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Amphiphilic poly-*N*-vinylpyrrolidone nanocarriers with incorporated model proteins

A N Kuskov[1], A L Villemson[2], M I Shtilman[1], N I Larionova[2], A M Tsatsakis[3], I Tsikalas[4] and A K Rizos[4,5]

[1] Department of Polymers, D I Mendeleyev University of Chemical Technology, 9 Miusskaya Square, Moscow 125047, Russia
[2] Department of Chemistry, M V Lomonosov Moscow State University, 119992 Moscow, Russia
[3] Medical School, University of Crete, Voutes, 71409 Heraklion, Crete, Greece
[4] Department of Chemistry and Foundation for Research and Technology—Hellas (FORTH), University of Crete, PO Box 2208, Heraklion 71003, Crete, Greece

E-mail: rizos@chemistry.uoc.gr

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Abstract

New nanoscaled polymeric carriers have been prepared on the basis of different amphiphilic water-soluble derivatives of poly-*N*-vinylpyrrolidone (PVP). The polymer self-assembly and interaction with model proteins (Bowman–Birk soybean proteinase inhibitor (BBI) and its hydrophobized derivatives) were studied in aqueous media. The possibility of inclusion of both BBI and hydrophobized oleic acid derivatives of BBI in amphiphilic PVP aggregates was investigated. It was ascertained that polymeric particles of size 50–80 nm were formed in certain concentrations of amphiphilic PVP and poorly soluble dioleic acid derivatives of BBI. Such polymeric aggregates are capable of solubilization of dioleoyl BBI with a concomitant prevention of its inactivation at low pH values.

1. Introduction

Nowadays, different types of polymers are used for the creation of drug delivery systems, for increasing protein stability and for providing prolongation and targeting of their effect. All these measures allow an increase in the therapeutic efficiency of medications [1–4]. Biocompatible and biodegradable polymers, which find applications as components of injection or implantation systems, are the most pertinent materials for the creation of such systems [5, 6]. Drug delivery formulations, created on the basis of amphiphilic water-soluble polymers, are provoking increasing interest as they allow the solubilization of poorly soluble drugs. The accumulation of preparations in different organism tissues and organs can be controlled,

[5] Author to whom any correspondence should be addressed.

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depending on the aggregate size and properties [4, 7, 8]. Another advantage of micellar forms is the ability of their simple, quick and reproducible preparation in large quantities. In this study we will try to find pathways for the creation of new nanoscaled carriers for protein drugs, which are now widely used for treating most diseases.

The physicochemical and biological properties of protein and peptide drugs (PPDs) are different from those of conventional ones, such as molecular size, biological half-life, conformational stability, physicochemical stability, solubility, oral bioavailability, dose requirement and administration. Therefore, the design and manufacture of PPD delivery systems has been a challenging area of research. So far, some of the conventional colloidal drug delivery systems, such as liposomes and nanoparticles, have been developed and tested for PPDs [9–11]. However, these conventional carriers are rapidly cleared from the systemic circulation, and they end up almost exclusively in the mononuclear phagocyte system (MPS), mainly the macrophages in liver and spleen [12]. Of course, this extensive uptake is advantageous for treating illnesses of the reticuloendothelial system (RES) because they provide high local concentration of pharmacotherapeutic agents. In the past few years, there has been an increasing interest in developing stealth nanoparticles as drug carrier systems to meet all the requirements. One of main methods for preparation of stealth nanoparticles or long-circulating nanoparticles is to modify their surface with a hydrophilic, flexible and non-ionic polymer, such as, for example, poly(ethylene glycol) (PEG) [13–16]. The biodegradable PEG-coated nanoparticles have found important potential therapeutic applications as injectable colloidal systems for the controlled release of drugs and site-specific drug delivery [8, 17, 18]. The stealth nanoparticles compared to other long-circulating systems show better shelf stability and ability to control the release of the encapsulated compounds [13, 17], but information on the stealth nanoparticles used to deliver protein drugs has not been clear so far.

