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Investigation on the proliferation of Gram negative bacterial cells onto sol-gel carriers

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This work is related to the synthesis and characterization of new hybrid sol-gel materials. Several carriers based on poly (N-acryloylglycine) (NAGly) composed of poly (ethylene glycol) dimethacrylate (PEGDM) were prepared. The effectiveness of biofilms formation of bacterial strains *Escherichia coli* and *Pseudomonas fluorescents* on the matrices was investigated by biochemical methods.

Two types of hybrid gels were synthesized based on PEGDM, 2-hydroxyethyl acrylate (HEA) and N-acryloxysuccinimide ester (NAS) with incorporation of inorganic precursors. The rheological properties of the gels and formed biofilms were investigated by quartz crystal microbalance (QCM).

The experimental results demonstrated that the obtained matrices are appropriate for biofilms formation.

Key words: biofilms, matrices, hybrid gels.

INTRODUCTION

Biofilms are defined as microbial communities adhered to surfaces and encased within an extracellular polymeric substance (EPS) produced by the microbial cells themselves. Biofilms may form on a wide variety of surfaces, including natural aquatic systems living tissues, indwelling medical devices and industrial/potable water system piping [1].

A major fact influencing the biofilm development in water treatment system is the surface area. Industrial water systems, unlike most natural environments (lakes and rivers), offer a tremendous amount of surface area for attachment. Different polymeric membranes, resins, storage tanks, cartridge filters, and piping systems all provide surfaces suitable for bacterial attachment and growth [2]. The topography of the surface to which a microbial cell attaches is also fundamental to biofilm formation. Generally, as the roughness of a surface increases, bacterial adhesion will also increase [3]. In addition, the physicochemical properties of the surface effects bacterial adhesion. Microorganisms attach more rapidly to hydrophobic, non-polar surfaces such as teflon and other plastics than to hydrophilic materials such as glass or metals [4–6].

Hybrid materials obtained by the sol-gel method are effective carriers of enzymes, cells, amino acids, etc. Development of a good biocompatible matrix for immobilization of cells is very crucial for improving the performance of functional biohybrids. Synthesis of solid inorganic materials from alkoxide, aqueous and polyol-modified silanes routes, as well as the incorporation of organic polymers, is further areas being developed to improve the viability of encapsulated cells [7]. Depending on the precursor (SiO_2, TiO_2, SnO_2, etc.) introduced in the system, the matrices obtained by the sol-gel technology possess different physical and mechanical properties. These multifunctional materials are used in different fields, including construction of chemical and biological sensors, in optics and catalysis [8–10].

According to type of bonding between organic and inorganic part, the hybrid materials can be clas-

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sified in two groups: Class I includes matrices based on non-covalent bonding (donor-acceptor, H-bonds, etc.) between organic and inorganic parts. They are quite simple to obtain because they consist of a mixture of two different types of polymers. Class II requires covalent bonds between the two parts [11].

In order to investigate the rheological properties of obtained sol-hybrid materials and biofilms, thickness shear mode (TSM) resonators was used. (TSM) resonators use transversal waves in the ultrasound range for investigation of the properties of complex materials and nanostructures at different scale [12–15]. The Quartz Crystal Microbalance (QCM) TSM resonator, is a simple, low cost effective, high-resolution mass sensing technique, based upon the piezoelectric effect. The QCM can be applied for solution measurements mainly in analytical chemistry and in electrochemistry due to its sensitive solution–surface interface measurement capability. The technique possesses a wide detection range [16–18]. At the low mass end, it can detect monolayer surface coverage by small molecules or polymer films. At the upper end, it is able to detect a larger masses bound to the surface. Such kind of masses can be complex arrays of biopolymers and bio-macromolecules, or even whole cells. This model and technique have been used to characterize the micro-viscoelastic properties of gels and biomaterials particularly during their formation [19]. The efficiency of this new micro-rheometer is sufficiently high to ensure a complete and a reliable on-line follow-up of the intrinsic characteristics at microscopic scale of both organic and inorganic materials [20, 21].

