SYNTHESIS OF N-(3-NITROBENZOYL)-GLUTAMIC ACID AND ITS IMMOBILIZATION BY ESTERIC BOND ON GELLAN

Daniela Mihaela DUMITRAŞ,[*a] Marcel POPA[a] and Valeriu ŞUNEL[b]

[a] Technical University "Gh. Asachi" of Iaşi, Department of Macromolecules, Bd. D. Mangeron 71 A, 700050 Iaşi, Roumania
[b] University "Al. I. Cuza" of Iaşi, Department of Organic Chemistry, Bd. Copou 11, 700506, Iaşi, Roumania

The paper studies N-(*m*-nitrobenzoyl)-L- glutamic acid (NBGA) synthesis by acylation of glutamic acid with *m*-nitrobenzoyl chloride and subsequent immobilization of the product by esteric bond on gellan, in conditions of activation with dicyclohexyl carbodiimide (DCCI). Based on a centered, rotatory, composed, second order experimental program, the regression equation describing the dependence of the amount of NBGA chemically bonded to the support, upon the reaction's parameters (active principle/support ratio, activator/active principle ratio and process duration) was obtained. The efficiency of the immobilization reaction gets maximal after 34 hours, for a DCCI/NBGA ratio of 1.34 and for maximal activator/active principle ratio. NBGA and the immobilization product have been studied by elemental analysis and FT –IR ^{1}H-NMR spectroscopy. It has been studied the active principle's release from the immobilization product, by acidic and basic hydrolysis, which proves the fact that reaction takes place according to a "zero order" kinetics, fact which confirms the biologically active product's controlled/sustained release capacity.

INTRODUCTION

The acylation of aminoacids with *m*- and *p*-nitrobenzoyl chlorides performed in order to obtain new biological actives compounds is a well known process. For example, L-asparagic acid and its acylated derivatives with *m*- or *p*- nitrobenzoyl radicals have a remarkable biological activity.[1-4]

Glutamic acid is a natural dicarboxylic aminoacid, having, alone or in his derivatives, valuable antimetabolitic, antimicrobial and antifungical properties. Also, this compound is considered to be useful for the future treatment of neurological conditions, ulcers, hypoglycemic coma, muscular dystrophy, epilepsy, Parkinson's disease and mental retardation.

Considering this we have proposed to improve the active principle 's chemo-therapeutic properties by means of its immobilization to macromolecular supports, especially of polysaccharidic nature ones.[5]

By condensing the L- glutamic acid with *m*-nitrobenzoyl chloride it was provided some protection for the amino- group and then the product has been immobilized by esteric bonds on gellan. The esteric bond is easily hydrolyzed in human body's digestive tractus, ensuring the controlled release of the active principle.

The paper presents the results concerning the synthesis and the description of the aminoacid derivative, as well as of the esterification product with gellan, showing their influence provided by them upon an amount of immobilized biological active product in the coupling product.

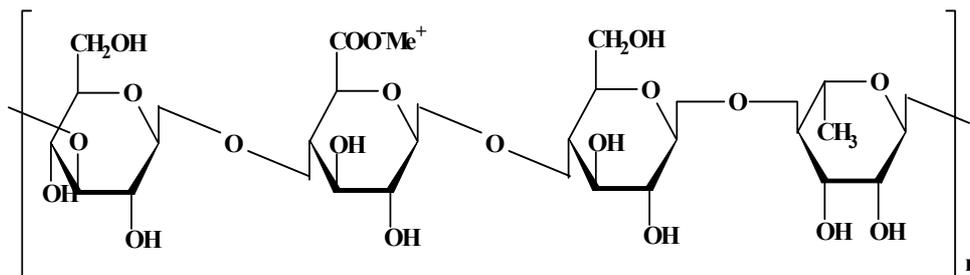## EXPERIMENTAL PART

Materials

L-Glutamic acid, from Merck.
m-Nitrobenzoyl chloride, from Merck.
Dicyclohexyl carbodiimide (DCCI), from Merck.
Gellan- provided by KELCOGEL Company, is a polysaccharide obtained from a microbial culture[10], with the following formula:

---

* Corresponding author: E-mail: danielamdumitras@yahoo.com

Gellan is recommended as being a suitable functional material, because of its excellent properties such as biocompatibility, biodegradability and non-toxicity.[6-8]

Gellan gum is an exocellular heteropolysaccharide, discovered by the screening of thousands of bacteria and prepared commercially by aerobic submerged fermentation from *Sphingomonas* elodea;[9] it is used as gelling agent in: disintegrable tablets and capsules, adhesive mucosal delivery, transdermal delivery, film coating, controlled release tablets, etc. It is a linear tetrasaccharide 4-L-rhamnopyranosyl-(α-1 $\rightarrow$ 3)-D-glucopyranosyl-(β-1 $\rightarrow$ 4)-D-glucuronopyranosyl-(β-1 $\rightarrow$ 4)-D-glucopyranosyl-(β-1$\rightarrow$ with O(2) L- glyceryl and O(6) acetyl substituents on the 3-linked glucose.

Methods

Synthesis of N-(m-nitrobenzoyl) –L–glutamic acid (NBGA)

In a 2 L reactor equipped with stirrer and a cooling system are to be introduced 250 mL of distilled water, 10 g (0.068 mol) of L-glutamic acid and 46 g (0.55mol) of $NaHCO_3$. Mixture is to be stirred until all components convert in solution, the reactant mixture is then cooled at 10^0C and then is added, by dropping, a solution made of 12,44 g (0.08 mol) m-nitrobenzoyl chloride in 80 mL anhydrous benzene. The stirring is continued for another 60 minutes, maintaining the constant temperature. The mixture is vacuum filtered and the benzene layer is then removed (after approximately 20-30 minutes) with a separation funnel. The aqueous layer is acidified with 37% HCl, stirring until pH=1.5, when a white precipitate separates. Vacuum filtration is performed and after hot water purification purifying, the dried product is weighted and characterized by elemental analysis (Kjeldhal method), melting point, FT-IR and ^{1}H-NMR spectroscopy.