The aim of the present work was to assess the merits of nanoparticles on the basis of another amphiphilic non-ionic polymer, poly-*N*-vinylpyrrolidone (PVP), as a protein drug carrier. For this purpose, amphiphilic poly-*N*-vinylpyrrolidones of molecular weight (MW) 2600, 4000 and 6500 Da with one end being the hydrophobic octadecyl group (PVP-OD) were synthesized using previously developed methods [19, 20]. Bowman–Birk soybean proteinase inhibitor (BBI) and its hydrophobized oleic acid derivatives (Ole-BBI and Ole_2-BBI) were chosen as model proteins. BBI can be used for simultaneous inhibition of trypsin and chymotrypsin [21] or for inhibition of human leucocyte elastase [22]. Also, BBI actively suppresses cell transformation *in vitro* and carcinogenesis *in vivo* [23]. However, the high therapeutic potential of such an inhibitor is limited by its rapid excretion from the organism [24]. Earlier it was shown that BBI preparations modified by derivatives of fatty unsaturated acid (oleic, linoleic or α-linolenic acid) possess greater membrane-acting activity in comparison with native protein [25] and have increased affinity to chymotrypsin and leucocyte elastase [26]. Still, the solubility of hydrophobized BBI derivatives is very low, and this prevents their application in medicine. All the BBI preparations were encapsulated within nanoparticles made of PVP-OD. The particles were characterized in terms of size, size distribution, shape, *in vitro* release of the protein and antitryptic activity.

2. Materials and methods

2.1. Materials

N-vinylpyrrolidone (VP), azobisisobutyronitrile (AIBN), mercaptoacetic acid (MAA), *N*,*N*-dicyclohexylcarbodiimide (CCD), octadecylamine, pyrene, pyridine, muriatic ethyl ester of *N*-benzoyl-*L*-arginine, ethyl ester of *N*-benzoyl-*L*-tyrosine, *N*-hydroxysuccinimide ester of

oleic acid and Bowman–Birk soybean proteinase inhibitor were purchased from Sigma, USA; citraconic anhydride, trypsin and chymotrypsin were purchased from Merck, Germany. The substrate for electron microscopy, 0.2% polyvinylformal, was from Sigma-Aldrich, USA. All solvents and components of buffer solutions were analytical grade preparations.

2.2. Methods

Amphiphilic poly-*N*-vinylpyrrolidones were synthesized in two steps, similar to our previous experiments [19, 20]. The BBI, modified by one (Ole_1-BBI) or two (Ole_2-BBI) oleic acid residues, was prepared using previously developed methods [27–29]. The investigation of amphiphilic PVP self-assembling processes in the absence and in the presence of BBI preparations was carried out by addition of appropriate amounts of protein to the PVP-OD solutions with different concentrations in distilled water or in a physiological solution (0.15 NaCl, pH 7.4). The solution was sonicated at 10 W for 15 min using a Sonic Desmembrator 60 (Fisher Scientific, USA); the cycle was repeated three times with 1 min intervals. Then the samples were taken and analysed. To obtain protein isolation from the colloid system, it was precipitated by a triple volume of acetone ($-20\,^{\circ}$C). The sediment was centrifuged and dried out. The purity of the protein was controlled electrophoretically. The electrophoresis of protein and mixtures of BBI and PVP-OD was carried out in 15% polyacrylamide gel (PAAG) according to Reisfeld at pH 4.5 [30]. The load of protein on one track was 6.5 μg.

To estimate the critical aggregation concentration (CAC) values for different PVP derivatives, the method used was based on the solubilization of the water-insoluble fluorescent dye, pyrene, in nano-aggregates. For this purpose, aliquots of 10 μl of pyrene solution in acetone (10 mg ml^{-1}) per test tube were dried under vacuum. The tubes were supplemented with 2 ml of serial dilutions (10^{-4}–10^{-10} M) of various PVP-OD samples, and shaken overnight at room temperature. The samples were filtered through 0.2 μm filters to remove the non-solubilized pyrene, and the fluorescence intensity of the solubilized pyrene was measured at an excitation wavelength of 340 nm and emission wavelength of 385 nm using a Hitachi 650-10 S spectrophotometer (Hitachi Instruments Inc, Japan). The average size of polymeric nano-aggregates obtained in water and in physiological solution and their size distribution were determined by dynamic light scattering [31–37] at a temperature of 25 °C. A multi-bit, multi-τ full digital correlator (ALV-5000) was used that covered a dynamic range of about ten decades. For each sample, the mean diameter of six determinations was calculated by applying multimodal analysis. The form of amphiphilic PVP aggregates was determined by transmission electron microscopy (TEM) using a JEOL JEM-1000 apparatus (Germany) at a voltage of 20 kV.