Hybrid organic-inorganic materials are deeply investigated as host substrate for bioapplication. Innovative applications of microorganisms embedded in sol-gel hybrid materials have strongly increased. One of those applications is the assessment of water quality to detect traces of chemical or biological pollution [22]. To increase the efficiency of these tests, it is important to carry out early detection of the different microorganisms involved. One can note the interest of the early detection of bacteria such as *Escherichia coli* involved in pathogenic human diseases such as gastroenteritis, urinary tract infections, or meningitis [23–25] or *Pseudomonas aeruginosa* presents in septicemia or nosocomial infections [26].

Several microbial strains which can form biofilms onto various matrices are well known: *Bacillus cereus, Bacillus licheniformis, Halomonas salina, Bacillus pumilus, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas species, Candida albicans, E.coli* [27–32].

Although current knowledge of bacterial biology owes much to work done on planktonic cultures of laboratory strains of *E. coli*, many isolates also have the capacity to form biofilm structures *in vivo* and *in vitro*. Indeed, *E. coli* is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract, where it thrives in an environment with structural characteristics of a multispecies biofilm [33, 34]. Biofilm formation of *E.coli* has mainly studied with non-pathogenic laboratory strains. The authors demonstrate that *E. coli* forms biofilms on multiple abiotic surfaces in a nutrient-dependent fashion [35–39].

Pseudomonas fluorescents is known to form biofilms and consequently the surface adhesion of a number of isolates has been investigated. *Pseudomonas fluorescens* is a ubiquitous, Gram-negative, motile, biofilm-forming bacterium commonly-encountered in soil and water habitats. The organism plays an important role in food spoilage, drinking water quality and plant disease [40]. Cossard et al. determined that the adherence properties of four *Pseudomonas fluorescens* isolates were independent of their ecological habitat [41].

A biofilm formation from cells of *Pseudomonas aeruginosa* and *Pseudomonas fluorescents* on different surfaces: glass, copolymers of cellulose, poly-dimethylsiloxane, etc. was reported [42].

This study is devoted to the synthesis and characterization of different hybrid materials based on pure organic material N-acryloylglycine (NAGly). Two microorganisms *Escherichia coli 52170* and *Pseudomonas fluorescens FFD16* were selected to be involved in the formation of biofilms on newly synthesized hybrid matrices as follows. The dynamics of proteins and polysaccharides production of biofilms were examined. Two types of gels based on poly(ethylene glycol) dimethacrylate with incorporation of inorganic precursor was also synthesized and characterized.

EXPERIMENTS AND EQUIPMENT

Reagents

2,2'-azobis(2-methyl proponitrile) (AIBN) and glycine were provided by Acros Organics. Poly(ethylene glycol)dimethacrylate (PEGDM 550, Mw= 550 g/mol), N, N-dimethylacetamide (DMAc) acryloylchloride, 2-hydroxyethyl acrylate (HEA), tetramethylorthosilicate (TMOS), 3-(dimethylamino) propionitrile (DMAPN), sodium persulfate (PSS), 2-hydroxy succinimide (NHC), Triethylamine (TEA), hydrochinon, ethyl acetate and hexane were purchased by Sigma-Aldrich. All products were used without any further purification. N-acryloxysuccinimide ester (NAS) was synthesized in Laboratory for Systems

and Applications in Information and Energy Technologies (SATIE), University of Cergy Pontoise.

Escherichia coli 52170 and Pseudomonas fluorescens FFD16 microbial strains were supplied by the Collection of Pasteur Institute of France. Salts for nutrient medium were obtained from Merck (Germany). Glucose and bovine serum albumin (BSA) were obtained from Fluka (Switzerland). Agar and LB for nutrient microbial growth mediums were obtained from Sigma-Aldrich.

Carriers for biofilm formation

The following four different polymeric matrices and hybrid gels were used for biofilm formation during the experiments:

The matrices were synthesized by co-polymerization between NAGly and PEGDM-like organic crosslinker. For biofilms formation we used four matrices with different ratio of NAGly and PEGDM as follows: NAGly/ PEGDM: 10/90, 20/80, 30/70, 50/50 w*t*%. Polymerization was obtained using 25 mg AIBN.

Preparation of NAGly

The polymer carriers for biofilms formation were synthesized according to Ringeard et al. [43].

