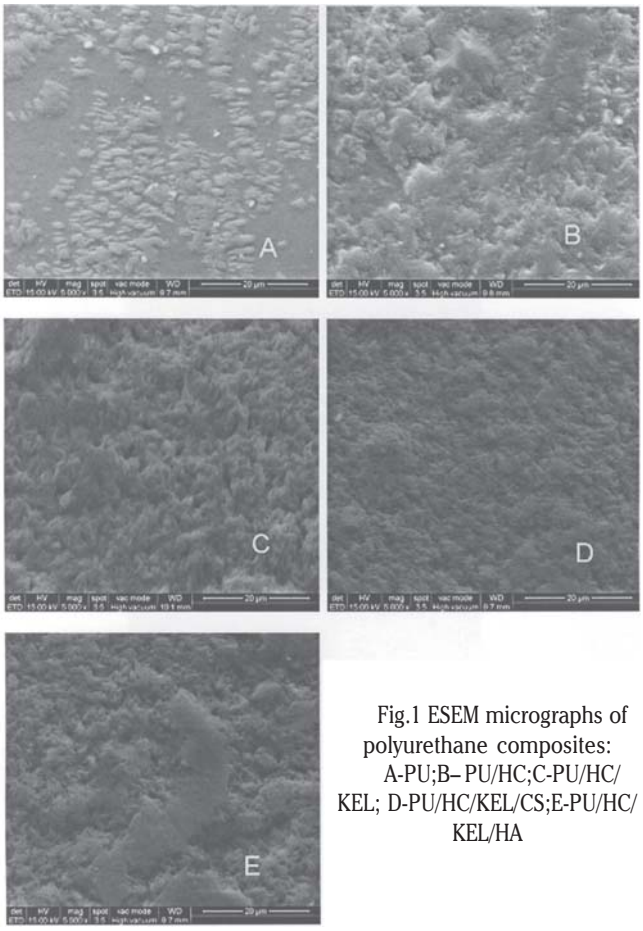

Fig.1 ESEM micrographs of polyurethane composites: A-PU;B– PU/HC;C-PU/HC/KEL; D-PU/HC/KEL/CS;E-PU/HC/KEL/HA

filaments called hyphae forming a mycelium. This species can grow under refrigeration temperatures, and can be cultivated in the laboratory onto different culture media: Sabouraud-agar, malt-agar, potato-dextrose-agar and Czapek-agar. For the study purpose Sabouraud-agar nutrient medium was selected and the inoculum generates fungal felty luxuriant grown colonies (figs. 2 and 3), varying in color from light silver grey to dark grey and thickness ranged between 1-15 mm. Literature [48] specifies that the mycelium is represented by nonsepted unicellular branched and multinucleated siphonoplast which is fixed with the help of rhizoids. From the side of the rhizoids arise three-five long unbranched sporangiophores, usually across the vegetative hyphae. At ends it shows globular sporangia of 80-200 μm dimension, dark grey color at maturity, in which unicellular light yellowish hyaline sporangia spores ovoid or cylindrical in shape of 10-12.5 x 3.5-8.5 μm, with a smooth epispore, are formed. It is specified that sporangia presents a thick ornamented membrane saturated with crystals of calcium oxalate [49]. An extension of the sporangiophore called the columella (cylindrical, ovoid or pyriform) protrudes into the sporangium. Through rupturing or solubilization of the sporangium membrane and release of sporangiospores it is observed the collar. Sexual reproduction involves the formation of brown zygospores evidencing verruca epispore. Following inoculation and incubation (at 25°C for 14 days) of the polymer samples the absence of hyphae and of the germinated spores of *Mucor mucedo* onto polymer samples surface, were remarked. The images of the control sample and fungal growth after 72 h of inoculation are presented in figure 2.

Although simple organisms, fungi are extremely adaptable to diverse environments. These microorganisms can cause allergic reactions, infections, odors, stains and surface degradation [46]. The only requirements for growth are a small amount of organic substrate and moisture.