The antitryptic activity of BBI preparations was determined by the relative esterase activity of trypsin that stayed free after its incubation with inhibitor preparations. In order to accomplish that, 0.1 ml of 0.1 mg ml^{-1} trypsin solution in 0.001 M hydrochloric acid was placed in the cuvette, 0.1–0.7 ml of amphiphilic PVP solution of different concentrations was added, and the total volume mixture was brought up to 0.8 ml with 0.05 M Tris-HCl, pH 8.0. The mixture was kept at 25 °C for 10 min. After that, 0.2 ml of 1.5 μM BAEE solution in Tris-HCl (pH 8) were added and the change in optical density was registered during 1–5 min at a wavelength of 253 nm with a Shimadzu spectrophotometer UV-265 FW (Japan).

3. Results and discussion

3.1. Nano-aggregates from amphiphilic PVP

Since all synthesized amphiphilic PVP derivatives are water soluble and contain significantly larger hydrophilic fragments than hydrophobic ones, it was assumed that hydrophobic

Table 1. Synthesized *N*-vinylpyrrolidone amphiphilic polymers and parameters of their aggregation in aqueous medium (C_p: amphiphilic polymer concentration).

			Particle average diameter (nm)		
			$C_p \approx$ CAC	$C_p = 5$ mg ml^{-1}	
Polymer code	Mn (Da)	CAC (mg ml^{-1})	Water[a]	Water[b]	Physiological solution[b]
PVP-OD 2600	2600	0.015	30	420	260
PVP-OD 4000	4000	0.029	40	450	280
PVP-OD 6500	6500	0.064	50	500	310

[a] The average diameter error is ±3 nm.
[b] The average diameter error is ±9 nm.

fragments, minimizing their contact with water, may 'fold' themselves into the globule shape surrounded by the hydrophilic block. This leads to the formation of nanosized particles. Generally, amphiphilic polymers are able to associate in solutions at a concentration higher than some threshold concentration limits (the so-called critical micelle concentration). For all samples of synthesized PVP amphiphilic polymers the obtained aggregates are of complex structure and their size exceeds the size of simple micelles corresponding to polymers of given MW. That is why in this study we use the term 'critical aggregation concentration' (CAC) to describe such a concentration limit.

A method of measuring a fluorescent probe (poorly soluble in water pyrene) was adapted for CAC estimation. After introduction of polymeric aggregates into the system, the amount of fluorescent probe that has passed into the aqueous medium can be measured after separation of the undissolved part of the probe. CAC values (see table 1) are in the micromolar range of concentrations. This means that the obtained amphiphilic polymers form aggregates at relatively low concentrations. The ability of water-soluble amphiphilic polymers for self-organization is deeply connected with interactions of their hydrophobic fragments. As is shown in table 1, the tendency to aggregate formation in these polymers rises by increasing the hydrophobic fragment share in the amphiphilic polymer. The polymer with the lower molecular weight in the water-soluble PVP block has the lower CAC. This can be explained by the fact that the interactions of the polymer molecules in aqueous media depend not only on the relative share of hydrophobic fragments in the macromolecule but also on their accessibility.

The size of polymeric nanoparticles obtained in aqueous environment was studied using dynamic light scattering. All PVP derivatives tested formed micelle-like aggregates in water solutions at concentrations near CAC values with the average size between 30 and 50 nm and narrow size distribution, as follows from the particle size measurement (table 1). At higher polymer concentrations, the nanoparticles associate, with the formation of larger aggregates with complex structure (table 1). The particle size distribution (figure 1(A)) obtained for amphiphilic PVP with MW 4000 Da and *n*-octadecyl hydrophobic group shows that the produced dispersion contains particles from 160 to 340 nm in size with the average diameter of 240 nm. Average diameters of aggregates in different media are shown in table 1. In all cases, the average size of the obtained particles was smaller for physiological solution than for water. Such lowering of particle size can be explained by the strengthening of hydrophobic interactions in solution with higher ionic force, which leads to the formation of a more compact structure.

The morphology of amphiphilic polymer aggregates is quite diverse. In our study, the form of produced nano-aggregates was investigated by using transmission electron microscopy (TEM). Experimental data, obtained by TEM, provide evidence of spherical particle formation with a wide range of size from 80 to 500 nm (figure 2(A)).

