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Comparative investigation of the feasibility of bacterial biofilms formation on the surface of the hybrid material UREASIL

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The purpose of this study is to determine the possibility of biofilm formation from different bacterial strains on the UREASIL surface as compared with the formation of biofilms on glass surface.
Seven gram-positve and eight gram-negative bacterial strains were used in this study. The feasibility of microbial biofilm formation on the surface of the new material UREASIL and on the surface of glass (control samples) was detected by three methods: determination of the number of alive bacterial cells in the biofilms; determination of the protein content in the biofilms by a modified Lowry method; confocal laser scanning microscopy for detection/visualization of the biofilms.
The structure of UREASIL was more unfavorable for adhesion and breeding than that of the glass, but problematic species with strong production of capsule substance or slime, such as *Klebsiella pneumonie* and *Pseudomonas aeruginosa* formed thick biofilms after 48 h cultivation on UREASIL, which were detected by three methods: confocal fluorescence microscopy, modified Lowry method and counting the number of surviving bacteria that colonized the surface of the glass and the UREASIL.

Key words: UREASIL, ureasilicate, biofilm formation

INTRODUCTION

Biofilm is the microbial lifestyle in natural and manmade environments. The initial microbial adhesion to surfaces is a complex process dependent on the non-specific interactions between bacteria and the surface, including van der Waals interactions, electrostatic forces, Lewis acid-base and hydrophobic interactions, the latter being the strongest of all long-range non-covalent forces [1]. After initial attachment, the accumulation step in biofilm formation depends on the bacterial proliferation, exopolysaccharide matrix production and intercellular adhesion [2]. The cells in the microbial biofilm demonstrate many changes in their metabolism: a higher biochemical activity by producing more new enzymes and metabolic adaptation mechanisms to a new variant of growth display. The biofilm formations present a higher level of resistance to all physical and chemical factors in comparison to their planktonic forms. Many genetic mechanisms of the cells play a role for the adaptation to the biofilm, a wide range of characteristics provide a number of advantages over planktonic bacteria [3-5].

The dynamically developing industry constantly needs new products which satisfy different specific requirements. Very often the growth of biofilm formations is around a foreign body in patients, around medical implants and can progress to development of an infection. It has been estimated that the subjects from bacterial biofilms are generally about 1000-fold less susceptible to the effects of commonly used antimicrobial agents than their analogous planktonic cells and are highly resistant to the phagocytosis of the immune system phagocytes. In addition, the various attacks of the antimicrobial immunity are neutralized by the same formation. That is a reason for the development of chronic infections mediated by biofilms and it is a problem for their eradication [3,6,7].

In search of promising new materials we turned to ureasils: sol-gel materials. Sol-gel process is one of the most versatile methods for the preparation of organic-inorganic hybrid materials due to the low temperature of synthesis [8]. The incorporation of inorganic materials into organic matrices ensures physical rigidity, photophysical and thermal stability of the obtained hybrid materials [9]. It is

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widely used for preparation of glassy matrices for optical applications. However, flexibility of pure inorganic glasses obtained by the sol-gel method is limited and they are often susceptible to cracking during a drying stage. Including organic polymers in an inorganic silica framework makes the final material more flexible [10]. This approach was used for the synthesis of new organic-inorganic materials based on polyether chains covalently linked to a silica framework by urea bridges, referred to as ureasilicates or ureasils [11]. These materials were initially used as a host matrix for highly luminescent europium salts [12-15], ionic conductive lithium salts [16,17], magnetic nanoparticles (NPs) [18,19] and organic dyes [20]. Recently, it was demonstrated that optical functionalities, such as semiconductor [21-24] or metal [25] NPs, can be successfully incorporated into ureasilicates, which makes them promising materials for fabrication of non-linear optically active devices.

Ureasilicates are obtained by hydrolysis and condensation of a ureasilicate precursor prepared by reaction between a silicon ethoxide modified by isocyanate group (referred to as ICPTES) and a polyoxyalkyleneamine (referred to as Jeffamine) [26].

The purpose of this study is to determine the possibility of biofilm formation from different bacterial strains on the surface of the new hybrid material UREASIL in comparison with biofilm formation on other materials. This is done to investigate possible biomedical application of UREASIL.