NAGly was prepared by adapted method of *Bentolila et al*. [44]. It was synthesized by a Schotten-Baumann reaction in aqueous phase as follows: 4.50 g of glycine (60 mmol) were dissolved in 60 ml of 2M solution of potassium hydroxide. The mixture was cooled at 4°C in water ice bath for about 10 minutes. 6 ml of acryloyl chloride (73.6 mmol) were added to the mixture drop wise using a dropping funnel. At the end of the reaction (TLC monitoring) the solution was washed with 2x40 ml of diethyl ether and the separated aqueous phase was acidified to pH = 2. The aimed product was extracted by 3x40ml ethyl acetate. After drying the organic phase over $MgSO_4$, the residue was concentrated using a rotary evaporator. The yield of this synthesis is 74%. mp 130 °C (lit. 132 °C) [45]; H NMR (250 MHz, dimethyl sulfoxide [DMSO]-d_6, (δ): 6.25 (dd, 1H, C(γ)H), 6.08 (dd, 1H; C(β)H), 5.67 (dd, 1H; C(α)H), 3.95 (s, 2H, Et); infrared (IR) (ATR): ν=3350 (s), 1740 (s), 1650 (s), 1600 (s), and 1530 (s) cm^{-1} [43].

Preparation of matrices based on NAGly/PEGDM

Four different types of matrices based on NAGly were synthesized according to the methodology described above [43]. The overall composition of the networks was varied between 10/90, 20/80, 30/70 and 50/50 wt% of NAGly and PEGDM. All investigated co-networks were reported as PNAGly/ PEGDM (x/y). The numbers between brackets (x/y) correspond to the PNAGly and crosslinker weight proportions, respectively. For example, a co-network obtained from a mixture of 450 mg of NAGly and 50 mg PEGDM was noted PNAGly/ PEGDM (90/10 wt%). The synthesis of co-network is performed in a flask containing *x* wt% of NAGly, *y* wt% of crosslinker and 600 µl of DMAc. The mixture was stirred and degassed to remove all traces of oxygen (radical inhibitor). Finally, 25 mg of AIBN were added at the last moment to avoid the rapid decomposition of the initiator. The content of the flask was taken with a pipette and placed between two glass plates separated by a teflon film (*e*=500 µm) and held together by a clamp system to ensure the sealing of the experimental device. The purpose was to achieve the shape of thin polymer membranes. The device was placed in an oven and treated according to the following thermal program: 2,5 h at 60 °C to complete polymerization and then one hour at 120 °C to achieve a post-curing. After polymerization, the cross linked polymer was detached from the device and vacuum dried at 60 °C [43].

Preparation of hybrid gels based on PEGDM and NAS

In the experiments for biofilms formation from two bacterial strains were used gels without NAS and with NAS synthesized in laboratory SATIE according the following procedure:

In 250ml two-necked flask in water ice bath (4 °C) were added 11,5 g NHC, 11 g TEA (three ethyl amine) and 10 g acryloylchloride. Further 150 ml chloforme was added drop wise using a dropping funnel during 20 minutes. After stirring the reaction mixture was washed three times with 80 ml cold water. The organic phase was dried over $MgSO_4$. After filtration it was added 50 mg hydrochinone. The solvent was removed to obtain 25 ml volume. The traces of hydrochinone were removed by filtration. Ethyl acetate and hexane 1.5ml/10ml respectively were added to the mixture. The aimed product was precipitated after several hours in ice bath conditions. The sediment (white precipitate) was recovered by washing with solution of hexane/ethyl acetate (9:1 V/V).

Two types of gels were synthesized based on PEGDM and HEA. 60.48 mg – 2-hydroxyethyl acrylate (HEA) and 10.27 mg poly(ethylene glycol)dimethacrylate (PEGDM) were dissolved in 1059.5µl H_2O. 79.75 mg TMOS was added to the mixture. 10% DMAPN and 7% PSS were used as gelling agents. To synthesize gel with NAS to the mixture was added 8.46 mg NAS.

