0273-2289/98/7402—0067$12.50

Immobilization of α-Chymotrypsin into the Poly(*N,N*-Diallyl-*N,N*-Didodecyl Ammonium Bromide)/Surfactant Nanocapsules

YURY E. SHAPIRO[1,*] AND ELENA G. PYKHTEEVA[2]

[1]*Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel;*
and [2]*Bogatsky Physico-Chemical Institute, National Academy of Sciences, Odessa 270080, Ukraine*

Received January 26, 1998; Revised April 17, 1998; Accepted May 21, 1998

ABSTRACT

The biocatalytic systems from nanocapsules containing α-chymotrypsin in the inner aqueous cavities have been prepared. They can act in both the organic solvent and the aqueous medium. For such encapsulation, the reversed hydrated micelles from *N,N*-diallyl-*N,N*-didodecyl ammonium bromide (DDAB) in cyclohexane (w_0 = 22), including α-chymotrypsin, have been polymerized by UV initiation. After precipitation by acetone, these nanocapsules were moved into the aqueous medium with the aid of ionic, AOT, or nonionic, Brij-97, surfactants. In this case, the unilamellar liposomes were formed. They have the inner monolayer from the poly-DDAB network, and the outer one predominantly from surfactant molecules. According to the light-scattering data, the average outer diameter of nanocapsules equals to 20 nm. The vesicular "coated" α-chymotrypsin was used for study of enzymatic activity. It has been shown using the integral form of the Michaelis-Menten equation, that by encapsulation of α-chymotrypsin the value of the Michaelis constant, K_m, increases by a factor of 1.8 by the ATEE hydrolysis. However, the value of the maximal velocity, V_{max}, decreases by a factor of 1.7. Encapsulated α-chymotrypsin has a high thermostability keeping its own activity up to 80°C. The polymer network blocks the conformational transitions of enzyme molecule by heating of a system.

*Author to whom all correspondence and reprint requests should be addressed. E-mail: yury@physchem.kth.se

Index Entries: α-Chymotrypsin immobilized; biocatalyst; enzyme in the net; nanocapsules; monomer surfactants; AOT; Brij-97; enzymatic activity; thermostability.

INTRODUCTION

A high catalytic activity of enzymes, their ability to realize the chemical processes under mild conditions, and the matchless selectivity of action open up broad prospects for use of biocatalysts in processes of the fine organic synthesis *(1)*. However, enzymes themselves satisfy the desired properties only in aqueous solution within the close ranges of pH and temperatures. The transition from water as a reaction medium to an organic solvent, or going out the optimal pH and temperature ranges in the aqueous phase are accompanied either by enzyme denaturing or by the dramatic drop of enzyme activity owing to the disappearance of substrate specificity. This fact essentially limits their application area.

A full or partial avoidance of these problems is possible in water or organic soluble biocatalytic systems like enzymes immobilized on the surface of a polymer vehicle *(2)*, inside reversed micelles from cationic *(3)*, anionic *(4,5)*, or nonionic *(5,6)* surfactants, and within various liotropic mesophases *(7,8)* or liposomes *(8,9)*. In these cases, enzyme is protected from denaturing action of medium, and its activity is only somewhat reduced *(3,10)*. The last does not hinder an application of such biocatalytic systems. There is another restriction. Reversed micelles encapsulating enzyme molecules are normally formed in a nonpolar solvent. Therefore, such systems are mainly restricted to organic-soluble substrates. On the other hand, the enzyme-containing liposomes exist only in the aqueous phase. Therefore, they are useful mainly for the water-soluble substrates.

Making biocatalytic ensembles capable of working in both aqueous and non-aqueous media with a good efficiency and without necessity of the special conditions for their upkeep is thus of high interest. We believe that the systems as polymer nanocapsules containing the enzyme molecules could fulfill these demands completely. Polymer coating protects an enzyme from denaturing under the influence of organic solvent, allows widening of the useful temperature and pH ranges of the enzymatic activity, and preserves enzyme in a solid state for a long time.

Two methods are established at present to prepare the polymer nanoparticles. The first method is more common. It consists of forming the polymer nanogranules through polymerization of the reversed micelles from the AOT molecules modified by methacryl groups. Such micelles must also contain the enzyme molecules modified by acrylonitrile, acrylamide, or *N,N'*-methylene-*bis*(acrylamide) *(11)*. In this case, the enzyme molecule is incorporated into polymer particles by the covalent bonds. The

average diameter of polyacrylamide granules depends on the hydration degree of micelles, $w_0 = [H_2O]/[surfactant]$, the acrylamide concentration, and is within the range from 40 to 100 nm. However, the necessity of a chemical modification of enzyme makes this method too laborious. Also, enzymatic activity is reduced to a great extent by this modification.

The second method is based on the use of micelles and vesicles from the monomer surfactants *(12–14)* polymerized under the UV irradiation or chemical initiation *(12,15)*. Such vesicle-forming surfactants contain vinyl, methacryl, diacetylene, isocianate, and styrene groups in their hydrocarbon chains or head groups. Accordingly, vesicles could be polymerized inside their bilayer or through their head groups.

Cationic pyridinium and ammonium surfactants having polymerizable head groups are also of high interest *(12–21)*. They can form unilamellar vesicles in aqueous solution provided their molecules contain two hydrophobic alkyl chains *(17–21)*. On the other hand, single-chain surfactants of this type form micelles *(12–16)*.

