JOURNAL OF Applied Polymer SCIENCE

Design of Poly(*N*-acryloylglycine) Materials for Incorporation of Microorganisms

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ABSTRACT: To incorporate microorganisms and to preserve their integrity, new matrices of poly(*N*-acryloylglycine) have been designed under appropriate conditions. To understand the interactions between the microorganisms and the organic part of the matrices, different conetworks of poly(*N*-acryloylglycine) have been synthesized and characterized. Copolymerization with two crosslinkers was performed with different compositions. The thermal and swelling properties of conetworks are specifically controlled and compared. These investigations show that the swelling ratio of these materials is compatible with the incorporation of biomolecules in these matrices. They successfully permit *Pseudomonas species 1625* bacteria incorporation. The biological activity of bacteria is also preserved, allowing the use of these materials for innovative biological applications. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2013

KEYWORDS: biomedical applications; crosslinking; films; biocompatibility; microscopy

Received 26 November 2012; accepted 25 February 2013; published online
DOI: 10.1002/app.39242

INTRODUCTION

The use of biomolecules (BMs) embedded in organic materials has strongly increased for innovative applications like bacterial detection for biosensor. In some industries such as cosmetic[1] and pharmacology,[2] incorporation of BM has become an original research topic with a wide field of applications. The assessment of water quality to detect traces of chemical or biological pollution[3] is one. To increase the efficiency of these tests, it is important to carry out early detection of the different BMs involved. Indeed, these pollutants can be hazardous for health and they can also cause a chemical or bacterial corrosion occasioning the degradation of storage equipment. One can note the interest of the early detection of bacteria such as *Escherichia coli* (*E. coli*) involved in pathogenic human diseases such as gastroenteritis, urinary tract infections, or meningitis[4–6] or *Pseudomonas aeruginosa* presents in septicemia or nosocomial infections.[7] Biosensors are particularly well adapted to detect this type of biological entities in a complex environment. The combination of a biological recognition element with a transduction element[8] leads to a selective and quantitative or semi-quantitative analysis system.[9–12] A major challenge of these sensors is the implementation for *in situ* and real-time detection of bacteria in aqueous media. One of the technological barriers in the manufacturing of these sensors is the immobilization of BM on their surface.[13,14] In most cases, a matrix is used to optimize the linkage of the biological entities on the sensor.[15] Lee et al.[16] have also reported the elaboration of optical biosensor for *E. coli* detection based on tetraethyl orthosilicate and mercaptopropyl triethoxysilane (MPS). Furthermore, Liang et al.[17] have reported the formation of an amperometric biosensor for the hepatitis B virus detection based on MPS. Most materials made from acrylamide appear as hydrogels, showing a particularly high swelling ratio.[18,19] In this context, such materials present an interest for the anchorage of BM in matrices. In this article, the hydrophilicity of the host material has been enhanced. To optimize the network's formation, a tight control of the interactions between the organic network and the BM has to be performed.[20–22] To achieve this, an acrylamide with hydrophilic function can be crosslinked to obtain a copolymer, material that can be conveniently handled. This pathway is particularly interesting: on one hand, it increases the mechanical properties of the material and on the other hand it prevents dissolution of the polymer. It should also reduce undesirable swelling. For example, poly(2-acrylamido-2-methyl-1-propanesulfonic acid) crosslinked with

Additional Supporting Information may be found in the online version of this article.

N,N-methylenebisacrylamide (BIS) forms a hydrogel with a very high swelling behavior.[23,24] This study is devoted to the synthesis and characterization of new three-dimensional conetworks based on purely organic *N*-acryloylglycine (NAGly). Through its glycine function, we supposed that NAGly can form Van der Waals interactions with amino acids constituting the BM. Therefore, we can assume that this type of interaction will enhance the biocompatibility of the host matrices. These conetworks must have hydrophilic, thermal, and biocompatible properties to permit the incorporation of bioactive entities. We work on the hydrophilic nature of poly(*N*-acryloylglycine) by modifying the ratio between the monomer and the crosslinker used for the copolymerization. The conetworks were realized with two different crosslinker. The first one is more hydrophilic than the second one but it limits the formation of large pore. The hydrophilic and thermal properties of the three-dimensional networks have, therefore, been studied. To investigate the incorporation and viability of BM in these matrices, *Pseudomonas species 1625* bacteria is used as model. The influence of the composition of the conetworks was also studied for this model.