Cell culture

Escherichia coli 52170 and *Pseudomonas fluorescens FFD16* were cultivated on solid agar nutrient medium containing yeast extract, peptone, glucose, NaCl at 30 °C for 24 h. After incubation the colonies were picked up and suspended in liquid nutrient medium containing LB and 10% glucose for 24h in bath shaker at 30 °C, pH 7. Further the cells were suspended in fresh liquid medium, containing LB and 10% glucose and the biomass was used for formation of biofilms onto different matrices.

Formation of biofilm

The obtained matrices were autoclaved for 20 min in 0.8 atm then placed in cell suspension with nutrient medium containing LB and 10% glucose to form biofilms with cell adhesion. The binding of cells was carried out at pH 7 and temperature 30 °C under continuous stirring in bath shaker (220 rpm). Biofilms formation was studied at 24, 72, and 120 h. Every 24 h the matrices were washed up by 0.9% NaCl and suspended in the fresh nutrient medium.

Methods

Biochemical analyses

The absorbance of the biomass of free cells and this produced by biofilms was measured at 590 nm with a "Biochrom, Libra S12", (Germany). The renovation of the biofilm was monitored microscopically as well as by means of the turbidity (OD-590) of the effluent.

The extracellular protein content attach to the matrices was measured using a modification of the Lowry method [46] according to Raunkjaer et al. [47]. The exopolysaccharide content was measured using the reaction with 3,5-dinitrosalicylic acid [48].

Thickness shear mode (TSM) resonators and Quartz Crystal Device (QCM)

It was observed the rheological properties of synthesized hybrid gels and formed biofilms by quartz crystal microbalance (QCM).

The experimental setup was presented in details according to *Ould Ehssein et al*. [19]. An AT-cut quartz crystal sensor resonating at 6 MHz was loaded on one side with the polymer gel. The other side was in contact with air. The polymer gel and the quartz were kept during the gelation at the desired temperature. The admittance of the quartz was measured within a 7 kHz band width near the resonance frequency using aHP4195A network analyzer. In order to observe the gelation process, the total admittance of the quartz was saved every 30s. The viscoelastic parameters are then computed online using an appropriate model developed in laboratory SATIE, University of Cergy Pontoise, Paris, France [19].

RESULTS AND DISCUSSION

The formation of biofilm from *Escherichia coli* and *Pseudomonas fluorescens* onto different polymer matrices has been shown by the determination of the extracellular polymeric substance (EPS) concentration. It was followed by measuring of extracellular proteins and exopolysaccharides present in the biofilm.

Initially, we started with investigation of biochemical properties of formed biofilms onto synthesized hybrid membranes. The dynamics of proteins and extracellular polysaccharides production by biofilms were studied.

Dynamics of extracellular proteins production from biofilms of Escherichia coli and Pseudomonas fluorescens onto different polymer matrices

After the culture was developed and a biomass was accumulated, the sterilized polymer carriers were added to the cell suspension for biofilms formation. The kinetics of proteins and polysaccharides produced by biofilms formed on four different types of polymer carriers based on NAGly/PEGDM in different ratio for 5 days was tracked and the 24th hour was assumed as initial period for the incubation. The quantity of proteins synthesized from biofilms of *Escherichia coli* and *Pseudomonas fluorescens* on four different types of matrices after 24, 72 and 120 h of incubation time is shown on Figures 1, 2 and 3. A biofilm is constituted of a mixture of polymeric compounds, primarily polysaccharides, generally referred to as EPS. These substances are characteristic of the formation of a biofilm formed by bacteria.

The figures show that a larger quantity of proteins is produced from biofilms formed on NAGly/PEGDM matrix in 50/50 wt% ratio of the components during the incubation time.

The concentration of proteins from biofilms increased proportionally to the quantity of NAGly in the matrices.