The main goal of this work was the immobilization of the unmodified α-chymotrypsin within nanocapsules from the reversed micelles of the polymerized *N,N*-diallyl-*N,N*-didodecyl ammonium bromide (DDAB) $[(H_2C = CHCH_2)_2\ N^+]\ [(CH_2)_{11}\ CH_3)_2]Br^-$, in cyclohexane. Activity of such biocatalytic systems obtained under various conditions, which we can determine as "an enzyme in the net," had been measured in comparison with activity of the nonimmobilized α-chymotrypsin. These biocatalytical systems can act in both an organic solvent and an aqueous phase after precipitation of nanocapsules by acetone and further addition of ionic, AOT, or nonionic, Brij-97, surfactants. In this case, the unilamellar liposomes are formed. They have the inner monolayer from the poly-DDAB network, and the outer one predominantly from surfactant molecules. An enzyme molecule remains as encapsulated in the inner aqueous cavity of vehicle. The proposed method of nanocapsule formation combines the advantages of the two above-mentioned methods and does not have their drawbacks.

MATERIALS AND METHODS

Materials

We used commercial α-chymotrypsin (EC 3.4.21.1, Sigma-Aldrich, USA), *bis*(2-ethylhexyl)sodium sulfosuccinate, AOT (Merck, Germany), and 10-oleoil ether of poly(oxyethylene), Brij-97 (Sigma-Aldrich, USA) without the further purification. Phosphate buffer (Merck, Germany) and *tris*(hydroxymethyl)aminomethane hydrochloride (TRIZMA) (Sigma-Aldrich, USA) were used for keeping of pH 7.2. *p*-Acetyl-L-tyrosine ethyl ester (ATEE) was used as the specific substrate and *p*-nitrophenyl acetate

(NPA) (both from Sigma-Aldrich, USA) was used as nonspecific substrate for α-chymotrypsin.

Experimental details for the new synthesis of DDAB were published earlier *(21)*. Cyclohexane, chloroform, acetone, and twice-distilled deionized water were used as solvents. Deuterium oxide, D_2O (99.9% D, ISOTEC Inc., USA) was used in the NMR experiments.

Preparation of Vesicles

For preparation of the biocatalytic system, DDAB was dissolved in the mixture of cyclohexane:chloroform = 50:1 v/v up to 10 m*M*. To achieve the required hydration degree of micelles, w_0 = 22 mol/mol, an aqueous buffer solution of α-chymotrypsin was added to the transparent solution of DDAB. A mixture was well shaken for 10 min until a transparent opalescent solution was obtained. The absence of sediment by further ultracentrifugation indicated the full inclusion of this amount of α-chymotrypsin into the reversed DDAB micelles. The inclusion of enzyme was also tested using the ordinary color reactions for proteins.

The radical polymerization of DDAB monomers was carried out under the influence of the nonfiltered UV-irradiation by continuous mixing of the colloid system for 15 min *(20,21)*. The prepared polymer nanocapsules with or without α-chymotrypsin were precipitated by a 10-fold excess of acetone. The white sediment was separated by ultracentrifugation (3×10^3 min^{-1}, 15 min), washed by acetone, and dried thoroughly.

For the formation of unilamellar bilayer vesicles, the dry polymer nanocapsules with or without enzyme were transferred in an aqueous buffer solution (pH 7.2) with aid of surfactant. The molar ratio of the added surfactant to initial DDAB varied from 1:4 to 4:1 mol/mol. To produce the stable opalescent dispersion suitable for NMR experiments, this colloid system was ultrasonicated after vigorous shaking (2 × 30 s, 35 kHz). A sonicated dispersion was stable and did not precipitate during a span of 4 mo.

Methods of Measurements

The ^1H and ^{13}C NMR spectra for checking of polymerization degree were performed using a Bruker AM 250 spectrometer at 250.13 and 62.90 MHz. The polymerization degree was also checked using the IR spectra measured in the cyclohexane solution by use of spectrophotometer Specord 75 IR (Jena, Germany) and standard cells from KBr.

The ^1H NMR spectrum of the vesicular nanoparticles from poly-DDAB/Brij-97 in D_2O shows overlapping signals from a polymer chain and surfactant, terminal CH_3 (0.93 ppm), $(CH_2)_n$-chain (1.33 ppm), as

well as the signals from individual poly-DDAB N^+CCH_2 (1.62 ppm), N^+CH_2 (3.54 ppm), and Brij-97, the most intensive signal OCH_2 (3.74 ppm), CH_2C = (2.06 ppm), = CH (5.36 ppm). An assignment of signals in the ^1H NMR spectra was done by means of 2D NMR experiment COSY-45, and in accordance with the previous assignment for similar compounds *(16)*.

The determination of the enzymatic activity was performed by the use of a UV-VIS spectrophotometer Hewlet-Packard Lambda-9 and the standard cells of 1 cm. The Tris-buffer solution was used as a solution of comparison. Activity of α-chymotrypsin in a free form and in nanocapsules regarding *p*-nitrophenyl acetate had been measured at 19°C by the discharge of the reaction product, *p*-nitrophenol, having the absorption maximum at 400 nm. This band did not coincide with the absorption band of *p*-nitrophenyl acetate itself. Scanning was performed every 30 s during 1 h of the process.

By the use of ATEE as a substrate, the measurements of activity of α-chymotrypsin in its free form or in nanocapsules were made at 25°C by exhausting of a substrate having the characteristic absorption band at 237 nm. Substrate concentrations were in range of 0.1–1 m*M*. The concentrations of α-chymotrypsin were in range from 1×10^{-3} to 4×10^{-2} m*M*.

For the determination of thermostability of α-chymotrypsin, the samples were kept for 20 min at 60 and 80°C, cooled up to the temperature of the activity measurement, and moved to the spectrophotometer cell.

The average hydrodynamic radius of nanocapsules was determined by the light-scattering method using nefelometer FEKM-57 (LOMO, Russia) at 20°C and the wavelength of 650 nm.

RESULTS AND DISCUSSION

Formation and Structure of the Vesicular Nanocapsules

Electron micrographs showed that the structures of the initial micelles from DDAB in water *(19)* as well as of the bilayer vesicles obtained during ultrasonication of the 0.1 *M* aqueous dispersion of this surfactant *(15)* were kept by the UV-initiated polymerization. Such a polymer is a closed two-dimensional network situated on the surfactant/water-phase border. As a rule, the hydrophobic alkyl fragments were packed within micelles or bilayer like it was established for phospholipid vesicles *(22)*. If enzyme globules were included into colloid particles before polymerization, then after polymerization, one could obtain biocatalytic nanocapsules in which enzyme was protected by the double polymer network.