EXPERIMENTAL

Experimental Part

Reactants. 2,2′-Azobis(2-methyl proponitrile) (AIBN, 98%) and glycine (98%) have been provided by Acros Organics. Poly(ethylene glycol) dimethacrylate (PEGDM, M_n = 550 g/mol) and acryloyl chloride (98%) were purchased from Aldrich. BIS (99%) and *N,N*-dimethylacetamide (DMAc, 99.5%) have been provided by Sigma-Aldrich. All products have been used without any further purification.

Preparation of NAGly. To increase yield, NAGly is synthesized by a Schotten–Baumann reaction in aqueous phase and prepared by a different method to that described by Bentolila et al.[25] NAGly is synthesized by a Schotten–Baumann reaction in aqueous phase. Typically, 4.50 g of glycine (60 mmol) was dissolved in 60 mL of a solution of potassium hydroxide (2*M*). The mixture is cooled at 0°C with ice water bath for about 10 min. In breif, 6 mL of acryloyl chloride (73.6 mmol) was added dropwise to the mixture using a dropping funnel. After addition, the mixture is stirred for 90 min at 0°C and then again 90 min at room temperature. The solution is then washed with diethyl ether (two times with 40 mL) and the aqueous phase is acidified to the pH value of 2. The product is extracted with ethyl acetate (three times with 40 mL). After drying the organic phase over $MgSO_4$, the residue is concentrated with a rotary evaporator. The yield of this synthesis is 74%. mp 130°C (lit. 132°C); ^{1}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}.

Preparation of Conetworks Based on Poly(N-acryloylglycine). All conetworks were synthesized according to the same pathway.

The overall composition of the networks was varied from 90 to 10 wt % of each compound. All investigated conetworks are reported as poly(*N*-acryloylglycine) (PNAGly)/PEGDM (*x*/*y*) or PNAGly/BIS (*x*/*y*). The numbers between brackets (*x*/*y*) correspond to the PNAGly and crosslinker weight proportions, respectively. For example, a conetwork obtained from a mixture of 450 mg of NAGly and 50 mg of PEGDM will be noted PNAGly/PEGDM (90/10). The synthesis of conetwork is performed in a flask containing *x* wt % of NAGly, *y* wt % of crosslinker and 600 μL of DMAc. The mixture is stirred and then degassed to remove all traces of oxygen (radical inhibitor). Finally, 25 mg of AIBN is added at the last moment to avoid the rapid decomposition of the initiator. The contents of the flask are 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 device is placed in an oven and baked according to the following thermal program: 2.5 h at 60°C for complete polymerization and then 1 h at 120°C to achieve post-curing. After polymerization, the crosslinked polymer is detached from the device and vacuum dried at 60°C.

Incorporation and Growth of Pseudomonas. *Pseudomonas species 1625* (*P. 1625*) microbial strain from the National collection for industrial and cell cultures (NBIMCC; Bulgaria) were grown from a glycerol stock solution on solid agar medium for 24 h at 30°C. After this incubation, colonies were picked up and suspended in liquid nutrient medium at pH of 7.0 (yeast extract, 14 g/L; potassium aspartate, 15 g/L; KNO_3, 8 g/L; $MnSO_4$, 0.025 g/L; $FeCl_3{\cdot}6H_2O$, 0.060 g/L; $(NH_4)_6MoO_{24}{\cdot}4H_2O$, 0.025 g/L) and then supplemented with 10% of glucose. The culture of free cells was done under agitation at 28°C for 24 h. In total, 150 mg of each matrix was autoclaved for 20 min in 0.8 atm, then placed in the cell suspension with nutrient medium to form the biofilms by cell adhesion.

Methods of Characterization

Spectroscopic Analyses of NAGly. The product is characterized by nuclear magnetic resonance (NMR) spectroscopy ^{1}H and IR spectroscopy. The NMR spectrum is performed on a Brucker AC250 spectrometer at 250 MHz using DMSO as solvent. The IR measurement is carried out on a Bruker spectrometer transmittance mode between 4000 and 600 cm^{-1} with a resolution of 2 cm^{-1}.