The figures also reveal that considering the produced proteins, the polymer matrix based on NAGly/PEGDM in 50/50wt% ratio is a better carrier for biofilm formation. The most quantity of proteins

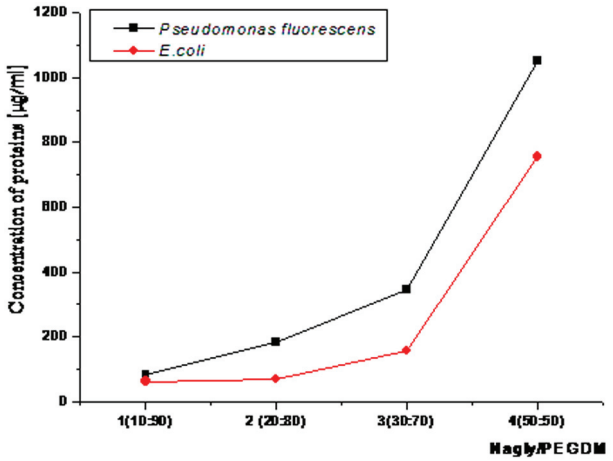

Fig. 1. Kinetics of proteins production from biofilms formed on NAGly/PEGDM matrices after 24 h

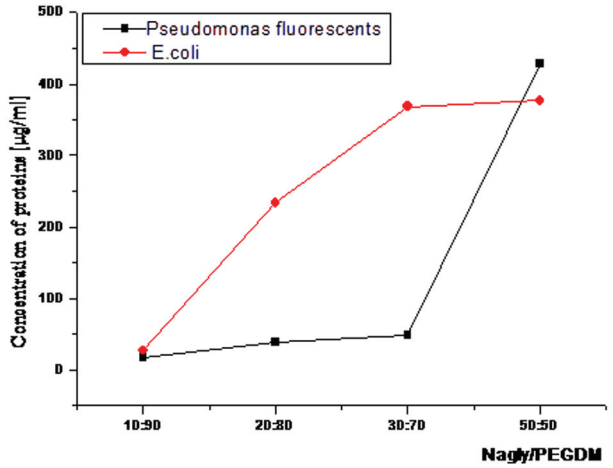

Fig. 2. Kinetics of proteins production from biofilms formed on NAGly/PEGDM matrices after 72 h

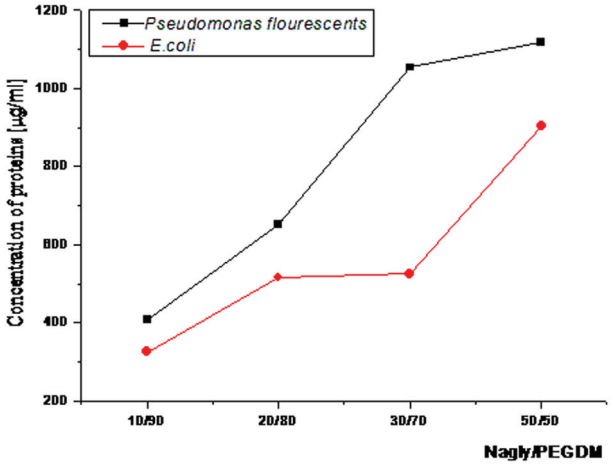

Fig. 3. Kinetics of proteins production from biofilms formed on NAGly/PEGDM matrices after 120

is produced of biofilm formed from *Pseudomonas fluorescents* cells.

Dynamics of extracellular polysaccharides production from biofilms of Pseudomonas fluorescents and Escherichia coli formed onto different polymer matrices

Kinetics of polysaccharides production from the formed biofilms is presented on Figures 4, 5 and 6.

The figures show that the quantity of polysaccharides produced from biofilms formed on a matrix of NAGly/PEGDM in 50/50 wt% ratio is higher.

It can be clearly observed that polysaccharides production of biofilms formed onto NAGly/PEGDM matrix is increased proportional of the concentration of NAGly in the matrices. These results can

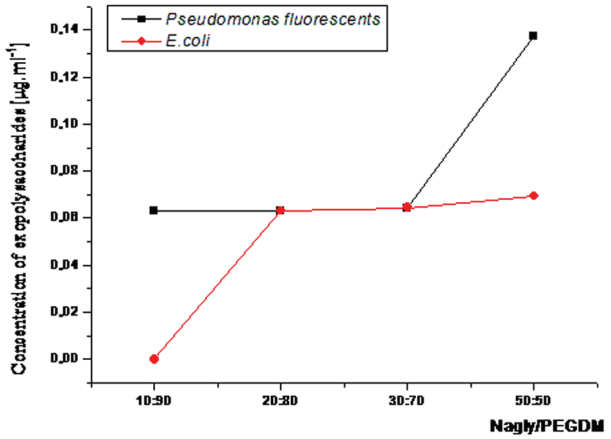

Fig. 4. Kinetics of the polysaccharides production from biofilms formed on NAGly/PEGDM matrix after 24 h