It should be noted that such a catalytic system has three substantial shortcomings. The first one is the necessity of the gel-filtration process for

separation of the nonentrapped enzyme from nanocapsules. This procedure on the whole is simple for phospholipid liposomes, but it gives great trouble in the case of solid polymer nanoparticles. Second, the double-polymer network essentially increases the *trans*-membrane diffusion coefficients of substrate and products of reaction in spite of the relatively large dimensions of cells. This one can substantially decrease the effective parameters of enzyme kinetics, mainly because of the specific inhibition by reaction products. Third, the liposomal dimensions for the main fraction of the sonicated *N,N*-diallyl-*N,N*-dialkyl ammonium bromide dispersion are extremely high and vary within a wide range. The outer diameter of liposomes is from 60 to 230 nm *(23)*. Of course, it decreases by polymerization, but only a little *(13)*.

The use of reversed micelles prepared from the monomer surfactant containing the solubilized water as an inner monolayer by the formation of the bilayer unilamellar vesicle allows variation of the diameter of the inner aqueous cavity within a wide enough range *(4,7)*. We can diminish the diameter to the average diameter of the α-chymotrypsin globule, 4.3 nm. Earlier it was shown *(7)* that α-chymotrypsin encapsulated into reversed micelles has the most activity just by the combination of dimensions of the inner micellar cavity and the solubilized protein globule. Enzyme does not dissolve in the nonpolar cyclohexane. Thus, it is fully included into the inner aqueous phase solubilized by reversed micelles.

The poly-DDAB network is formed around an enzyme globule after the UV-initiated polymerization of DDAB (Fig. 1). The observation of alterations in the IR and ^{13}C NMR spectra of the UV-irradiated dispersions allows control of the degree of polymerization.

The absorption band at 1636 cm^{-1} in the IR spectrum corresponding to the valent vibrations of the allyl double bond is the most informative for the determination of the DDAB polymerization degree in the structure of reversed micelle. The ratios of the intensity of this band to the intensities of bands corresponding to the symmetric, ν_s = 2838 cm^{-1}, and asymmetric, ν_{as} = 2912 cm^{-1}, valent vibrations of CH bond for the cyclohexane CH_2-groups, used as the inner reference of intensity, reduce to one-third by polymerization. The same result can be obtained if the intensities of bands corresponding to the valent vibrations of CH-bonds for the terminal methyl groups of the dodecyl fragments, ν_s = 2888 cm^{-1} and ν_{as} = 2945 cm^{-1}, are used as references. Therefore, we can positively maintain that 2/3 of all the DDAB allyl groups in reversed micelles are polymerized, forming a network encapsulating the α-chymotrypsin globules.

After the UV-initiated micellar polymerization of DDAB, the total intensity of CH_2 = CH—signals in the ^{13}C NMR spectrum decreases by a factor of 2.4, the intensities of C10, C11, and C12 signals are not changed

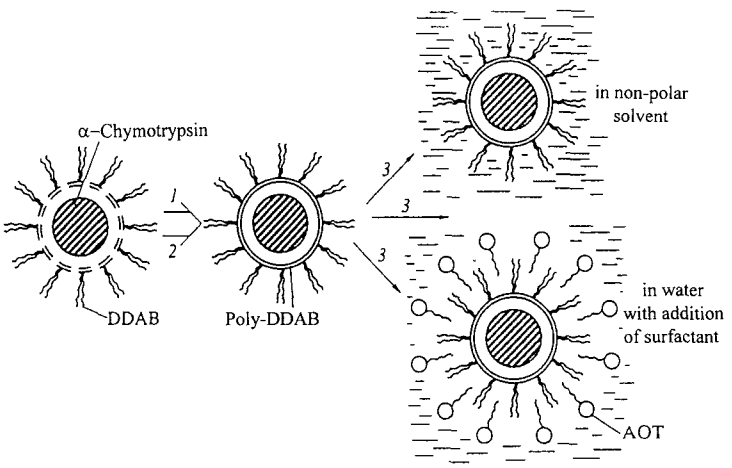

Fig. 1. Scheme of preparation of the enzyme containing nanocapsules. Designation of operations: 1—free radical polymerization, 2—precipitation by acetone, 3—dissolving.

really at all, but the new signals from $CH_2CHCH_2N^+$-groups of poly-DDAB appear at 21.3–32.6 and 60.1–62.0 ppm (Table 1). Therefore, according to the ^{13}C NMR spectroscopy data, up to 60% of monomer molecules in micelles are polymerized. This fact is in conformity with the IR spectroscopy data showing up to 67% of monomer molecules are polymerized.

The anionic or nonionic surfactant molecules were applied as the outer monolayer by the formation of unilamelar vesicles. Especially stable bilayer is formed from the anionic AOT molecules that interact with the inner polymer monolayer containing ammonium fragments. Figure 1 shows that the reversed micelles from poly-DDAB, including enzyme, were moved from cyclohexane into aqueous phase after precipitation, addition of surfactant, and the further ultrasonication. Polymer nanocapsules save enzyme quite well from ultrasound destroying.