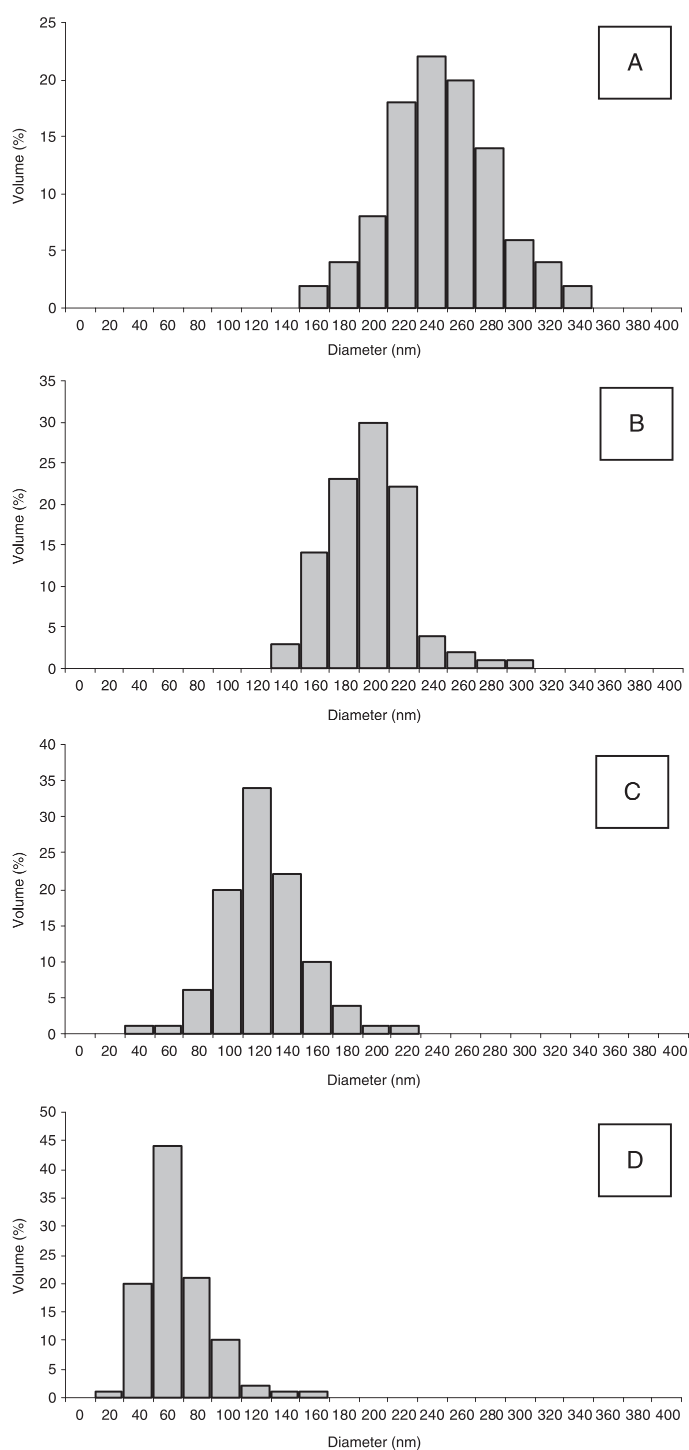

Figure 1. Size distribution of PVP-OD 4000 particles in physiological solution. (A) PVP-OD 4000 (0.5 mg ml^{-1}); (B) PVP-OD 4000 (0.5 mg ml^{-1}) + BBI (2.0 mg ml^{-1}); (C) PVP-OD 4000 (0.5 mg ml^{-1}) + Ole_1-BBI (2.0 mg ml^{-1}); (D) PVP-OD 4000 (0.5 mg ml^{-1}) + Ole_2-BBI (2, 0 mg ml^{-1}).

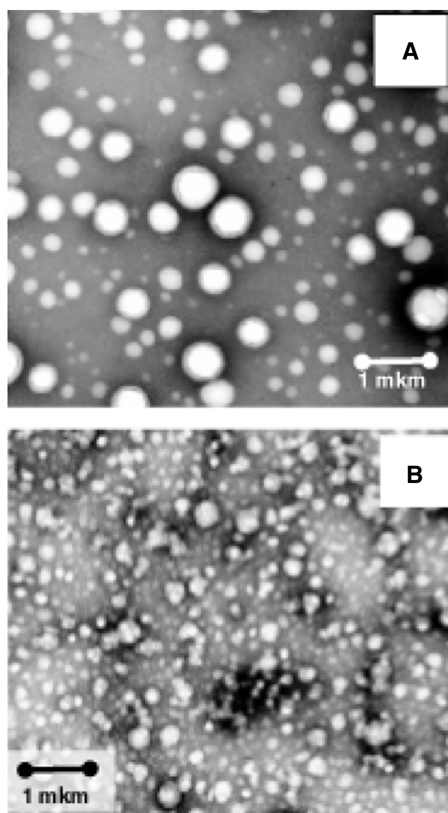

Figure 2. Photomicrography of prepared particles obtained by the TEM method. (A) PVP-OD 2600 (C = 0.5 mg ml^{-1}); (B) PVP-OD 2600 (0.5 mg ml^{-1}) + Ole$_2$-BBI (2.0 mg ml^{-1}).