Characterizations of Conetworks

Soluble fraction in the final material. To determine the proportion of impurities (monomers, oligomers, initiator, and solvent) present in the network and consequently ensure the good crosslinking, a known mass of polymer is washed using a Soxhlet extractor with dichloromethane for 3 days. After extraction, the film is placed under vacuum at 60°C for 2 days to remove the dichloromethane. The extracted content (EC) is given as follows:

$$\mathrm{EC}(\%) = \frac{W_0 - W_E}{W_0} \times 100$$

where W_0 and W_E are the weights of samples before and after extraction, respectively.

Swelling behavior. The hydrophilic nature of the materials is determined by the measurements of swelling in water. To achieve these measurements, a dry sample of a known mass W_0

is immersed in water at 25°C with a pH of 7. The variation of mass versus time is measured. The following formula gives access to the swelling ratio SR (%):

$$SR(\%) = \frac{W - W_0}{W_0} \times 100$$

where W_0 and W are the sample weights before and after soaking in water, respectively.

Thermogravimetric analysis. Thermal properties (thermal resistance, purity) are studied by thermogravimetric analysis (TGA). TGA measurements were carried out using a TGA Q50 model (TA Instruments). Samples were heated at 20°C/min from ambient temperature to 600°C under air (slope, 10°C/min).

Scanning Electron Microscopy. Scanning electron microscopy (SEM) images of the materials surfaces were obtained in a Jeol JSM-6300 microscope, with the samples previously sputter-coated under vacuum with gold, 15 kV of acceleration voltage, and 15 mm of distance working.

Biochemical Analyses. The extracellular proteins content attached to the matrices were measured using a modified Lowry method,[26] as described by Raunkjær et al.[27] The exopolysaccharide content was measured using the anthrone method,[28] as modified by Raunkjær et al.[27] to eliminate the effect of a non-anthrone-specific color development.

RESULTS AND DISCUSSION

Synthesis of NAGly

NAGly was prepared in good yield by a method adapted from Bentolila et al.[25] (Figure 1). The FTIR spectrum (Supporting Information Figure S1) of NAGly displayed a peak at 3350 cm^{-1}, assigned to amine bond stretching. Peaks at 1650 and 1530 cm^{-1} were assigned to CO bending and stretching for the amide, whereas signals owing to CO for acid stretching appeared at 1740 cm^{-1}. Finally, the spectrum displayed peaks of vinyl group at 1600 cm^{-1}. The ^1H NMR spectrum (Supporting Information Figure S2) for NAGly showed characteristic vinylic proton signals at 5.67 (dd), 6.08 (dd), and 6.25 (dd) ppm, together with a signal at 3.95 (s) ppm that was assigned to the methylene group of glycine. NAGly did not respond to ninhydrin or picrylsulfonic reagents, indicating the absence of a free amino group.

Formation of Conetworks

The conetworks were synthesized by free-radical copolymerization of NAGly with PEGDM or BIS as crosslinker. This crosslinker is chosen to obtain a different chain length with various

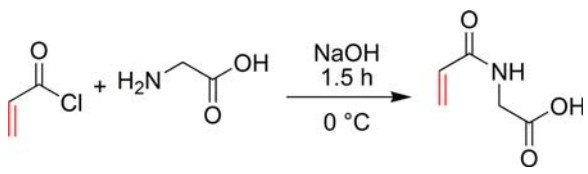

Figure 1. Synthesis of NAGly. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

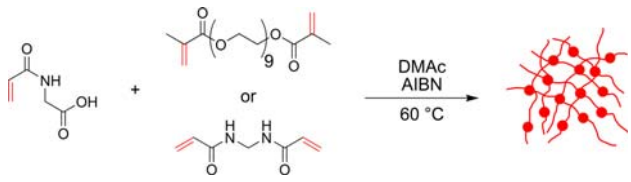

Figure 2. Conetwork formation for homopolymers based on two different crosslinkers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

molecular weights (Figure 2). The copolymerization was initiated by radicals generated at 60°C by AIBN as thermal initiator. The copolymerization reaction carried out in DMAc as common solvent (NAGly, 0.25 mL/g). Indeed, water cannot be used because, contrary to NAGly, PEGDM is not soluble enough in this solvent. The overall PNAGly/crosslinker composition of the conetworks was varied from 10 to 90 wt % of each partner amount. To check that NAGly and the crosslinker are quantitatively copolymerized and crosslinked, the extractible products contained in the conetworks were measured in dichloromethane. The extracted content after this stage shows a good crosslinking of each network regardless of the composition used for the synthesis (<12%). ^1H NMR analyses of the dichloromethane solution fraction confirm the presence of oligomers from NAGly and show a small quantity of DMAc used for the polymerization. The different PNAGly conetworks should thus be considered as correctly crosslinked.