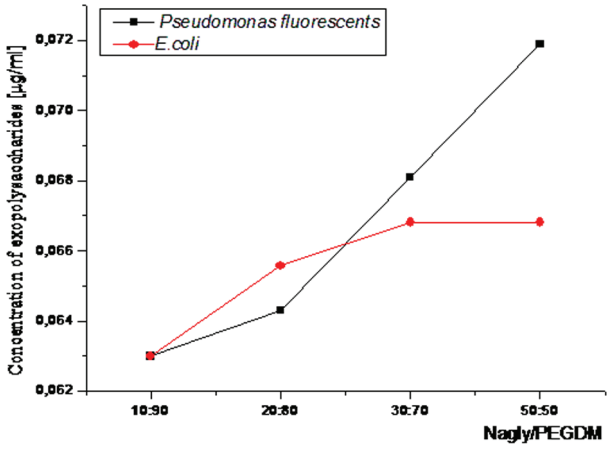

Fig. 5. Kinetics of the polysaccharides production from biofilms formed on NAGly/PEGDM matrix after 72 h

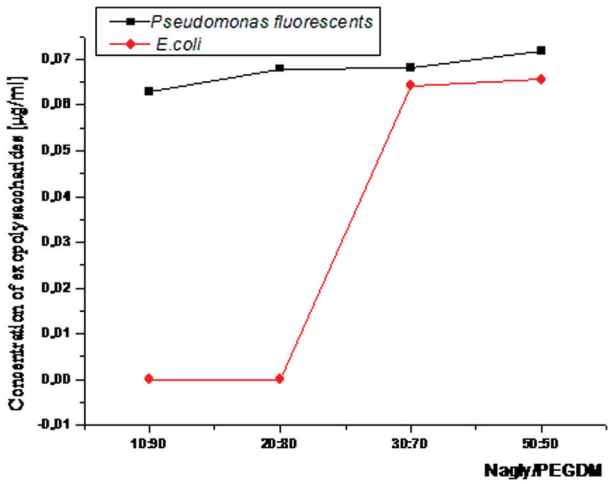

Fig. 6. Kinetics of the polysaccharides production from biofilms formed on NAGly/PEGDM matrix after 120 h

be explained by the preference of bacteria to form biofilms in the presence of higher concentration of NAGly. The production of extracellular substances is more significant for *Pseudomonas fluorescents* compared to *Escherichia coli.*

Comparing the proteins and polysaccharides production from biofilms on the four types of carriers we could conclude that the most appropriate matrix for biofilm formation is NAGly/PEGDM in 50/50 wt% ratio. The adhesion of bacterial cells is expected to be higher for conetworks with a large amount of NAGly due to the increase of interactions between glycine function and bacterial cells.

Visualization of biofilms by optical microscopy

Some representative images of the formation of biofilms from *Pseudomonas fluorescents* and *Escherichia coli* on NAGly/PEGDM (50/50 wt%) are shown in Figures 7 and 8 (a and b), respectively. The composition for NAGly/PEGDM (50/50 wt%) has been chosen to promote interactions between microorganisms and materials, as already explained. These images are representative of the whole sample. They demonstrate that the formed biofilm are concentrated on the surfaces of matrices based on NAGly and the growth is more efficient for Nagly/PEGDM (50/50wt%).

Microscopic images show that *Pseudomonas fluorescents* and *Escherichia coli* cells are visible in biofilms onto matrices uniformly onto surfaces. The microscopic analysis presents that the cells from *Pseudomonas fluorescents* are more onto the surface than cells of *Escherichia coli*.