Evaluation of the Vesicular Dimensions

The hydrodynamic radii of the poly-DDAB/AOT nanocapsules were determined by the light scattering method. It is well known *(24)* that the hydrodynamic radii of the dispersed particles, r_0, are connected with turbidity of a dispersion, τ, according to equation:

$$r_0 = (\lambda/8\pi^2)(9[\tau]\lambda/\alpha^2)^{1/3} \quad (1)$$

Table 1
The Assignment of the ^{13}C NMR Signals of the DDAB Reverse Micelles in Cyclohexane Encapsulating α-Chymotrypsin in the Inner Aqueous Cavities Before and After Polymerization of Allylic Segments by the UV-Irradiation

	Before polymerization		After polymerization	
Carbon atom[a]	δ, ppm	Relative group intensity	δ, ppm	Relative group intensity
C2′	130.6		130.8	
	128.2		128.1	
C3′	118.2		118.0	
	115.7	0.112	115.5	0.046
C1′	64.8		64.9	
	62.5		62.7	
	—		62.0	
	—		60.1	
C1	59.9		59.7	
	57.7	0.098	57.4	0.099
C10	32.5		32.6	
	31.9		32.0	
$(CH_2)_8$	30.3		30.4	
C11	23.3		23.3	
	21.3	0.713	21.3	0.779
C12	15.2		15.2	
	13.3	0.077	13.3	0.076

[a]$\{(H_2C = CH-CH_2)_2N^+[CH_2(CH_2)_8CH_2CH_2CH_3]_2\}\ Br^-$
3′ 2′ 1′ 1 10 11 12.

where $[\tau] = \tau/c$ is the characteristic turbidity, c is the volumetric concentration of surfactant, and α is the parameter characterizing the deviation of the optical properties of particle from those for medium:

$$\alpha = (3/4\pi)/(m^2 - 1)/(m^2 + 2) \quad (2)$$

where m is the relation of the refractive index of particle $n^{20}_D = 1.4614$ (was took for *N,N*-didodecyl-*N,N*-dimethyl ammonium bromide, which is similar to used DDAB) to the refractive index of the medium $n^{20}_D = 1.3335$, an $\lambda = 650/1.3335 = 487.4$ nm is the wavelength of light in the measured dispersion.

Equation 1 is valid if the parameter characterizing the relationship of the dimension of a particle to the wavelength, $z = 8\pi r_0/\lambda < 2$ *(24)*. Accord-

ing to Eq. 1, the average hydrodynamic radii of the empty poly-DDAB/AOT vesicles and the enzyme-containing vesicles equal (9.2 ± 0.3) and (10.0 ± 0.8) nm, correspondingly.

Activity of α-Chymotrypsin Immobilized within the Vesicular Nanocapsules

Activity of α-chymotrypsin entrapped into nanocapsules was determined by the use of the specific ATEE, and nonspecific, NPA, substrates. It is known, that these substrates in an aqueous media are liable to the spontaneous hydrolysis. Nevertheless, the constants of the nonenzymatic hydrolysis velocity are less than the constant of velocity for enzymatic process with α-chymotrypsin, k_{+2}, by factors of 150 and 80 for ATEE and NPA, correspondingly. Therefore, it can be asserted that the spontaneous hydrolysis does not make significant contributions to the activity parameters of α-chymotrypsin. However, we shall note that parameters showed in Tables 2 and 3 have to a certain extent only an approximate character.

Processing of the spectrophotometrical data was made using the integral form of the Michaelis-Menten equation by the consideration of inhibition by the product of reaction *(25)*. This equation can be rearranged in the Foster-Niemann coordinates as:

$$p/t = (vK_p)/(K_p - K_m) - K_m (K_p + S_0)/(K_p - K_m)\, 1/t \ln/(S_0/[S_0 - P]) \quad (3)$$

where S_0 and P are the initial and current concentrations of substrate and product, K_m and K_P are Michaelis-Menten and inhibition constants, and v is the velocity of a process.

Figure 2 shows the Foster-Niemann plots $P/t = f\{1n[S_0/(S_0 - P)]/t\}$, where t and P were determined experimentally and S_0 had discrete values in the range of the experimental series from 5×10^{-5} up to 5×10^{-4} mol/L for the buffer solution of α-chymotrypsin and for α-chymotrypsin entrapped into the bilayer liposomes of poly-DDAB/AOT. These plots result in the line of slope:

$$K_m (K_p + S_0)/(K_m - K_p) = x \quad (4)$$

which we can transpose in the linear equation for K_m and K_P determination:

$$S_0 = x (1 - K_p/K_m) - K_p \quad (5)$$

Figure 3 shows these secondary plots constructed using the Foster-Niemann linearization for the same samples as were referred to Fig. 2. Using the plots in Fig. 2, the x values were determined for each concentra-

Table 2
Enzymatic Activity of Biocatalytic Systems by the NPH (pH 8.0, 19°C)

No.	Biocatalytic system	K_m* 10^4, mol/L	V_{max}* 10^8, mol/L*s	k_{cat}* 10^2, s^{-1}	k_{eff}*, L/s × mol	K_P* 10^5, mol/L
1.	α-Chymotrypsin in an aqueous buffer	3.8 ± 0.3	9.9 ± 0.5	1.2 ± 0.2	26 ± 4	1.1 ± 0.1
2.	α-Chymotrypsin after ultrasonication	9.6 ± 1.3	33 ± 9	4.8 ± 0.7	50 ± 14	1.1 ± 0.1
3.	Vesicles from poly-DDAB/AOT containing α-chymotrypsin	3.9 ± 0.3	3.4 ± 0.5	1.0 ± 0.3	26 ± 9	0.69 ± 0.05
4.	Empty vesicles from poly-DDAB/AOT	0.029 ± 0.004	0.004 ± 0.001	—	—	0.59 ± 0.04
5.	Mixture of vesicles from poly-DDAB/AOT and α-chymotrypsin in an aqueous buffer	0.9 ± 0.1	2.7 ± 0.6	0.6 ± 0.1	6 ± 1	0.17 ± 0.02
6.	Mixture of nanocapsules from poly-DDAB and α-chymotrypsin in cyclohexane after its following movement to aqueous solution by addition of AOT	2.6 ± 0.3	0.17 ± 0.05	0.13 ± 0.09	5 ± 4	5.4 ± 0.5

tion of S_0. The values of K_m and K_P were estimated from the secondary plots, and then the V_{max} value was calculated from Eq. 3.