Immobilization of NBGA on gellan

According to the experimental program, 0,5 g NBGA is to be dissolved in 5 mL DMSO under stirring, and then gellan is added (according to the amounts listed within the experimental program), stirring being continued for 15 minutes, as to provide the polysaccharide's swelling and the L-glutamic acid derivative's diffusion towards the support.

Apart, the activator (DCCI) solution is prepared by dissolving the amount stated in the experimental program in 2 mL DMSO. DCCI solution is added over the support and NBGA solution; this is the reaction zero moment. The mixture is maintained under stirring at a temperature of 18^0C, on a period defined within the experimental program.

After the reaction's end, the product is precipitated with 10 mL acetone, under vigorous stirring for 10 minutes. In order to provide a first wash, the obtained suspension is to be centrifuged for 5 minutes at 5000 rpm.; afterwards the supernatant is removed. For the second and third wash, another 10mL acetone are to be added and the first wash's operations are to be repeated.

At the end, 25 mL acetone are to be added over the product and stirring has to be provided until the next day. Then the immobilization product is filtered and dried under high vacuum.

Experimental program

Preliminary studies concerning the drugs immobilization using different polysaccharidic supports,[11] as xanthan[12,13] or gellan[14] have shown that the efficiency of the immobilization reaction is influenced by several factors. For the present system there have been selected, as being of crucial influence, the following parameters: the activator/active principle ratio, the active principle/support ratio and the process' duration.

In order to gather information upon the manner in which immobilization's efficiency (expressed by the chemically bond amount of NBGA) is controlled by these factors, there has been used an experimental centered, rotatory, composed, second order program, which considerably reduced the number of experiments and finally, made possible the full optimization of the process.

This program provided the codification of variables in order to facilitate the results' processing. The studied variables and their codification are presented in Table 1.

The equation used to describe the dependence of the amount of immobilized NBGA (Y,%) upon the considered parameters has the form shown below:

$$Y = a_0 + \sum a_i x_i + \sum a_{ij} x_i x_j \quad \text{cu } i \leq j$$

where: a_0 – free term
a_i, a_{ij} – regression coefficients
x_i, x_j – variables expressing the process parameters.

Table 1

Codification of variables and their variation domain

Real variable	Codified variable				
	-1.682	-1	0	1	1.682
DCCI/NBGA mol/mol –x_1	1.1	1.18	1.3	1.42	1.5
NBGA/Gellan mol/mol –x_2	2	2.6	3.5	4.39	5
Time (h) – x_3	8	14.5	24	33.5	40

The experimental results listed in Table 2, have been processed by the multiple regression method.

Table 2

Experimental values: the content of immobilized NBGA

Nr. crt	X_1	X_2	X_3	% N	% NBGA immob.
1	-1	-1	-1	4.65	49.18
2	+1	-1	-1	5.60	59.80
3	-1	1	-1	5.41	57.19
4	+1	1	-1	6.44	68.08
5	-1	-1	1	5.4	57.08
6	+1	-1	1	5.65	59.72
7	-1	1	1	6.5	68.70
8	+1	1	1	6.7	68.39
9	-1.682	0	0	4.6	48.62
10	1.682	0	0	5.4	57.07
11	0	-1.682	0	4.68	49.46
12	0	1.682	0	7.2	76.1
13	0	0	-1.682	5.3	56.02
14	0	0	1.682	5.9	62.36
15	0	0	0	6.25	66.07
16	0	0	0	6.25	66.07
17	0	0	0	6.35	67.12
18	0	0	0	6.5	68.71
19	0	0	0	6.5	68.71
20	0	0	0	6.5	68.71

Release of the active principle from the support

The ester was studied in terms of the drug's *in vitro* release kinetics. The impossibility to dose NBGA by the means of spectral methods (UV) or by other available methods constrained us to indirectly assess the release process, that is to monitor the temporal evolution of the pH variation within the acidic or basic hydrolysis of the immobilization product.

The NBGA fixing on gellan by esteric bonds is making possible its gradual release by the immobilization product, in acidic or basic medium capable to hydrolyze these bonds. Therefore, it has been determined the temporal variation of pH (controlled by the consumption of acid or base within the hydrolysis of the esteric bonds).

In order to study the active principle's release, from the generated system, there has been selected a product having a content of 76.1%NBGA in the case of release in acidic environment, respectively 68.7% NBGA in the case of release in basic environment.