MATERIALS AND METHODS

UREASIL

O,O-bis(2-aminopropyl)-polypropylene glycol-block-polyethylene glycol-block-polypropylene glycol-500 (Jeffamine ED-600, Fluka) was dried under dynamic vacuum before use. 3-Isocyanate propyltriethoxysilane (ICPTES, Aldrich), tetrachloroauric acid (Aldrich), trisodium citrate dihydrate (Aldrich), absolute ethanol (Riedel-de Haën), citric acid monohydrate (Merck) were used as received. Distilled water with a resistance around 18 MS/cm was used for the preparation of dilute aqueous solutions.

Microbial strains: Seven gram-positive and eight gram-negative strains were used for the experiments in this study. Two control strains from American type culture collection *Staphylococcus aureus* MSSA (ATCC29213) and *Escherichia coli* (ATCC25922) and the following clinical isolates from patients, more of them multidrug resistant (MDR) were used: *Enterococcus faecalis* (n=3), *Staphylococcus aureus* MRSA (n=3), *Moraxella catarrhalis* BRO+ (n=1), *Escherichia coli* ESBL (n=1), *Klebsiella pneumoniae* ESBL (n=1), *Enterobacter aerogenes* MDR (n=1), *Morganella morgannii* MDR (n=1), *Pseudomonas aeruginosa* MDR (n=1), *Acinetobacter baummannii* MDR (n=1), *Stenotrophomonas maltophilia* (n=1). The strains were stored in skim milk at -70°C. Before laboratory testing they were three times subcultivated, reproduced and after that they were used in the experiments.

Chemical materials: Na_2CO_3, NaOH, Na tartrate and $CuSO_4$ with chemical purity; 2N Folin-Ciocalteu reagent.

EXPERIMENTAL PROCEDURE

Synthesis of the ureasilicate monoliths

The synthesis of the ureasilicate monoliths included several steps. At the first step stoichiometric amounts of Jeffamine and ICPTES (1:2;R=2.0) were mixed in a glass vessel under stirring at 200 rpm for 10 min, so that the rapid uncatalyzed reaction between amino and isocyanate groups forming polyurea linkages took place [11]. The obtained material will be referred to hereafter as a conventional ureasilicate precursor. At the second step an additional amount of ICPTES was introduced in order to adjust the desired molar ratio between ICPTES and Jeffamine in the final mixture. Ethanol was used as a homogenizing agent and was added 5 min later. The third step consisted of the catalyzed hydrolysis/condensation of the mixture by addition of ammonia or citric acid aqueous solution. The mixture was stirred for 10 min more and poured into a polystyrene cell, covered with Parafilm ^{R}, which was pin-holed after gelation at room temperature. The gelation time varied from 1 h to 3 days depending on the catalyst used and the R value. During the final step the cell with the resulting gel was kept in an oven at 40ºC for two weeks, which assured completion of hydrolysis/condensation reactions and evaporation of residual liquids. This drying process led to sample shrinkage of about 30% of total volume [26].

Counting CFU/ml of surviving bacteria

The bacterial strains were grown on a Brain heart infusion (BHI) agar (Oxoid microbiology products, Cambridge, UK) at 35°C overnight. The suspension of each of them was prepared in BHI broth (Oxoid microbiology products, Cambridge,

UK) with bacterial density of at least 1.5-2×10^8 colony-forming units (CFU/ml). It was inoculated with 100 µl of a pure microbial culture on the UREASIL fragments in a 96-well polystyrene microtiter plate (Nunc) and on the wells without Ureasil and was incubated at 35°C for 48 h. After the incubation the infected broth from the wells was aspirated under sterile conditions and the samples were washed with 200 µl of PBS. Using serial 10-fold dilution and counting the value of CFU/ml on the BHI agar, the number of living bacterial cells and the concentration of biofilm were determined. The controls used were: three controls of UREASIL with sterile PBS, sterile broth and sterile water; and the same controls on the wells without UREASIL.

Modified Lowry method

The protein content was measured using a modified Lowry method [27]. The principle behind the Lowry method of determining protein concentrations [28] lies in the reactivity of the peptide nitrogens with copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids.

10 µl of the sample washings were diluted to 1 ml with distilled water. First, reagent A: 2% Na_2CO_3 in 0,1N NaOH, reagent B: 1% $CuSO_4.5H_2O$ and reagent C: 2% sodium tartrate, were prepared. Reagent D was prepared by mixing reagents B and C in the ratio 1:1. Reagent D was prepared just prior to use. Thereafter 5 ml of mixed reagents A and D in a ratio of 1:50 were added and stirred for 140 min. Then 0.5 ml of 1N Folin-Ciocalteu reagent (phosphomolybdotungstate) were added. After 45 min the absorbance was measured at 750 nm against a control containing the same components without the microbial culture. The amount of protein was determined by the standard. As a reference a solution of bovine serum albumin was used.