3.2. Interaction of amphiphilic PVPs with model proteins

The results of dynamic light-scattering investigations for different BBI derivatives and PVP-OD mixtures are shown in tables 2 and 3. Figure 4 shows experimental correlation functions of PVP-OD 6500 (0.5 mg ml^{-1}), and PVP-OD 6500 (0.5 mg ml^{-1}) + Ole$_2$-BBI (2.0 mg ml^{-1}) at a scattering angle of 90° along with their normalized distributions of relaxation times. The increase of native BBI abundance in solution from 0.5 to 2.0 mg ml^{-1} leads to the small decrease in particle size (table 2). Also, the introduction of BBI lowers the average size of particles compared to empty polymeric aggregates (figure 1(B)). Probably, the presence of protein compacts and regulates the structure of colloid aggregates. Using TEM it was shown that the obtained particles are of spherical shape. It should be mentioned that in contrast to the native BBI, Ole$_1$-BBI and Ole$_2$-BBI initiate the formation of complex aggregates at low concentrations of PVP-OD in water. Both hydrophobized BBI derivatives decreased the average size of the particles and constricted their size distribution (figures 1(C), (D)). The most pronounced effect was observed for Ole$_2$-BBI. This difference can be explained by the increased affinity of protein to amphiphilic PVP-OD due to the strengthening of hydrophobic interactions between them after the attachment of one or two oleic acid residues.

It should also be emphasized that at a certain ratio of PVP-OD and BBI concentrations in the mixture, the protein becomes fully soluble in aqueous medium. As we can see from table 2, the average size of the particles even decreases to 50 nm. At the same time a

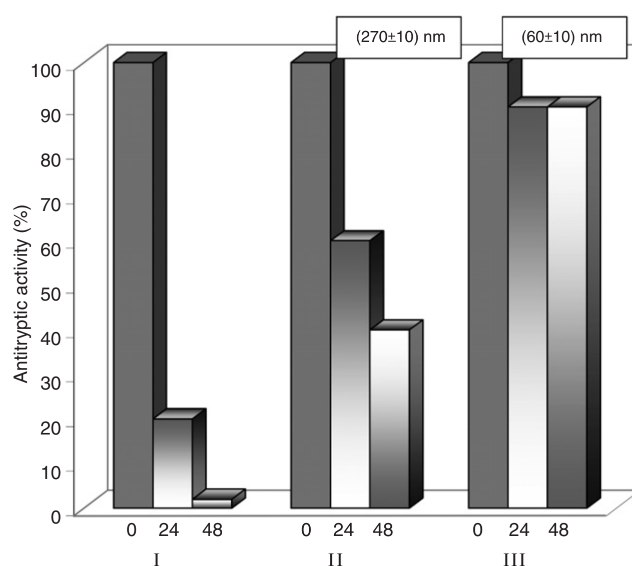

Figure 3. Change of antitryptic activity of Ole_2-BBI preparations in the presence and in the absence of PVP-OD 6500 at pH 1.4: (I) Ole_2-BBI (0.5 mg ml^{-1}); (II) PVP-OD 6500 (0.5 mg ml^{-1}) + Ole_2-BBI (0.5 mg ml^{-1}); (III) PVP-OD 6500 (0.5 mg ml^{-1}) + Ole_2-BBI (2.0 mg ml^{-1}).

size of 30–50 nm for these particles is comparable with the predicted micelle size for this polymer. We can assume that in this case the destruction of large polymeric ensembles and formation of micelle-like colloid particles takes place and that the obtained particles solubilize hydrophobized preparations. At low concentrations of Ole_2-BBI the strength of interaction between polymer chains prevails, but with increasing concentration of hydrophobized protein in the mixture, the main contribution comes from hydrophobic interactions between protein fatty acid residues and the polymer. As a result of this, small micelle-like aggregates are formed instead of larger complex ones (figure 2(B)).