RESULTS AND DISCUSSION

Synthesis and characterization of N-(*m*-nitrobenzoyl) –L–glutamic acid(NBGA)

N-(m-nitrobenzoyl) – glutamic acid (NBGA) was obtained by acylation of L-glutamic acid with 3-nitrobenzoyl chloride (Fig. 1).
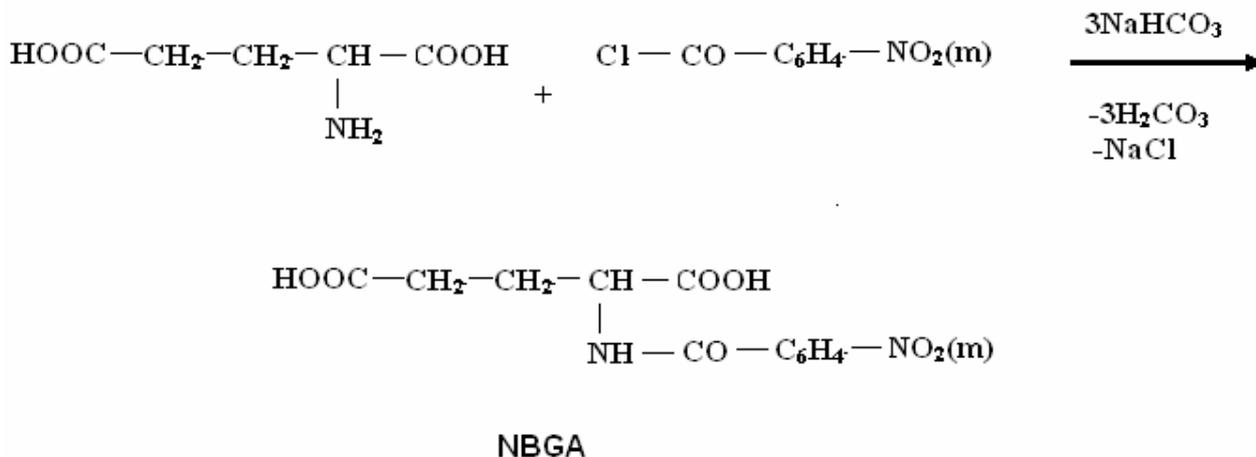
Fig. 1 – Acylation reaction of L-glutamic acid with *m*-nitrobenzoyl chloride.

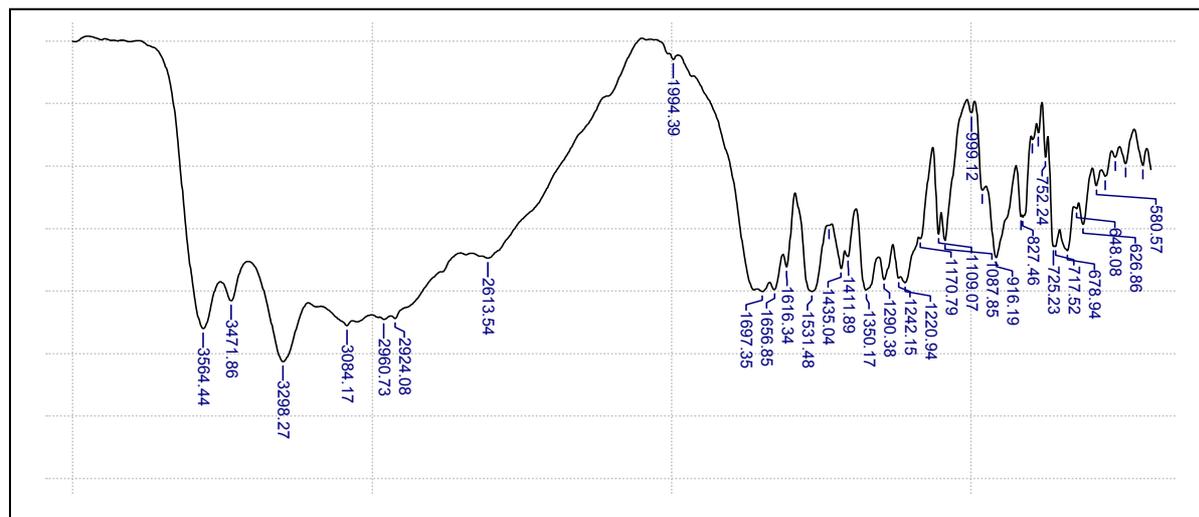NBGA is a white, crystallized product with m.p.= 97-98^{0}C. %$N_{practical}$ =9.45 (%$N_{theoretical}$ =9.46).

Fig. 2 – FT-IR spectrum for NBGA.

Formation of NBGA is proven by the FT-IR spectrum, which attests the presence of the amidic group at 1697.35 cm⁻¹, as well as of the carboxylic groups at 1220 cm⁻¹ and 1290 cm⁻¹, for CH_2- ,