Confocal laser scanning microscopy:

Confocal laser scanning microscopy is an important method for the study of biofilm structure. This is a non-destructive and non-invasive method [29]. Confocal laser scanning microscopy allows the detection and localization of the biofilm on the hybrid material UREASIL.

Glass coverslips covered with the hybrid material UREASIL and control glass coverslips were placed in a 6-well plate with BHI broth (Oxoid microbiology products, Cambridge, UK) and inoculated with 100 µl of a pure microbial culture. After 48 h incubation at 35°C for forming a biofilm the culture media was removed and the biofilms were fixed in 2.5% GA in PBS at 8°C for 24 h. The fixed biofilms were rinsed 5 times with PBS before staining with 0.1% (w/v) acridine orange (AO) (Sigma Chemical Co., St. Louis, MO, USA) in PBS at room temperature for 5 min. AO is a membrane permeant nucleic acid stain that intercalates dsDNA and binds to ssDNA, as well as to ssRNA through dye-base stacking to give a broad fluorescence spectrum when excited at 476 nm. The biofilms were rinsed again as described above and were mounted on glass slides using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Images were taken using a confocal microscope Leica DM2500 (Leica Microsystems, Wetzlar, Germany).

After immobilization, the QCM-resonator was put into 1 mL distilled water.

Table. 1. Microbial biofilm detected by counting surviving bacteria

Microbial species (number of strains)	Microbial number over UREASIL [cfu/ml]	Microbial number over glass [cfu/ml]
Staphylococcus aureus MSSA (ATCC29213)	7×10 000	3×100 000
Staphylococcus aureus MRSA (n=2)	8×100 000;	6×1 000 000;
	3×1 000 000	2×10 000 000
Streptococcus intermedius (n=2)	3×100 000;	9×100 000;
	2×1 000 000	7×1 000 000
Enterococcus faecalis (n=3)	9×10 000;	4×10 000 000;
	4×100 000	1×10 000 000
Mora×ella catarrhalis bro2+ (n=1)	8×1 000	5×10 000
Escherichia coli (ATCC25922)	5×10 000	3×100 000
Escherichia coli ESBL (n=1)	9×10 000	2×10 000 000
Klebsiella pneumoniae ESBL (n=1)	5×100 000	8×100 000 000
Morganella morgannii MDR (n=1)	6×10 000 000	8×100 000 000
Pseudomonas aeruginosa (n=1)	7×10 000 000	8×1 000 000 000
Acinetobacter baumannii	5×100 000	4×100 000 000
Stenotrophomonas maltophilia (n=1)	9×10 000	7×100 000 000

Stain	A. UREASIL	B. Glass
Staphylococcus aureus *MRSA*		
Streptococcus intermedius		
Enterococcus faecalis		
Moraxella catarrhalis		
Escherichia coli ESBL		