The measurements of activity show that the entrapment of α-chymotrypsin into vesicular nanocapsules changes the kinetical parameters of enzymatic hydrolysis. It is convenient to compare the process efficiency of various catalytic systems when operating the second-order efficiency rate constant, k_{eff},

$$k_{eff} = k_{cat}/K_m = k_{+2}k_{+1}/(k_{-1} + k_{+2}) \quad (6)$$

where k_{cat} is the catalytic constant identical to the rate constant, k_{+2}, of destruction of the enzyme–substrate complex, ES, by product P formation according to the Michaelis-Menten model:

$$k_{cat} = V_{max}/E_0 \quad (7)$$

where E_0 is the general enzyme concentration.

Table 2 shows that K_m is not changed by the *p*-nitrophenyl acetate hydrolysis, and V_{max} decreases by a factor of 2.9 after the α-chymotrypsin immobilization within vesicles of poly-DDAB/AOT (system 3) relative to

Table 3
Enzymatic Activity of Biocatalytic Systems by the *p*-Acetyl-L-tyrosine Ethyl Ester Hydrolysis (pH 7.2, 25°C)

No.	Biocatalytic system	$K_m * 10^4$, mol/L	$V_{max} * 10^2$, mol/L*s	k_{cat}, s^{-1}	$k_{eff} * 10^3$, L/s × mol	$K_p * 10^6$, mol/L
1.	α-Chymotrypsin in an aqueous buffer	2.3 ± 0.2	16.5 ± 1.5	0.25 ± 0.05	7.3 ± 0.3	5.0 ± 0.3
3.	Vesicles from poly-DDAB/AOT containing α-chymotrypsin	4.1 ± 0.3	9.8 ± 0.9	1.0 ± 0.2	2.4 ± 0.6	36.3 ± 0.1
7.	Vesicles from poly-DDAB/Brij-97 (10:1 mol/mol) containing α-chymotrypsin	31 ± 3	220 ± 24	22 ± 6	7.1 ± 1.9	15.6 ± 0.8
8.	Vesicles from poly-DDAB/Brij-97 (5:1 mol/mol) containing α-chymotrypsin	15.9 ± 1.6	168 ± 14	4.2 ± 0.4	2.6 ± 0.7	36.5 ± 1.2
9.	Vesicles from poly-DDAB/Brij-97 (1:1 mol/mol) containing α-chymotrypsin	16.4 ± 1.7	71 ± 8	1.8 ± 0.2	1.1 ± 0.4	122 ± 7
10.	Nanocapsules from poly-DDAB in cyclohexane containing α-chymotrypsin	6.2 ± 0.7	2.4 ± 0.3	0.12 ± 0.01	0.19 ± 0.06	40.6 ± 1.7

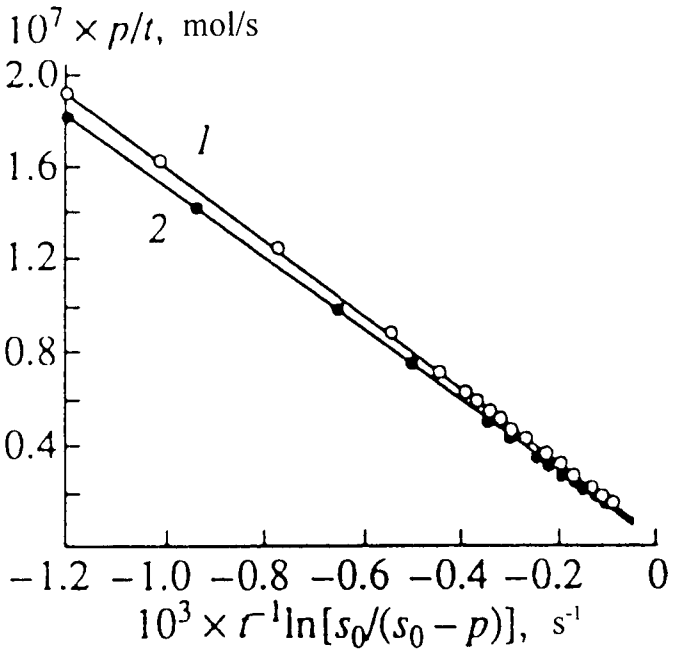

Fig. 2. The Foster-Niemann plots for: the buffer solution of α-chymotrypsin, 1, and for α-chymotrypsin entrapped into bilayer liposomes of poly-DDAB/AOT, 2, by the ATEE hydrolysis (pH 7.2, 25°C).

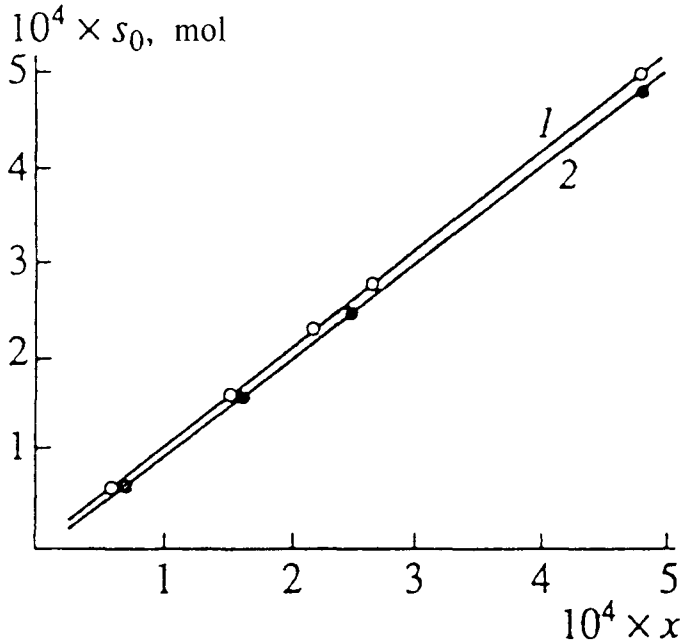

Fig. 3. Secondary plots constructed in coordinates S_0 - x by use of the Foster-Niemann linearization for the same samples as were referred on Fig. 2.

those constants for native enzyme (system 1). We shall note the E_0 values for system 3 are one-half those for other systems used. The constant of inhibition by the product of the reaction, $K_p = k^i_{-1}/k^i_{+1}$, decreases 1.6 times by the immobilization. This indicates the stabilization of a complex between the biocatalytic particle and the molecules of the product, *p*-phenolate-anion.