-CH- groups at 2960 cm⁻¹, 2924 cm⁻¹, respectively 1433.11 cm⁻¹ specific to L-glutamic acid, and for the benzenic ring at 3084 cm⁻¹ and for nitroarenes at 1531.48 cm⁻¹.
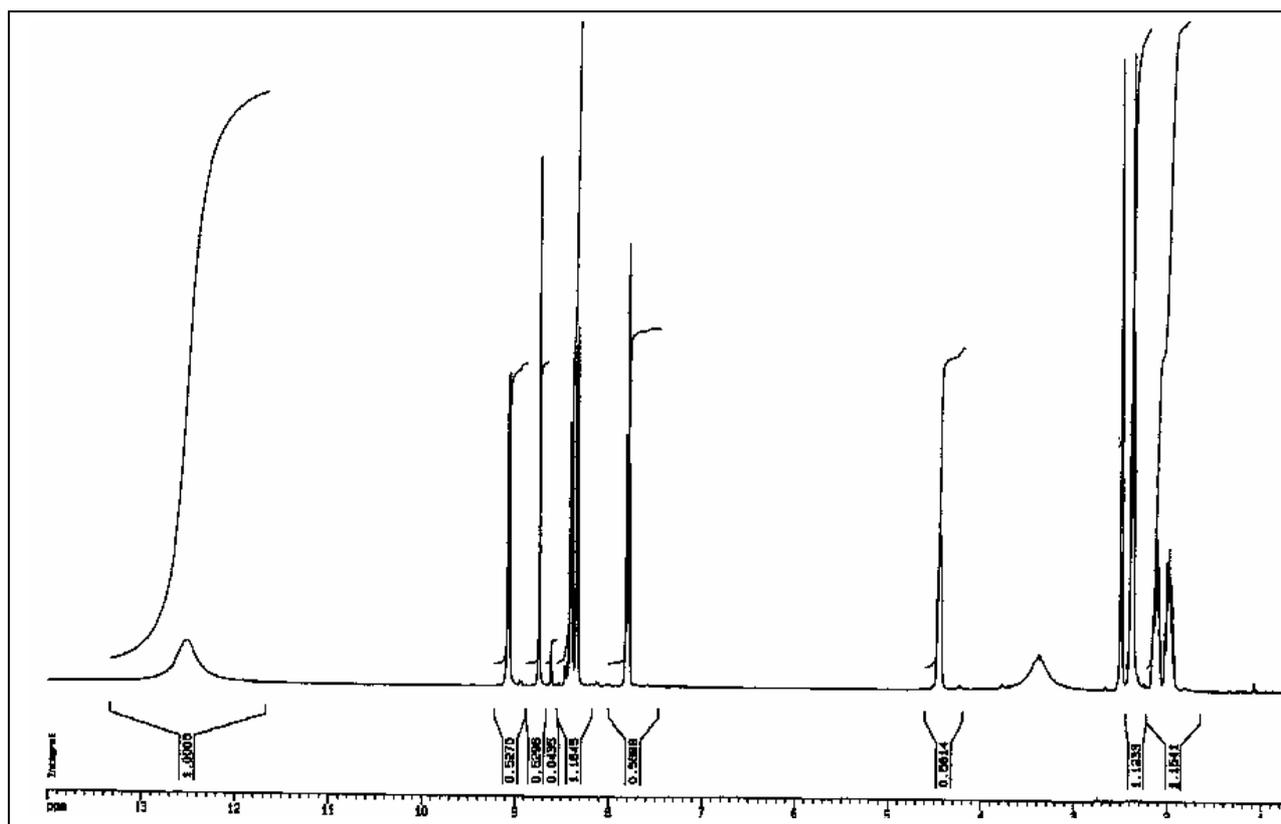
Fig. 3 – The ¹H-RMN spectra for NBGA.

¹H-NMR spectra for NBGA indicate acilated amino group formation by peak occurrence at the 6-8.5 region .[15]

¹H-NMR (DMSO d6, 400MHz) δ(ppm): 12.5 (s, H, COOH); 2.3 (t,2H,CH_2); 2-2.1 (C,2H,CH_2); 4.5 (t,1H,CH); 7.8 (s,2H,NH, Ar); 8.4 (d,2H,Ar); 8.72 (s,1H,Ar).

Immobilization of NBGA on gellan

NBGA coupling on gellan is based on esterification of carboxylic groups of active principle with hydroxyl groups of support, activated by DCCI, according to Fig. 4:
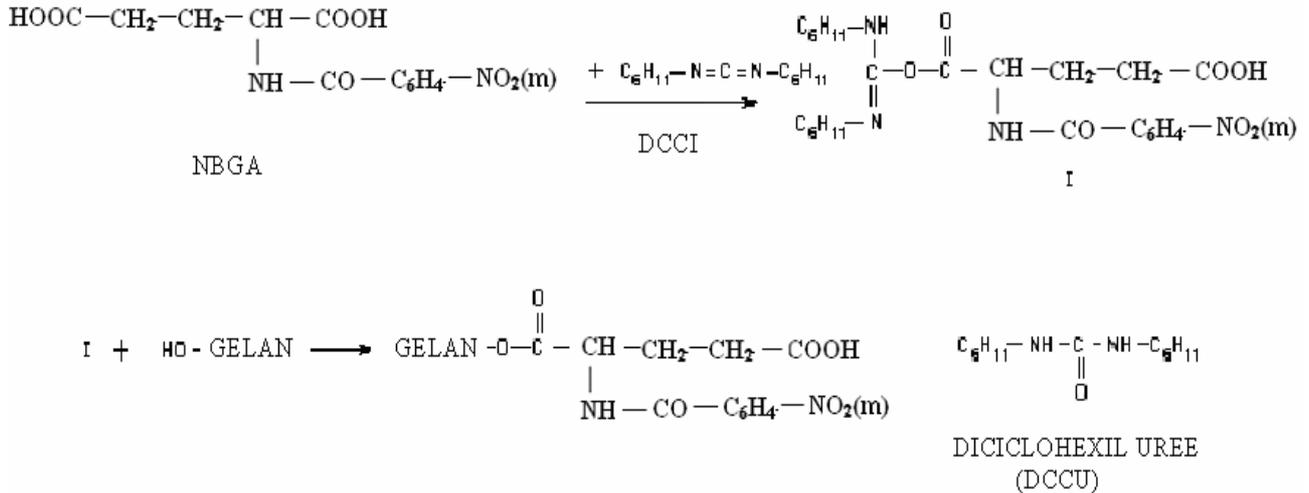

Fig. 4 – Immobilization reaction of NBGA on gellan.