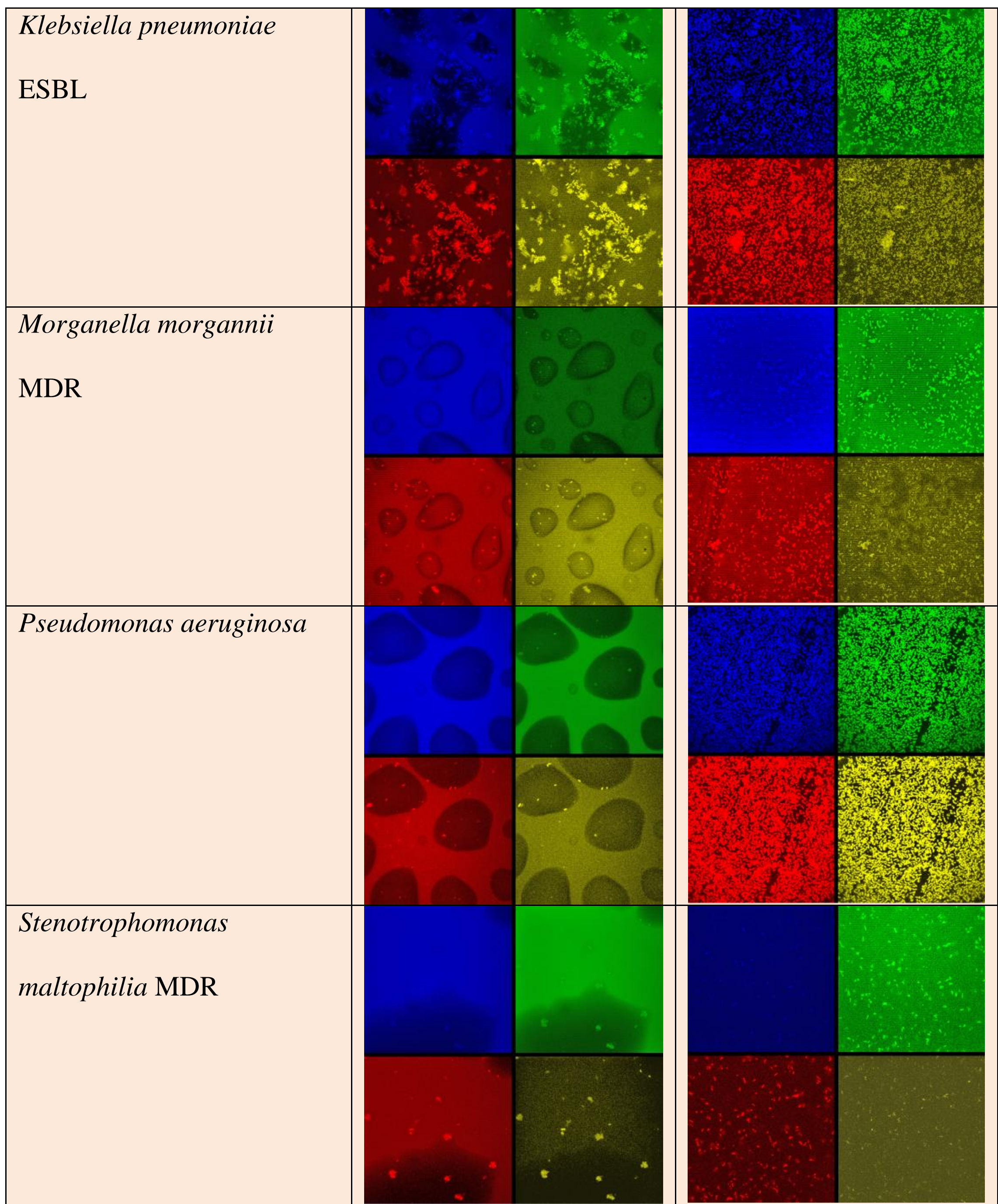

Fig. 1. Confocal fluorescence microscopy images:
A) biofilm formed on UREASIL, B) biofilm formed on glass.

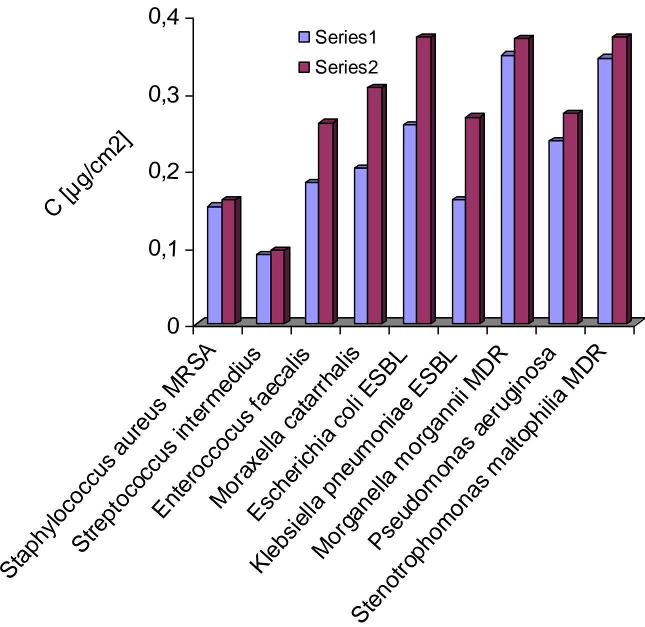

Fig. 2. Detection of microbial biofilm using a modified Lowry method for determination of protein quantity in washings: Series 1 - UREASIL; Series 2 - glass