Rheological properties of formed biofilms by QCM

The results obtained by QCM analysis of the starting organic materials based on gel HEA/TMOS

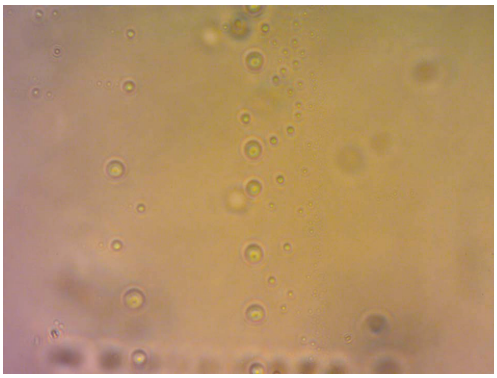

Fig. 7. Optical microscopy image of empty matrix from NAGly/PEGDM (50/50wt%), magnification 100x(oil)

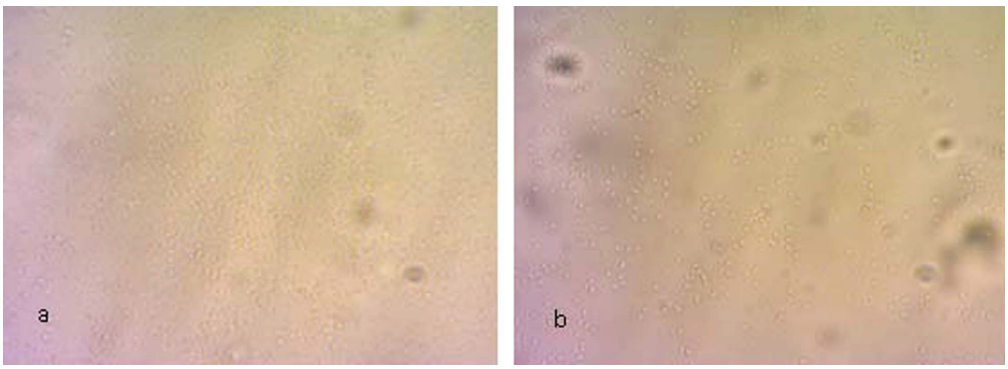

Fig. 8. Optical microscopic image of biofilm after 5 days from *Pseudomonas fluorescents* (a) and *Escherichia coli* (b) onto matrix NAGly/PEGDM (50/50wt%), magnification 100x(oil)

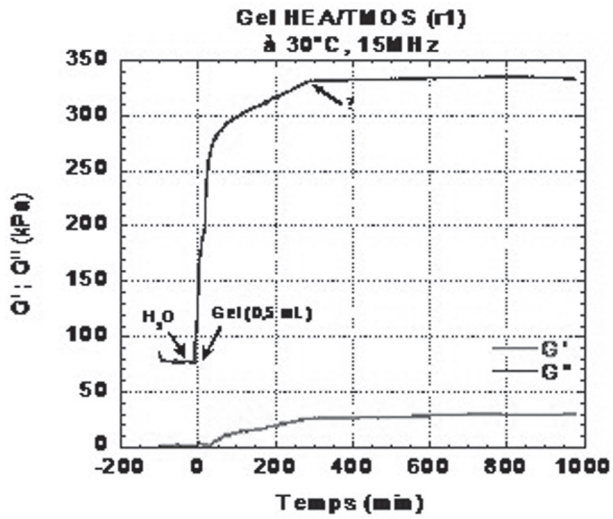

Fig. 9. Rheological properties of formed gels without microorganisms

and rheological properties of the formed biofilms are shown on the next figures. Figure 9 shows that the starting mixture presents a low elasticity evolution (G' is almost constant with time) but the viscosity (G") increases with time due to the incorporation of two parts of materials.

The QCM analyses (Figure 10 a and b) show that the viscosity of the formed biofilms from *E.coli* onto the gel without NAS, is lower than the biofilms formed from *Pseudomonas fluorescents* cells. The elasticity is higher in biofilm formed from *E. coli* cells than *Pseudomonas fluorescents* biofilm.

Concerning the viscosity of the biofilm from *E. coli* cells formed onto gel with NAS is significantly higher comparing with the other biofilm viscosity. In this case the elasticity profiles are the same for both biofilms.

According to the rheological properties it can be concluded that both biofilms possess more defined viscosity properties than elastic. This can be explained by viscose properties of extracellular polymeric substances (EPS) of formed biofilms.