Formation of the NBGA ester with gellan is proven by IR spectrum, which attests the presence of esteric groups at 1244 cm⁻¹, as well as the functional groups specific to NBGA: 3037.88cm⁻¹, attributed to –NH- group , 1535.33 cm⁻¹ attributed to nitroarenes, 1625.99 cm⁻¹ attributed to amidic groups, 1575 cm⁻¹ attributed to arenes, as well as functional groups specific to gellan: 3327.2 cm⁻¹ attributed to OH groups, 2929.86 cm⁻¹ for CH_3- and 1625.99 cm⁻¹ attributed to C=O group on carboxylate (Fig. 5).
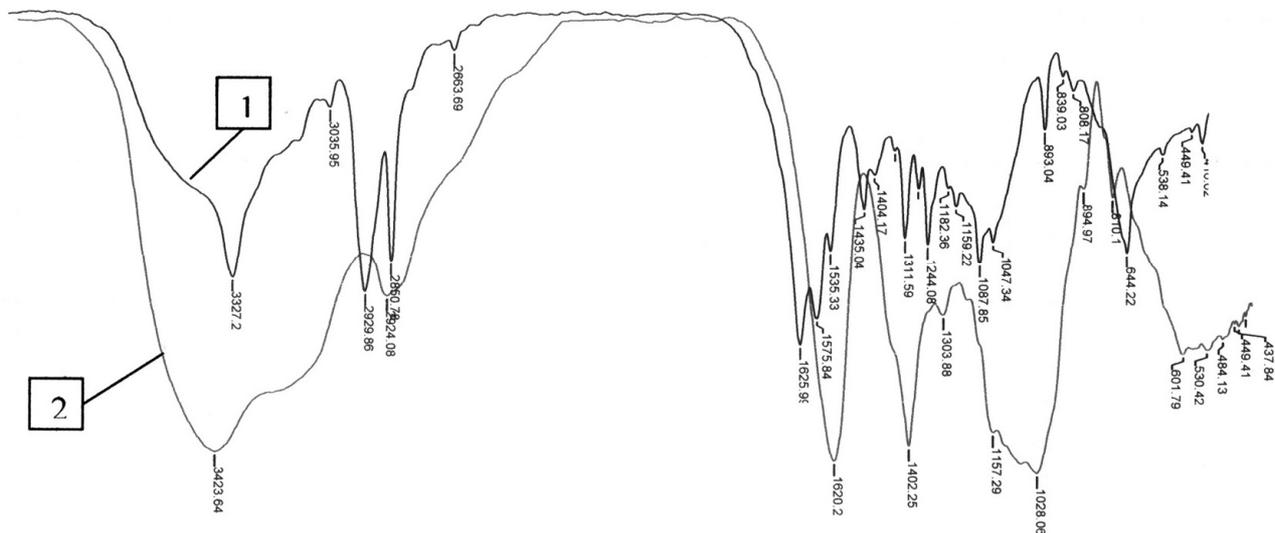

Fig. 5 – FT-IR spectrum for : 1 – NBGA-gellan system ; 2 – gellan itself.

The processing of experimental results led to the following regression equation:

$$\%NBGA = 67.45 + 2.78 X_1 + 5.95X_2 + 2.21X_3 - 4.50X_1^2 - 0.33X_2^2 - 2.39X_3^2 - 0.99X_1X_2 + 0.5X_1X_3 - 2.26 X_2X_3$$

If two of the variables are particularized at the center of the experimental domain, information on the influence of the third one may be obtained, as is shown in curves plotted in Figs. 6-8.

Fig. 6 shows the influence of the amount of activator, expressed as the DCCI/NBGA molar ratio, upon the efficiency of the immobilization reaction. It is to be noticed that when increasing of DCCI amount until the DCCI/NBGA molar ratio of 1.34, the percentage of NBGA in the reaction product increases too, continuously; at greatest values of the ratio the percentage of immobilized NBGA decreases. The effect can be explained by the fact that in the first part of the curve there occurs an intensive activation of the carboxyl functional groups, respectively of medicament.
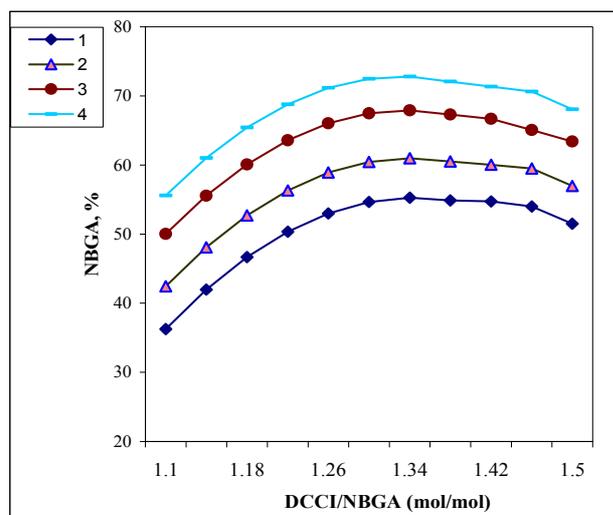
The decrease of the percentage of immobilized NBGA shown in the second section of the curve is explained by the fact that at a higher DCCI/NBGA molar ratio the carboxyl groups get activated within NBGA in ε position; then for one molecule of active principle two esteric bonds with the support can be formed.

Therefore, in order to ensure the greatest amount of NBGA immobilized on gellan, we must work always with an excess of activator, but not more then DCCI/NBGA molar ratio=1.34.

Fig. 6 – Influence of the DCCI/NBGA molar ratio on the immobilizated active principle ratio, at t = 24 hours: 1– NBGA/Gellan = 2 mol/mol; 2 – NBGA/Gellan = 2.6 mol/mol; 3 – NBGA/ Gellan = 3.5 mol/mol; 4 – NBGA/Gellan= 4.4 mol/mol.