Swelling Behavior

On the one hand, the water content of a hydrophilic material strongly affects its mechanical properties and dimensional stability. On the other hand, the water content promotes the incorporation of BM in the matrices. The hydrophilic property of the present conetworks is ensured by the lateral position, favoring the formation of intermolecular hydrogen bonds between acid group of PNAGly and the solvent. Figure 3 shows the swelling measurements carried out at 25°C for the different conetworks. The networks PNAGly/PEGDM (90/10), (75/25), (50/50), (25/75), and (10/90) show 180, 125, 90, 70, and 55% of swelling ratios, respectively, when soaked in water for 1 h. The swelling ratios increase with NAGly weight content in the conetwork series and reach a plateau corresponding to the SR. It is equal to 350, 250, 160, and 90% for PNAGly/BIS (90/10), (75/25), (50/50), and (10/90) conetworks, respectively, under the same conditions.

Molecular modeling force fields (MM2 field) can explain this result by the conformation of the carboxyl group in the polymeric chain. The lone pairs on the oxygen atom of the alcohol group are placed so as to form intramolecular bonds with the amido groups of the main chain. Consequently, the lone pairs become less available to form intermolecular bonds with the solvent. The swelling ratio for PNAGly/PEGDM (x/y) is about twice lower than that of a PNAGly/BIS (x/y) network with the same composition. In addition, if one looks at the effect of the crosslinker on the swelling ratio for a given PNAGly amount, it is perceptible. In fact, the amide functions of BIS form intermolecular hydrogen bonds with water. Thus, the swelling ratio is

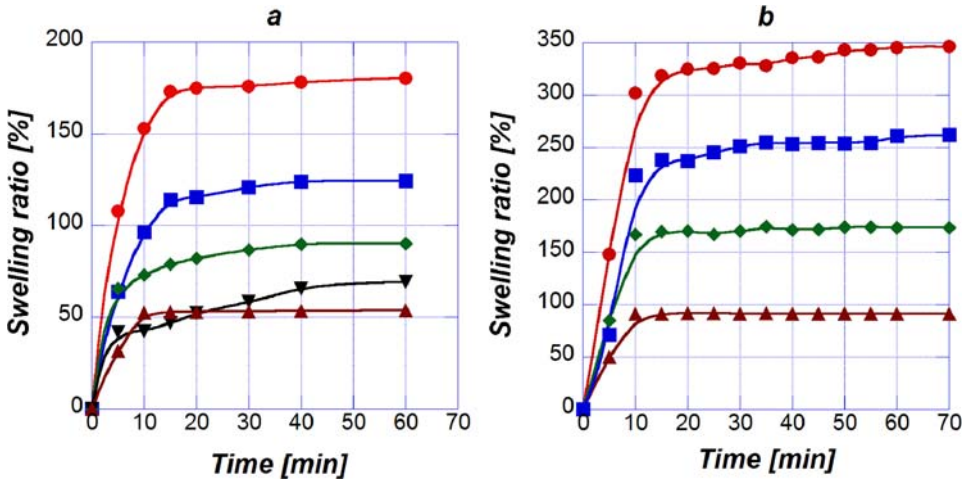

Figure 3. Swelling ratio versus time for different PNAGly contents (●: 90/10; ■: 75/25; ◆: 50/50; ▼: 25/75; ▲: 10/90). (a) PNAGly/PEGDM conetworks and (b) PNAGly/BIS conetworks. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mainly governed by the PNAGly content especially when it is higher than 25 wt %. For these networks, the swelling ratio is sufficient to develop the incorporation of BM while maintaining the physical integrity of the material. Consequently, a too high degree of crosslinking will reduce the range of use of materials (too low SR). The 90/10-ratio copolymer seems to be a good compromise between a reasonable SR and a good stability of the material in aqueous media. In all case, the swelling curves present a plateau that indicates the stability of the different networks after the swelling effect. The structural integrity of matrices is sufficient after swelling in water for the use of these networks as biosensors.