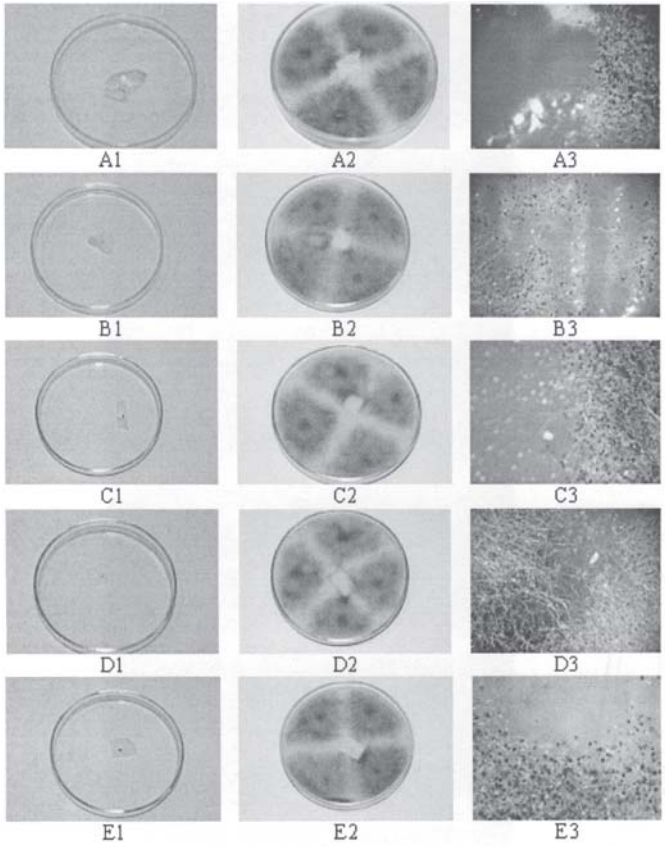

Fig. 2. The images of the control sample (1) and fungal growth (2) and detail (3, x18) after 72 h of inoculation: A-PU;B– PU/HC;C-PU/HC/KEL;D-PU/HC/KEL/CS;E-PU/HC/KEL/HA

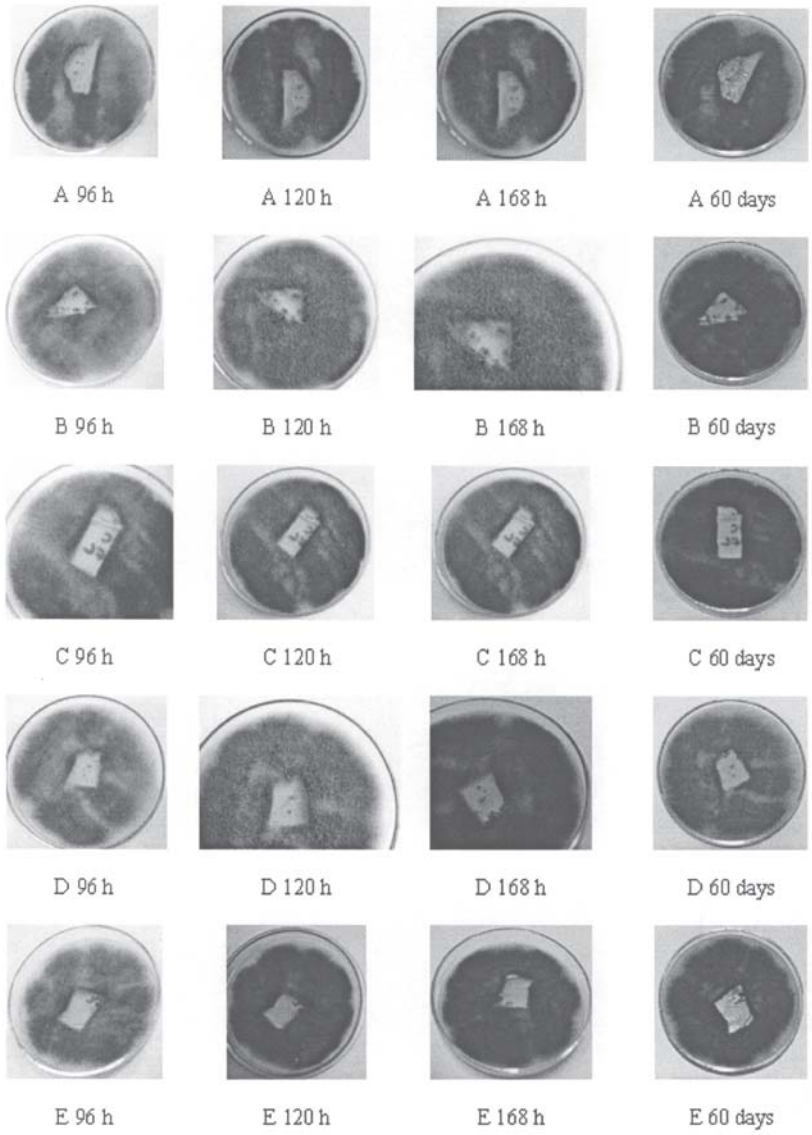

Fig. 3. The images of fungal growth after 96 h, 120 h, 168 h and 60 days of inoculation: A-PU;B– PU/HC;C-PU/HC/KEL;D-PU/HC/KEL/CS;E-PU/HC/KEL/HA

The organic components can be considered as potential carbon source for growth of fungi, if environmental conditions are adequate. Since fungi can utilise surface contaminants and products of their metabolism, they release aggresive metabollites such as organic acids and provoke damaged area or may penetrate deeper into the material. Surface composition and morphological microheterogeneity is important for attachment of fungal spores [50].