RESULTS AND DISCUSSION

The formation of a biofilm after 48 h of incubation was evaluated by using the method of counting CFU/ml (Table 1) with the help of confocal laser scanning electron microscopy images (Fig. 1) and with a modified Lowry method for detection of the protein content in the studied samples (Table 2).The 48-h incubation period proved enough for the tested bacterial strains to build well-formed biofilms on the investigated areas (Fig. 1), a fact which corresponded with the results from other our previous research [30,31]. From the results given in Table 1 it can be concluded that, defined by the microbial number, the surviving bacteria colonizing the surface of the glass outnumber the surviving bacteria in the biofilm coating the ureasil. The difference is about 10 fold in staphylococci and moraxella and nearly 100 fold in enterococci, i.e. in Gram-positive and Gram-negative cocci. Only in *Streptococcus intermedius* the difference is less than 10. In Gram-negative rods and multidrug-resistant bacteria the difference in the number of surviving bacteria in the biofilm coating the glass is even higher – up to 1000 times more than ureasil. The differences shown in Table 1 and Fig. 1 between the various strains (ranging from 10 to 1000 times) depended on the capabilities of the particular bacteria involved with the different surface structures of the cell wall in their adhesion to surfaces and biofilm formation [3,5]. In conclusion, from the initial results it can be argued that the structure of ureasil was more unfavorable for adhesion and breeding than that of the glass, but problematic species with strong production or overproduction of exopolysaccharide alginate, capsule substance or slime such as *Klebsiella pneumonie, Morganella morgannii*, *Pseudomonas aeruginosa* and other glucose non-fermenting bacteria: *Acinetobacter baummannii* and *Stenotrophomonas maltophilia* formed a thick biofilm after 48 h cultivation on ureasil. The biofilm formation from these bacteria is very strong and difficult to eliminate, because they have many surface biomolecules responsible for adhesion [5,30,31]. The formation of biofilm on the surface of a polystyrene plate, tested with *Pseudomonas aeruginosa*, showed results similar to those on the glass - a many times higher microbial number of this coating on the ureasil.

Sterile saline and broth cultivation without prior culturing microorganisms in them displayed no microbial growth.

The nine strains arbitrarily selected were quantified in the washings by the modified method of Lawry. The obtained results are shown in Fig. 2. The findings of this method confirm most of the results of the microbiological method. As a result of the studies it can be concluded that the number of surviving bacteria colonizing the surface of the hybrid material UREASIL is lower than that of the glass surface colony. These results are also confirmed by confocal microscopy, as can be seen in Fig. 1.

New strategies for prevention and treatment of clinically relevant bacterial biofilms are needed, because the biofilm-forming microbes are responsible for most of the chronic bacterial infections in humans and animals [3,5,30,31]. The results obtained support the conclusion that UREASIL is a material with good potential for biomedical applications.

This work explores the formation of a biofilm on the hybrid material UREASIL, as a first step in the study of the formation of a biofilm on a base material composite UREASIL with various types of nanoparticles. Further investigations regarding this theme are in progress.

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СРАВНИТЕЛНО ИЗСЛЕДВАНЕ НА ВЪЗМОЖНОСТТА ЗА ОБРАЗУВАНЕ НА БАКТЕРИАЛНИ БИОФИЛМИ ВЪРХУ ПОВЪРХНОСТТА НА ХИБРИДЕН МАТЕРИАЛ UREASIL

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(Резюме)

Целта на това изследване е да се определи възможността за образуването на биофилм от различни бактериални щамове на повърхността на UREASIL в сравнение с образуването на биофилм върху стъклена повърхност.

Седем грам-положителни и осем грам-отрицателни бактериални щама бяха използвани в експериментите в това проучване. Възможността за образуване на микробиален биофилм върху повърхността на новия материал UREASIL и на повърхността на стъклото (контролни проби) е установена по три метода: определяне на броя на живите бактериални клетки в биофилмите; определяне на белтъчното съдържание в биофилмите чрез използване на модифициран метод на Лоури; конфокална лазерно сканираща микроскопия за откриване и визуализация на биофилми.

Структурата на UREASIL е по-неблагоприятна за адхезия и размножаване от тази на стъклото, но проблемни видове със силно производство на капсулно вещество или слуз като *Klebsiella pneumonie* и *Pseudomonas Aeruginosa* формират дебел биофилм след 48 часа култивиране върху UREASIL, който е открит и от трите метода: конфокална флуоресцентна микроскопия, модифициран метод на Лоури и чрез преброяване на броя на живите бактерии, които колонизират повърхността на стъклото и UREASIL.