Thermal Behavior

Figure 4 shows the TGA spectra of PNAGly/PEGDM conetworks containing from 10 to 90 NAGly wt %. Single PEGDM 550 network is stable at temperature up to 350°C and its decomposition proceeds in a single step, whereas PNAGly/PEGDM conetworks are characterized by different TG profiles. A first weight loss is observed between room temperature and 150°C and it is assigned to the evaporation of moisture and solvent (≈8%) used in the synthesis and still present after the purification step. This value is in agreement with the extractable rate determined after washing. A second decomposition region, beginning at 250°C and ending at about 450°C is noticeable and owing to the decomposition of the polyamide structure of PNAGly in ketone and nitrile derivative. A third weight loss from 450 to 600°C is attributed to the decomposition of the PEGDM segments and the calcination of the organic part of the conetwork. By understating the effects related to this last degradation, the amount of NAGly presents into material can be calculated from the second weight loss. Results are summarized in Table I. The weight ratios deduced are in agreement with the initial weight ratio of the monomers used for the synthesis. Furthermore, the temperature of decomposition is high enough to consider future applications of the copolymer for BM incorporation.

Incorporation of *Pseudomonas*

The choice of the location of the BM in a biosensor depends on the desired application. When the biological response of the

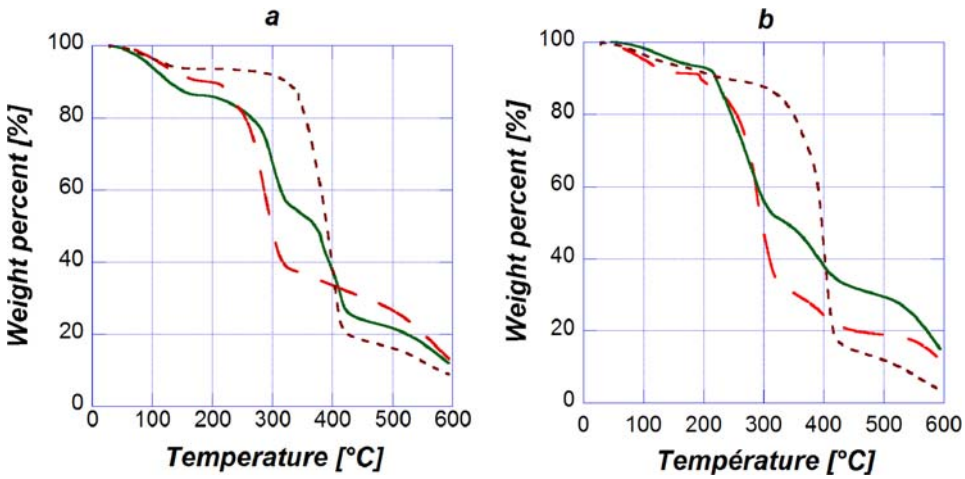

Figure 4. Weight percent versus temperature. (a) PNAGly/PEGDM conetworks, (b) PNAGly/BIS conetworks for different weight ratios (– –: 90/10; ——:50/50; - -:10/90). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. NAGly Weight Ratio Used (Use) and Calculated (Cal) of the Different Conetworks

Sample (x/y)	NAGly (wt %; use)	NAGly for PEGDM (wt %; cal)	NAGly for BIS (wt %; cal)
90/10	90	-[a]	92
50/50	50	55	48
10/90	10	7	9

[a]The value for this matrix could not be determined.

sensor is slow, the penetration of organism is preferred, whereas if it is rapid, the BMs are placed on the surface of biosensor.[29] Depending on the sensibility of the biosensor, a volume incorporation of BM will be more sensitive than a surface interaction. In this article, the penetration of *Pseudomonas* has been chosen to prove the ability of our new materials to encapsulate the BM. Some representative images of the formation of biofilms from *Pseudomonas* species on PNAGly/PEGDM (90/10) and PNAGly/BIS (90/10) at two different magnifications are shown in Figures 5 and 6, respectively. For both crosslinkers, the high composition for NAGly (90 wt %) has been chosen to promote interactions between BM and materials, as already explained. These images are representative of the whole sample. They demonstrate that the formed *Pseudomonas* species biofilm are concentrated in the pore interface for all conetworks based on PNAGly and the growth is more efficient for PEGDM than BIS. A biofilm is constituted of a mixture of polymeric compounds, primarily polysaccharides, generally referred to as extracellular polymeric substance (EPS). These substances are characteristic of the formation of a biofilm form by bacteria. The proliferation of *Pseudomonas* species has been shown by the determination of the EPS concentration. They have been followed by measuring extracellular protein and exopolysaccharide present in the biofilm. Figure 7(a,b) shows that substances produced by *Pseudomonas* anchored in the two types of matrices. In both cases, an increase of the two substances is observed, showing that the host matrices avoid the growth and preserve the biological activity of the bacteria. However, the production of extracellular substances is more significant for PNAGly/PEGDM than for PNAGly/BIS (1.5 times greater for PEGDM).