From Table 3, one can see that the K_m value increases by a factor of 1.8, and V_{max} decreases at 1.7 times by the ATEE hydrolysis after the α-chymotrypsin immobilization within vesicles of poly-DDAB/AOT (system 3) relative to these constants for system 1. This observation indicates the destruction of the enzyme–substrate complex by application of the specific substrate. However, by immobilization of enzyme, the k_{eff} values for both substrates are twice that for the free α-chymotrypsin action. The constant of inhibition by the product of reaction increases by a factor of 7.3 for the ATEE hydrolysis by the immobilization of enzyme. This also indicates destruction of the complex between the biocatalytic particle and product molecules, quite different from the *p*-nitrophenyl acetate hydrolysis.

The Michaelis, K_m, and inhibition, K_p, constants increase to a more considerable extent by the immobilization of α-chymotrypsin into poly-DDAB/Brij-97 vesicles than by the use of AOT as the outer shell. The value of V_{max} increases, too. Moreover, K_m and V_{max} reach the maximal values by

the low relative content of Brij-97 (1 molecule of Brij-97 to 10 monomer units of DDAB). The increase of the covering degree of nanocapsules by Brij-97 leads to the decrease of K_m and V_{max} values and to the significant increase of K_p. Nevertheless, all these parameters exceed by means of a nonionic surfactant the corresponding values for enzyme immobilized within the poly-DDAB/AOT vesicles. Therefore, the values of k_{cat} and k_{eff} are also more than for enzyme in the poly-DDAB/AOT vesicles.

The displayed effect of superactivity can be explained by the promoting influence of the ammonium groups *(26,27)* of poly-DDAB in the presence of a nonionic surfactant. On the other hand, the decrease in catalytic efficiency with the increase of the covering degree of nanocapsules can be explained by the influence of the *trans*-membrane diffusion of substrate and product molecules *(1,9)*.

Nanocapsules from poly-DDAB obtained after polymerization of the reversed DDAB micelles containing α-chymotrypsin (system 10) show a high activity in cyclohexane (Table 3). However, k_{eff} for the vesicular biocatalyst (system 3) is 13 times those for the system 10.

For checking whether the change of activity of α-chymotrypsin immobilized into liposomes is connected with UV irradiation or sonication of sample, the test experiment was performed. During this experiment, the buffer solution of α-chymotrypsin was subjected to irradiation and the following sonication at the facilities of the bilayer nanocapsule formation (system 2). Such a force leads to the increase of K_m by a factor of 2.5 (Table 2). K_p does not change by that. Therefore, it is more correct to compare systems 2 and 3 for the estimation of the kinetics parameter change. Thus, we can conclude using Table 2 data that K_m and k_{eff} decrease by factors of 2.5 and 1.9 by the immobilization of α-chymotrypsin within vesicles. This observation shows, on the contrary, the increase to some extent of stability of the Michaelis enzyme–substrate complex.

Inasmuch as bilayer vesicles themselves can sometimes act as catalysts because of so-called micellar catalysis *(27)*, the kinetics parameters were also measured for the empty poly-DDAB/AOT vesicles in the buffer solution. Indeed, vesicles themselves act as a weak catalyst by the NPA hydrolysis. However, the V_{max} value for system 4 is 850 times less that for the vesicles containing α-chymotrypsin or, accordingly, 2500 times less that for the nonimmobilized enzyme. The K_p values for systems 4 and 3 are practically the same. This allows us to suppose that the products of hydrolysis are rather absorbed on the surface of liposomes than inhibit the active site of enzyme. Indeed, the decrease of the formally evaluated K_m for empty vesicles in comparison to that for the α-chymotrypsin solution (system 1) brings evidence of the association of the initial substrate with vesicles.

For checking of location of the α-chymotrypsin globule within polymerized nanocapsules by preparation of the biocatalytic system according

to the proposed method, we have attempted to incorporate enzyme into polymerized reverse micelles in cyclohexane or into vesicles prepared in advance. Mixing of the α-chymotrypsin solution (system 2) and polymerized empty vesicles (system 4) results in system 5 (Table 2). It is seen that the K_m and K_P values are 4.3 times less than those for system 3. This observation can be explained by the dynamic adsorption of enzyme on the outer surface of a vesicle. Such an adsorption protects the active site by the formation of the Michaelis complex or the complex of enzyme with the product of reaction by inhibition. In such a manner, one can conclude that the α-chymotrypsin globules do not penetrate from aqueous solution into vesicles through polymer net. They also do not penetrate into polymerized reversed DDAB micelles in cyclohexane. Therefore, the longtime contact of α-chymotrypsin with cyclohexane leads to the inactivation of enzyme (system 6).

As a consequence, we can conclude that by encapsulation of α-chymotrypsin within reversed DDAB micelles, by their further polymerization and moving of nanocapsules to the aqueous phase as bilayer vesicles, using surfactants, such as AOT and Brij-97, the enzyme molecules are definitely situated within nanocapsules.

Profiles of the pH Activity for α-Chymotrypsin Encapsulated Within Vesicles

Figure 4 shows the pH activity profiles for α-chymotrypsin in an aqueous solution and immobilized one within nanocapsules from poly-DDAB/AOT or poly-DDAB/Brij-97. Activity was measured on the base of the optical density changes of a catalytic system at λ = 237 nm and 25°C after 5 min from beginning of the ATEE hydrolysis.