CONCLUSIONS

Our study reveals that it is possible to preserve microbial adhesion of *Pseudomonas fluorescents* and *E.coli* in newly obtained matrices. Additionally, the obtained results show that the most appropriate carrier for biofilm formation is the matrix based on NAGly/PEGDM in 50/50 wt% for both bacterial strains. Finally our results from rheological properties reveal that the matrices obtained by gel with and without NAS are also appropriate for biofilm formation.

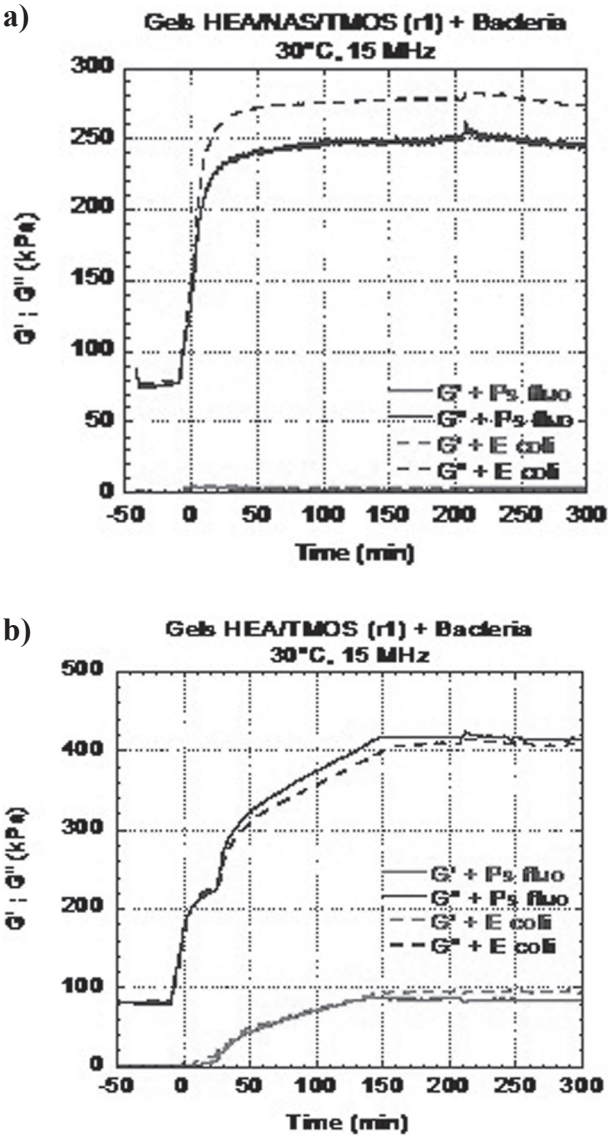

Fig. 10. Rheological properties of biofilms formed from *E.coli* and *Pseudomonas fluorescents* onto two kinds of gels (a-gel with NAS) and (b-gel without NAS)

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ИЗСЛЕДВАНИЯ ВЪРХУ ПРОЛИФЕРАЦИЯТА НА ГРАМ-ОТРИЦАТЕЛНИ БАКТЕРИАЛНИ КЛЕТКИ ВЪРХУ ЗОЛ-ГЕЛНИ ХИБРИДНИ НОСИТЕЛИ

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(Резюме)

Представената статия е свързана със синтез и характеристика на нови хибридни зол-гелни материали. Приготвени са няколко носителя на основата на поли (N-акрилоилглицин) (NAGly), съдържащи поли (етилен гликол) диметакрилат (PEGDM). Чрез биохимични методи е изследвано формирането на биофилми от клетки на щамовете *Escherichia coli* и *Pseudomonas fluorescents* върху синтезираните матрици.

Синтезирани са два вида хибридни гелове на основата на 2-хидроксиетил акрилат и поли (етилен гликол) диметакрилат с инкорпорирането на неорганични прекурсори. Чрез кварцово-кристална микро везна са изследвани реологичните свойства на получените гелове и формираните биофилми.

Експерименталните резултати показаха, че получените мнатрици са подходящи за формиране на биофилми.