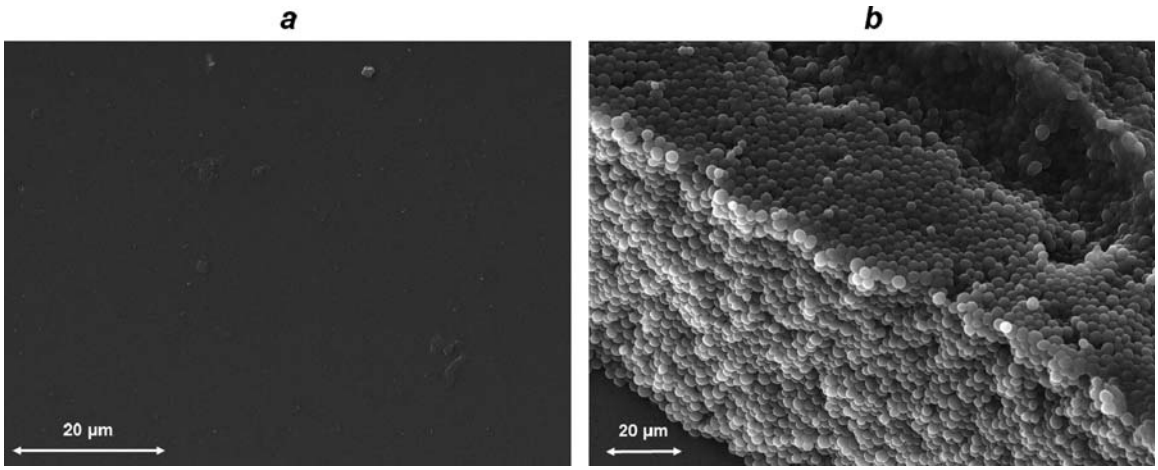

Figure 5. SEM images of the surfaces of PNAGly/PEGDM (90/10) sample without (a) and with (b) *Pseudomonas*.

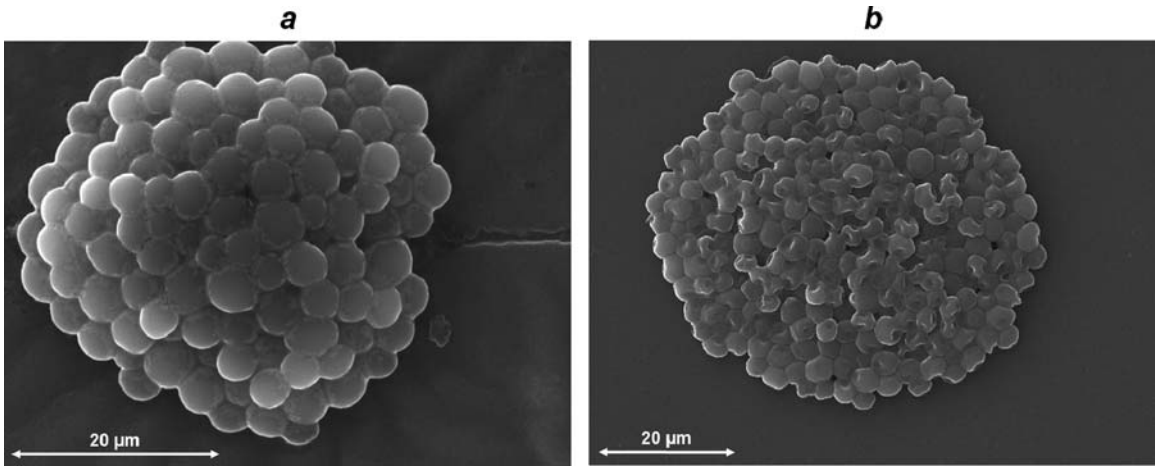

Figure 6. SEM images of the surfaces of the PNAGly sample with PEGDM (a) or BIS (b).