In our experiments, the images of fungal growth after 96 , 120 , 168 h and 60 days of inoculation are presented in figure 3. Generally, one can observe that *Mucor mucedo* was aggressive and caused rapid growth after 72 h of inoculation onto Sabouraud-agar nutrient medium, but the biopolyurethane composites samples relatively to the fungal attack particularly showed a different relationship.

From the visual examination of the inoculated samples with *Mucor muc*edo onto Sabouraud-agar medium the following observations resulted:

- for the PU sample the *Mucor mucedo* inoculum exhibits luxuriant growth, over Sabouraud-agar medium but it is found to be absent onto polyurethane surface after 72 h. After 96 h some isolated punctiform sporulated colonies are observed onto the surface of polyurethane and are stable up to 60 days. The polyurethane composite film sample can be considered as inhibition zone for Mucor mucedo growth;

- for the PU/HC sample the *Mucor mucedo* inoculum exhibits luxuriant growth, over Sabouraud-agar medium and after 72 h some isolated punctiform sporulated colonies are observed onto the surface of polyurethane and are stable up to 60 days. These results demonstrate that the low amount of hydrolyzed collagen (10 % w/w) is not nutritious enough for growing *Mucor mucedo* onto sample surface;

- for the PU/HC/KEL sample the *Mucor mucedo* inoculum exhibits luxuriant growth, over Sabouraud-agar medium but it is completely absent onto the fragments of PU/HC/KEL. Apparently the added elastin is in a very few amount (1 % w/w) it results for this test extracellular matrix a potential bioprotective effect. The result shows a complete protection of this biopolyurethane composite and no unsightly or detrimental fungal growth;

- for the PU/HC/KEL/CS sample the *Mucor mucedo* inoculum exhibits luxuriant growth, over Sabouraud-agar medium and after 72 h no fungal growth covering this biopolyurethane composite is observed, and as in case of PU and PU/HC some isolated punctiform sporulated colonies are observed onto the surface and are stable up to 60 days;

- for the PU/HC/KEL/HA sample the *Mucor mucedo* inoculum exhibits luxuriant growth , over Sabouraud-agar medium but it is completely absent onto the surface of PU/HC/KEL/HA sample. The biopolyurethane composite in which the hyaluronic acid was incorporated shows that there is a bioprotective effect by disrupting some key metabolic process thereby repulsing colonization onto surface by fungus *Mucor mucedo*.

All the samples revealed a luxuriant growth of *Mucor mucedo* mycelium and a high sporulation on Sabouraud-agar nutrient medium around the biopolymer film samples with no covering their surface. However the composite surface displayed a bleached appearance, which could potentially be explained by the etching effect of organic acids produced by the fungus. In particular it was remarked for PU/HC/KEL and PU/HC/KEL/HA the absence of colonies of *Mucor mucedo*, due to a potential biologically active bioprotective action. For biopolyurethane composites PU/HC and PU/HC/KEL/CS, and solely PU it was observed the presence of some isolated punctiform sporulated colonies, stable after 72 h of inoculation up to 60 days, which makes them inhibition zones for *Mucor mucedo* growth.

Conclusions

In the present study the relationship between the ordinary species *Mucor mucedo* isolated from air and cultivated onto Sabouraud-agar nutrient medium, and four new biopolyurethane composites, and polyurethane solely, was investigated. The fungal growth was monitored over 60 days.

It was remarked for PU/HC/KEL and PU/HC/KEL/HA a potential biologically active bioprotective action. The biopolyurethane composites PU/HC and PU/HC/KEL/CS, and solely PU film samples can be considered as inhibition zones for *Mucor mucedo* growth. Building up these extracellular matrices biocomposites particular responses against *Mucor mucedo* fungal attack were achieved, which means that some of them are more efficiently bioprotective and biologically active than others. In our opinion, PU as well as the corresponding biocomposites manifests a strong bioprotective activity vs. *Mucor mucedo* species, which makes these biomaterials to be utilized in various branches of medical bioengineering.

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