Fig. 7 – Influence of NBGA/ Gellan molar ratio on the immobilizated active principle ratio, at t = 24 hours: 1 – DCCI / NBGA=1.1 mol/mol; 2 – DCCI / NBGA = 1.18 mol/mol; 3 – DCCI / NBGA=1.5 mol/mol.
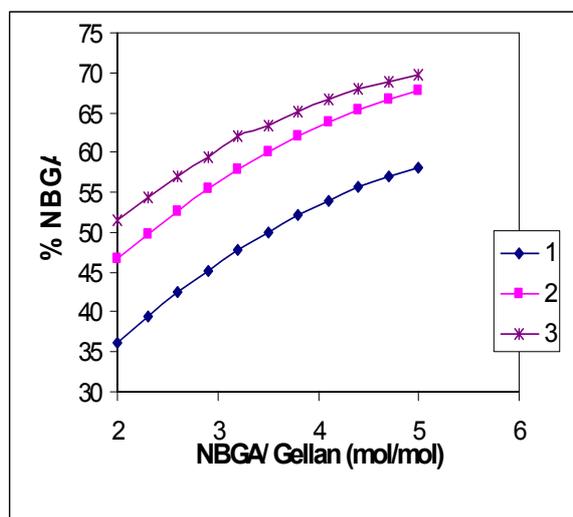

The amount of coupled NBGA increases with the NBGA/ Gellan molar ratio (Fig.7). It is obvious that, in order to reach maximal immobilization yields, a minimal amount of gellan should be considered. The high number of hydroxyl groups contained in one mole of polysaccharide (10 -OH groups) provides a sufficient number of reactive sites for NBGA's esterification, which explains

why the immobilized NBGA percentage increases with its percentage in the reaction substrate, in NBGA/gellan ratio respectively.

An important parameter in the synthesis is the duration of the esterification reaction. Its influence on the amount of fixed NBGA on support is shown in Fig. 8.

Fig. 8 – Influence of the reaction time upon the immobilized NBGA ratio, at a DCCI/NBGA molar ratio: **1**.- 1.1 mol/mol, **2**.- 1.18 mol/mol, **3**.- 1.26 mol/mol.
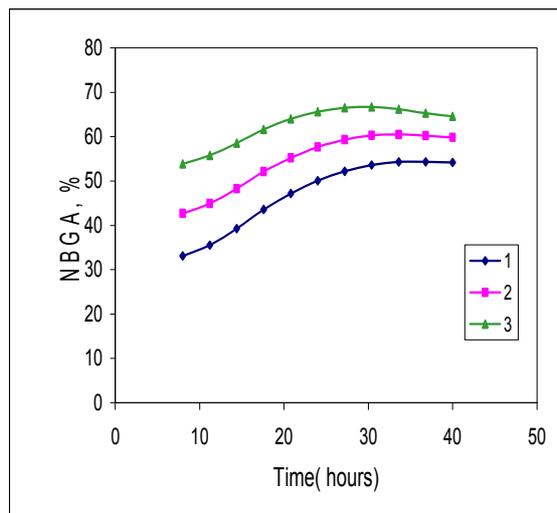
One should observe that, regardless of the other parameters values of the process, maximum yields are reached after about 33 hours of reaction time. A possible explanation might be that, along the whole duration of the synthesis, NBGA esterification to gellan's carboxylic groups is completed by the intermolecular reaction of polysaccharide's esterification. DCCI activates carboxyl groups of NBGA and of the support, too, being partially consumed in this reaction, which results in gellan's slight crosslinking.

Such explanation is supported, too, by the fact that, on increasing the duration of the synthesis, the reaction products become more and more rigid, manifesting a lower and a lower capacity of swelling in water and also due to hydrophobization through NBGA's bonding.

The results presented in Figs. 6-8 are confirmed in Fig. 9, which illustrates, in three-dimensional representation, the influence of each of the two parameters on immobilization's efficiency (estimated by the ratio of NBGA in the immobilization products).
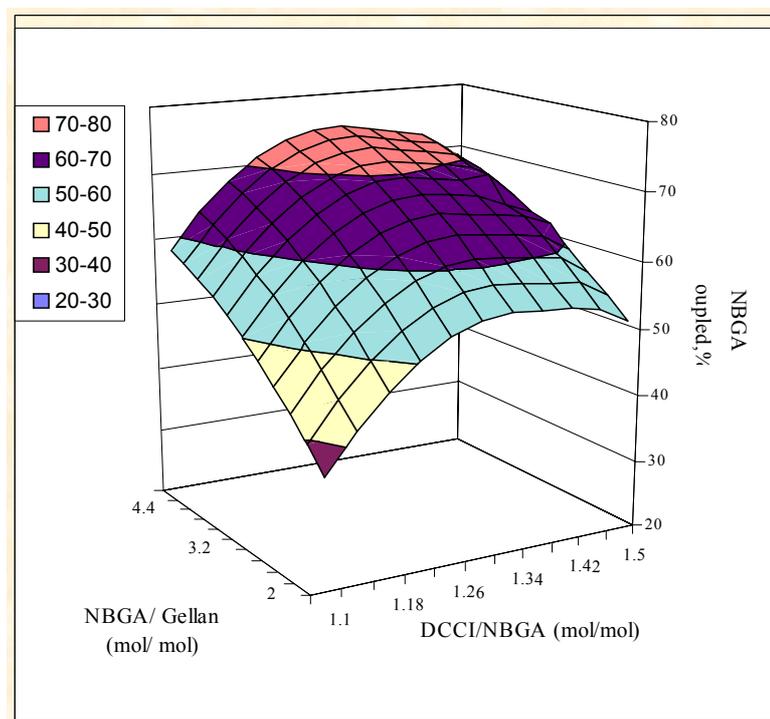
Fig. 9 – Influence of the DCCI/NBGA and NBGA/gelan molar ratio on the amount of drug immobilized at 24 hours.