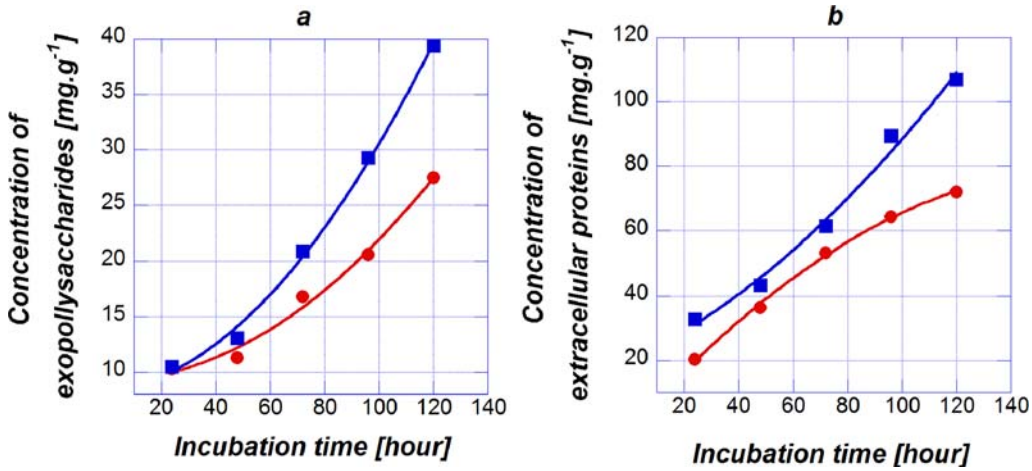

Figure 7. Concentration of exopolysaccharide (a) and extracellular proteins (b), produced by biofilms formed onto PNAGly/PEGDM (■) and PNAGly/BIS (●) 90/10 conetworks. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

These results can be explained by the preference of bacteria to form biofilm in the presence of amino acid function of the PNAGly. The *Pseudomonas* species incorporated via PNAGly/PEGDM are more numerous (on a micrometer scale) than the cells attached in PNAGly/BIS conetwork. Intuitively, the encapsulation of the *Pseudomonas* species is expected to be higher for conetworks with a large amount of NAGly (owing to the increase of interactions between glycine function and BM). However, experimental results suggest that for our sample the NAGly is not the only factor responsible for the incorporation of *Pseudomonas* species. The nature of the crosslinker (chain length/hydrophobicity) also plays an important role. The different results revealed that the incorporation is better for PEGDM than BIS. This study shows the influence of two parameters of the crosslinker: the length and the hydrophilicity. The swelling in water can be considered as the incorporation of small molecule in the material. Therefore, it appears logical that the best swelling is obtained for the most hydrophilic crosslinker (350 vs. 180%). When incorporating larger entities such as *Pseudomonas*, the spacer length becomes a more important factor. This fact can explain the best incorporation of the BM in PNAGly/PEGDM matrices. We supposed that the porosity may be responsible for the best incorporation and activity enhancements of the *Pseudomonas*.

CONCLUSIONS

This article describes the study of new matrices based on NAGly to incorporate BM. We have shown that it was possible to preserve the biological integrity of a living organism (*Pseudomonas*) in these matrices. Indeed, bacteria are able to be immobilized and to be grown on the matrices. The different properties of these materials have permitted a better understanding of the relationship between chemical structure and biological integrity of the network. The best results were obtained for a highly porous matrix, resulting from the copolymerization between NAGly and PEGDM-like organic crosslinker.

This study shows that it can be possible to use these materials for the design of a new *in situ* and real-time biosensor. To optimize these materials, the mechanical properties of these materials can be enhanced by adding an inorganic component. The use of sol–gel process using ORganically MOdified Silica (ORMOSILS®) can therefore be envisaged. Furthermore, the incorporation of BE in these matrices can be improved by changing the nature of organic or inorganic precursors (owing to the stability of the Si—C bond in ORMOSILS®).

ACKNOWLEDGMENTS

Authors thank Arnaud Brosseau from the *Laboratoire de Photophysique et Photochimie Supramoléculaires et Macromoléculaires* (PPSM) for conducting the thermal analysis of these materials and Rila Project 01/11 with NSF of Bulgaria.

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