The optimum of activity for the free α-chymotrypsin in an aqueous solution is as usually at pH 7.5 *(1)*. A shoulder at pH 5.5 on the pH activity profile is caused by the increment of the profile for the spontaneous ATEE hydrolysis in solution without α-chymotrypsin (Fig. 4). Encapsulation of enzyme within poly-DDAB/Brij-97 nanocapsules shifts the pH optimum toward the base region up to pH 8.3 and within poly-DDAB/AOT nanocapsules up to pH 10.8. Moreover, an activity value measured at the optimum point decreases twice as much as that for the free α-chymotrypsin.

We can qualitatively explain this shift of the pH optimum toward the base region using the general form of the Henderson-Hasselbalch equation *(28)*:

$$pH = pK + \log [(Base)/(Acid)] \quad (8)$$

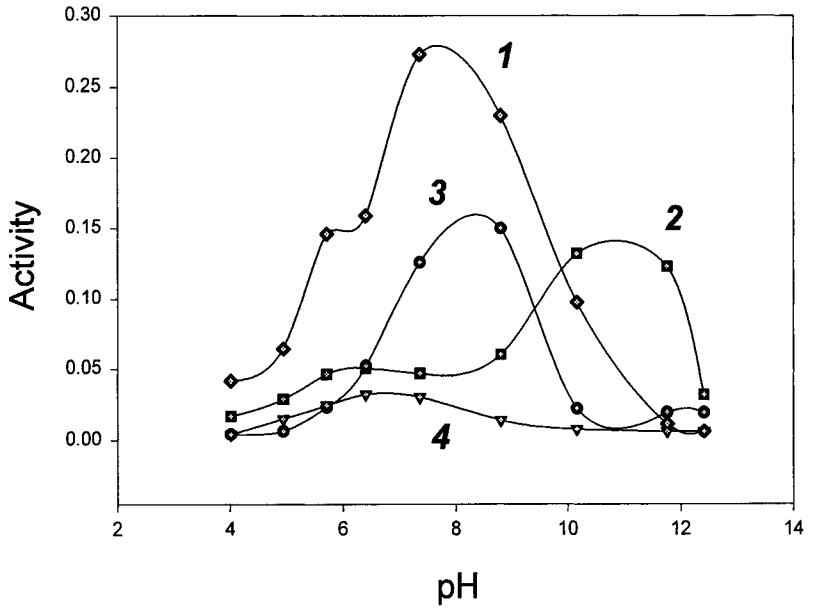

Fig. 4. The pH activity profiles for the ATEE hydrolysis at 25°C by the use of α-chymotrypsin in an aqueous solution (1), and immobilized one within nanocapsules from poly-DDAB/AOT (2) or poly-DDAB/Brij-97 (3). Curve 4 shows the spontaneous ATEE hydrolysis without α-chymotrypsin.

where pKs are in the studied case the values for amino acid residues situated in the active site of α-chymotrypsin, Ser-195, His-57, and Asp-102, and pH is the pH optimum value.

Encapsulation of enzyme within the basic poly-DDAB net leads to the appearance of the non-zero logarithmic increment in the proton–donor presence. The nonionic Brij-97 is a very weak proton acceptor. On the contrary, AOT is a strong proton donor. Therefore, the logarithmic term of Eq. 8 is more by the use of AOT as the outer monolayer than of Brij-97 at the same poly-DDAB concentration. The optimum is shifted in this case with $\Delta pH = 3.3$.

Thermostability of α-Chymotrypsin Immobilized Within Nanocapsules

It is well known that the native α-chymotrypsin in an aqueous solution loses activity at 45°C because of its denaturation. However, α-chymotrypsin immobilized in the polyacrylamide nanogranules can keep its activity on heating up to 80°C *(11)*.

For checking of the thermostability of α-chymotrypsin immobilized within nanocapsules systems 1, 3, and 5 were kept at 60 and 80°C for 20 min

Table 4
Enzymatic Activity of Biocatalytic Systems by the NPH Hydrolysis (pH 8.0, 19°C) after Keeping During 20 Min at Various Temperatures

Biocatalytic system	$k_{cat} \times 10^2$, s^{-1}			k_{eff}, L/s × mol		
	19°C	60°C	80°C	19°C	60°C	80°C
1. α-Chymotrypsin in an aqueous buffer	1.24	0	0	31.6	0	0
3. Vesicles from poly-DDAB/AOT containing α-chymotrypsin	1.02	0.55	0.07	25.6	7.5	1.5
5. Mixture of vesicles from poly-DDAB/AOT and α-chymotrypsin in an aqueous buffer	0.16	0.04	0	6.2	1.2	0

(Table 4). The immobilized α-chymotrypsin (system 3) preserved 31% of its activity measured without heating. Even after keeping the immobilized α-chymotrypsin at 80°C, biocatalyst preserved 8% of its initial activity. On the other hand, native α-chymotrypsin (system 1) lost enzymatic activity at 60°C. The activity of enzyme mixed with the empty poly-DDAB/AOT vesicles (system 5) diminished at 60°C up to 6% of initial one, and at 80°C vanished completely. This observation again corroborates the noninclusion of α-chymotrypsin into nanocapsules after their simple mixing.

To check the influence of the outer surfactant monolayer on the preservation extent of nanocapsules, the relationship of poly-DDAB/Brij-97 was varied by the activity measurements for the α-chymotrypsin containing nanocapsules after keeping with ATEE at 60°C for 20 min. Table 5 shows this increase of the Brij-97 contents promotes rising of the thermostability of enzyme.

Therefore, α-chymotrypsin immobilized within vesicular nanocapsules with the inner polymer monolayer has a high thermostability. We can conclude that the high thermostability is the common feature of enzymes encapsulated within polymer particles. We can suppose that polymer network sufficiently blocks the conformational transitions of an enzyme molecule and its denaturation by heating a system.