Analysis of the aforementioned data shows that, in order to reach a maximal content of NBGA in the immobilization compounds, the synthesis should be performed in the following conditions:

DCCI/NBGA=1.35 mol/mol;

NBGA/Gellan=5mol/mol;

t=34 hours.

A synthesis performed in these conditions generates a product having a content of 76.3% NBGA.

Release of the active principle from the support

In order to study the active principle's release capacity, hydrolysis of the esteric groups in acid, respectively basic medium, has been performed, starting from the idea that pH variation represents a suitable method for estimating the kinetics of drug release.

Release of NBGA from immobilization product in acid medium

The experimental data show that in acid environment the pH values increases in time, rapidly, in the first 7 minutes, and subsequently more slowly, until the reaching of a constant level at approximately 100 minutes after the process' beginning.

The explanation is that in the first interval the bioactive molecules on surface, respectively on a superficial layers of the immobilization product are released (the release rate of NBGA is higher). The constant level after 100 minutes indicates the starting of "zero order" release kinetics, which is specific to polymer-drug systems with controlled release of the active principle; the process is probably dominated by the immobilized NBGA diffusion phenomenon from the support.

Fig. 10 – Variation in time of pH values for acidic hydrolyze of the NBGA immobilization product on gellan (76.1%).
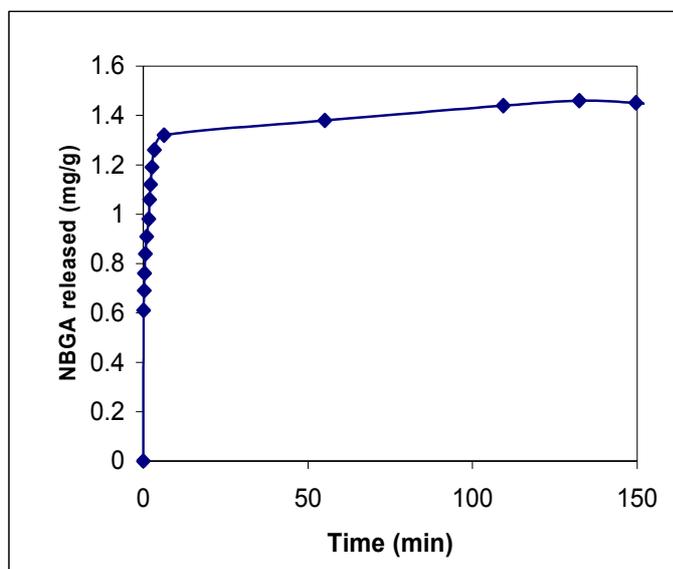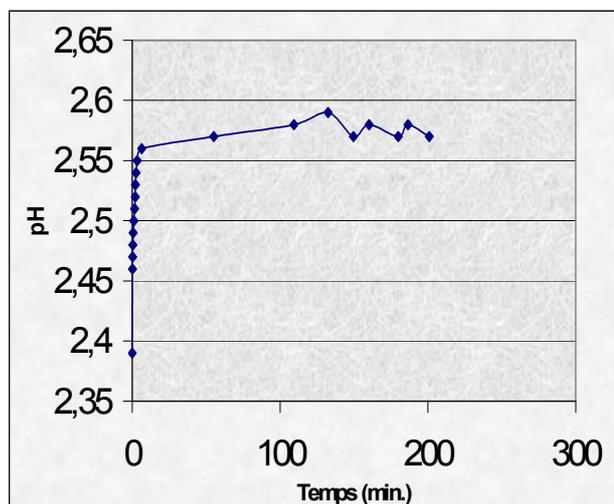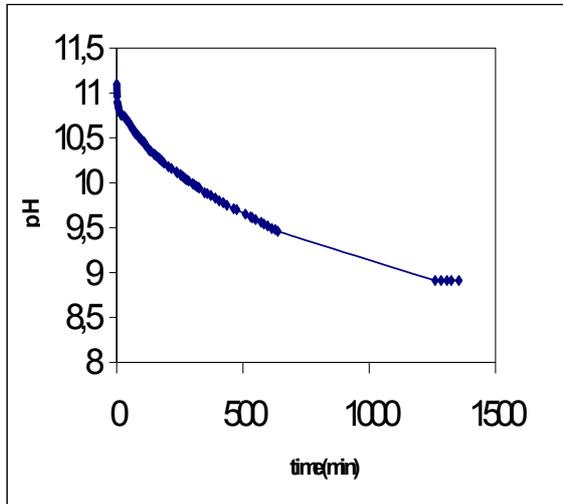
Fig. 11 – Amount of NBGA released in acidic medium by the immobilization product, at 37ºC (76.1% NBGA).

The NBGA release from the immobilization product in a basic medium

The study of the biological active principle release capacity was performed by hydrolyzing the

It is to be noticed that the fastest release of active principle takes place in the first 15 minutes; afterwards the release takes place more slowly for a 3-hour period, then reaching a constant level, typical for the "zero order" release kinetics.

esteric bonds in basic medium, too, when the reaction devolves more slowly. The results are presented in Fig. 12.

Fig. 12 – Variation in time of pH values for the hydrolyze in basic medium of the immobilization NBGA product on gellan at $T = 37^0C$ (68.7% NBGA).

The variation of the amount of released NBGA by the active principle-gellan system is presented in Fig. 13.