CONCLUSIONS

The present results indicate that "enzyme in the net" can act in both the organic solvent and the aqueous medium. Encapsulation within the polymerized unilamellar vesicles does not result in considerable dimin-

Table 5
Influence of Surfactant Ratio in Nanocapsules from Poly-DDAB/Brij-97 Containing α-Chymotrypsin on the Maximal Velocity of the *p*-Acetyl-L-tyrosine Ethyl Ester Hydrolysis (pH 7.2, 25°C) After 20 Min at 60°C

[DDAB]/ [Brij-97],mol/mol	$V_{max} \times 10^7$, mol/s		Percent to the initial velocity
	Before heating	After heating	
10:1	220	108	49
5:1	168	109	65
1:1	71	51	72

ishing of enzymatic activity. In addition to that, we can vary the activity levels and the position of an optimal point on the pH activity profiles using various ionic and non-ionic surfactants as the mild outer coat and the polymer/surfactant ratio.

Moreover, encapsulated enzyme has a high thermostability keeping its own activity up to 80°C. The polymer network blocks the conformational transitions of the enzyme globule by heating of a system.

ACKNOWLEDGMENT

One of the authors (Y. E. S.) gratefully acknowledges Swedish Institute and KTH for financial support that enabled his investigations in Sweden.

REFERENCES

1. Shuler, M. L. and Kargi, F. (1992), *Bioprocess Engineering. Basic Concepts.* Prentice Hall, Englewood Cliffs, NJ.
2. Johnson, J. C. (1979), *Immobilized Enzymes: Preparation and Engineering: Recent Advances.* Park Ridge, NJ.
3. Martinek, K., Levashov, A. V., Klyachko, N. L., and Berezin, I. V. (1977), *Proc. Acad. Sci. USSR* **236,** 920–923.
4. Khmelnitsky, Yu. L., Levashov, A. V., Klyachko, N. L., and Martinek, K. (1984), *Russian Chem. Rev.* **53,** 545–565.
5. Shapiro, Yu. E., Pykhteeva, E. G., Levashov, A. V., and Klyachko, N. L. (1994), *Biol. Membr.* **7,** 277–290, 379–390.
6. Puchkayev, A. V. and Metelitsa, D. I. (1994), *Biochemistry (Russia)* **59,** 45–54.
7. Klyachko, K., Levashov, A. V., Pshezetsky, A. V., Bogdanova, N. G., Berezin, I. V., and Martinek, K. (1986), *Eur. J. Biochem.* **161,** 149–154.
8. Lasic, D. D. (1993), *Liposomes: From Physics to Applications.* Elsevier, Amsterdam.
9. Shapiro, Yu. E., Smirnova, A. V., Makarevich, I. F., and Ulesov, A. V. (1997), *Biopolymers and Cell (Ukraine)* **13,** 213–218.
10. Kurganov, B. I., Tsetlin, L. G., Chernov, N. N., Chebotareva, N. A., and Rudakova, I. P. (1991), *Proc. Acad. Sci. USSR* **319,** 1488–1490.

11. Matveeva, E. G., Levashov, A. V., Shegolev, A. A., Ryzhova, V. V., Polyakov, V. A., and Martinek, K. (1985), *Biotechnology (Russia)* No. **6,** 84–93.
12. Shapiro, Yu. E. (1988), *Russian Chem. Rev.* **57,** 1253–1272.
13. Paleos, C. M. (1985), *Chem. Soc. Rev.* **14,** 45–68.
14. Buschl, R., Folda, T., and Ringsdorf, H. (1984), *Makromol. Chem.* (Supp.) 6, 245–258.
15. Paleos, C. M., Dais, P., and Malliaris, A. (1984), *J. Polym. Sci., Polym. Chem. Ed.* **22,** 3383–3391.
16. Shapiro, Yu. E., Orlov, Yu. N., Yegorov, V. V., and Zubov, V. P. (1985), *Proc. Moscow Univ., Chem.* **25,** 487–492; 595–600.
17. Lukin, Y. V., Adamenkova, M. D., Kartusov, S. N., Lanshina, L. V., Shapiro, Yu. E., Yegorov, V. V., et al. (1984), *Russian J. Phys. Chem.* **58,** 2233–2239.
18. Yegorov, V. V., Shapiro, Yu. E., and Zubov, V. P. (1987), *Proc. Moscow Univ. Chem.* **28,** 183–186.
19. Babilis, D., Dais, P., Margaritis, L. H., and Paleos, C. M. (1985), *J. Polym. Sci. Polym. Chem. Ed.* **23,** 1089–1098.
20. Iino, Y., Ogata, Y., Shigehara, K., and Tsuchida, E. (1985), *Makromol. Chem.* **186,** 923–931.
21. Shapiro, Yu. E., Pykhteeva, E. G. and Fedorova, G. V. (1997), *Russian J. Bioorganic Chem.* 23, 174–182.
22. Shapiro, Yu. E., Viktorov, A. V., Volkova, V. I., Barsukov, L. I., Bystrov, V. F., and Bergelson, L. D. (1975), *Chem. Phys. Lipids* **14,** 227–232.
23. Regen, S. L., Shin, Y. S., Hainfeld, J. F., and Wall, J. S. (1984), *J. Am. Chem. Soc.* 106, 5756–5757.
24. Van de Hulst (1957), *Light Scattering by Small Particles.* Wiley, New York.
25. Keleti, T. (1986), *Basic Enzyme Kinetic.* Wiley, New York.
26. Matyjaszewski, K. (1996), *Curr. Opinion Solid State Mater. Sci.* **1,** 769–774.
27. Rathman, J. F. (1996), *Curr. Opinion Coll. Interfase Sci.* **1,** 514–518.
28. Lehninger, A. L. (1970), *Biochemistry,* Worth, New York.