Fig. 13 – Variation in time of the amount of released NBGA by the active principle-gellan system (68.7%NBGA).
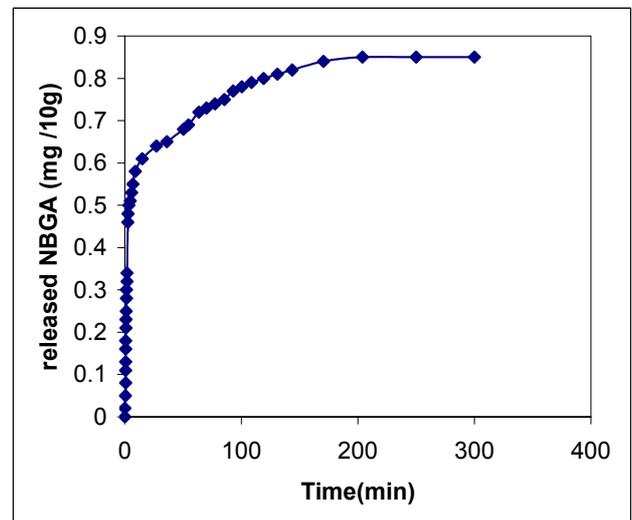
The NBGA's biological activity and the immobilization product on gellan tests

Since these two synthesized compounds (NBGA and NBGA-gellan system) can have antitumoral activity, acting as antimetabolites, toxicity tests for them have been performed. Toxicity has been evaluated on the basis of the observed mortality through LD5 determination.

Acute toxicity has been orientatively determined by intraperitoneal administration of the substances, as suspension in Twen 80, to groups of three mice (20-25g), according to classical laboratory methods (Korber method).

The animals were monitorized and their mortality has been assessed after 7 days (Table 3).

Table 3

Toxicity of the tested compounds

Compound	Lab animal	Administration way	LD50(mg/g body)
NBGA	mice	i.p.	1250
NBGA-gellan	mice	i.p.	2700

Studying the data from Table 3 we had proof that the products (NBGA and NBGA-gellan system) were featuring toxicity parameters favorable to the same drug products. Since the toxicity of the immobilization product is inferior than NBGA's, it is recommended to be tested as antimetabolite in the treatment of some experimental malignant tumors. Natural polymeric support decreases the active principle's toxicity and so, it can act as a carrier for the bioactive product (NBGA) towards the affected tissues, determining a certain release kinetics, too.

CONCLUSIONS

A new aminoacid derivative has been synthesized, that is N-(*m*-nitrobenzoyl)-glutamic acid (NBGA) respectively, which shows biological activity.

The chemo-therapeutically parameters of the active substance can be improved by its immobilization on macromolecular supports, especially of the polysaccharidic nature.

The imobilization reaction's efficiency is a controlled by the following factors: the activator/NBGA ratio, the NBGA/support ratio and reactions duration.

If the amount of DCCI increases up to a DCCI/NBGA ratio (mol/mol) of 1.34, the percentage of NBGA within the reaction product increases too continually; after this ratio level the NBGA percentage starts to decrease.

If the molar ratio DCCI/NBGA increases up to 1.34, the carboxyl groups and medicament groups get more and more active.

At the greatest DCCI/NBGA ratio the carboxyl group located in the ε position gets activated too; hence, for one molecule of active principle, two ester bonds to support can form subsequently.

The amount of copuled NBGA increases with the NBGA/Gellane molar ratio;

The release of active principle from the coupling product can be performed by the breaking of esteric bonds by the means of acid hydrolysis or by the means of basic hydrolysis, each one being specific for different sections of the digestive tractus.

REFERENCES

1. V. Sunel, M. Popa, A. Popa and C. Soldea, *Cellulose Chem. Technol*., **2000**, *34,* 269.
2. V. Sunel, C. Basu, C. Oniscu and C. Soldea, *Roum. Biotechnol. Lett*., **2000**, *5,* 393.
3. V. Sunel, C. Ciugureanu and M. Ungureanu, *Rev. Med. Chir. Soc. Med. Nat*., Iasi, **1993**, *98,* 273.
4. V. Sunel and C. Soldea, *Comp. Rend. Acad. Bulg. Sci*., **1993**, *46,* 61.
5. M. Popa, L. Rusu, V. Sunel and C. Basu, *Cellulose Chem. and Technol*., **2002**, *36*, 510.
6. B. T. Stokke, B. E. Christensen and O. Smidsrod, "Polysaccharides. Structural Diversity and Functional Versality'', in S. Dumitriu (Ed.), Marcel Dekker, New York, 1998, p. 433.
7. W. Y. Chuang, T. H. Young, C. H. Yao and W. Y. Chiu, *Biomaterials*, **1999**, *20*, 1479.
8. M. N. V. Kumar, *Reactive & Functional Polymers*, **2000**, *46*, 1-27.
9. R. Chandrasekaran and A. Radha, *Trends Food Sci.,* **1995**, *6*, 143-148.
10. S. Dumitriu, P. Vidal, E. Chornet, in S. Dumitriu (Ed.), "Polysaccharides in Medical Applications", Marcel Dekker, Inc., New York, 1996, p. 125.
11. M. Popa, L. Rusu, V. Sunel and C. Basu, *Cellulose Chem. and Technol*., 2002, *36*, 510.
12. S. Dumitriu, M. Dumitriu, in S. Dumitriu (ed.) "Polysaccharides in Medical Applications", Marcel Dekker, Inc., New York, 1996, p. 705.
13. M. Popa, V. Sunel, L. Balaita Rusu and C. Basu, *Eurasian Cem.Tech.Journal*, **2003**, *5*, 121-126.
14. C. Lionte, V. Sunel, O. Pintilie, O. Mungiu, I. Teleman and M. Popa, *Eurasian Cem.Tech.Journal,* **2002**, *4*, 201-222.
15. J. R. Dean, A. Jones, D. Holmes, R. Reed, J. Weyers and A. Jones, "Practical Skills in Chemistry", Prentice Hall (Eds.), 2002, 190-200.