The introduction of BBI and its mono-acylated derivative in 0.02 mg ml^{-1} solution of amphiphilic polymers (PVP-OD4000 and PVP-OD 6500) unlike Ole_2-BBI does not lead to the formation of polymeric aggregates at low concentrations of proteins. Probably, the degree of Ole_1-BBI hydrophobicity is insufficient for initiating the formation of small micelle-like aggregates by amphiphilic PVP-OD. However, similarly to the case of Ole_2-BBI preparations, a tendency of decreasing particle size with increasing Ole_1-BBI concentration in the mixture is observed. Thus, after the encapsulation of mono-acylated BBI derivatives in polymeric aggregates, both large complex aggregates and small colloid particles are present in aqueous medium.

3.3. Antitryptic activity

During the solubilization process the substance is incorporated in the particle core and its interaction with the environment is practically impossible. For indirect evidence of protein solubilization, the denaturing effect of the low-pH solution was measured against the plain Ole_2-BBI preparation in aqueous medium and on Ole_2-BBI which was preliminarily encapsulated in polymeric aggregates of different size. As one can see in figure 3(I), plain Ole_2-BBI preparations lose 80% of their antitryptic activity in 24 h and are fully inactivated in 48 h. In contrast, protein encapsulation in polymeric aggregates leads to preservation of its activity

Table 2. The size of PVP-OD aggregates in water in the presence of BBI preparations. The average diameter error is ±8 nm.

PVP-OD2600 (mg ml^{-1})	BBI (mg ml^{-1})	Average diameter (nm)	Ole_2-BBI (mg ml^{-1})	Average diameter (nm)
0.02	0	70	0	50
	1.0	70	1.0	60
	2.0	50	2.0	50
0.5	0.5	380	0.5	310
	1.0	340	1.0	100
	2.0	260	2.0	50
5.0	0.5	410	0.5	350
	1.0	370	1.0	130
	2.0	290	2.0	80
PVP-OD4000 (mg ml^{-1})	**BBI (mg ml^{-1})**	**Average diameter (nm)**	**Ole_2-BBI (mg ml^{-1})**	**Average diameter (nm)**
0.02	0	No particles	0	No particles
	1.0	No particles	1.0	90
	2.0	No particles	2.0	60
0.5	0.5	430	0.5	370
	1.0	360	1.0	120
	2.0	290	2.0	90
5.0	0.5	430	0.5	390
	1.0	400	1.0	160
	2.0	340	2.0	110
PVP-OD6500 (mg ml^{-1})	**BBI (mg ml^{-1})**	**Average diameter (nm)**	**Ole_2-BBI (mg ml^{-1})**	**Average diameter (nm)**
0.02	0	No particles	0	No particles
	1.0	No particles	1.0	110
	2.0	No particles	2.0	80
0.5	0.5	470	0.5	400
	1.0	390	1.0	230
	2.0	330	2.0	100
5.0	0.5	520	0.5	440
	1.0	460	1.0	200
	2.0	380	2.0	130

(figure 3(II)). In the mixture of amphiphilic polymer with protein consisting basically of large aggregates, the protein activity decreases by 60% in 48 h. It can be assumed that in this case the preparation, which is not included in the particle core and is localized between polymer chains of large aggregates and so can interact with the aqueous medium, becomes inactivated. At the same time the formation of small micelle-like polymeric particles and encapsulation of protein in these particles leads not only to an increasing solubility but to practically total preservation of inhibitor antiproteinase activity (90%) (figure 3(III)). This protective effect can be explained by the full absence of interaction between hydrophobized protein preparations with solvents and serves as evidence of total Ole_2-BBI solubilization. The small loss of antitryptic activity (10%) can be associated both with conformational changes in Ole_2-BBI preparation structure during the process of aggregate formation and further embedding in hydrophobic particle core and with possible protein partial contact with the aqueous medium.

Table 3. The size of PVP-OD aggregates in buffer 0.05 M Tris-HCl, 0.15 M NaCl, pH 8.5 in the presence of BBI hydrophobized derivatives. The average diameter error is ±8 nm.

PVP-OD2600 (mg ml^{-1})	Ole_1-BBI (mg ml^{-1})	Average diameter (nm)	Ole_2-BBI (mg ml^{-1})	Average diameter (nm)
0.02	0	50	0	50
	1.0	50	1.0	40
	2.0	40	2.0	40
0.5	0.5	200	0.5	160
	1.0	140	1.0	70
	2.0	90	2.0	50
PVP-OD4000 (mg ml^{-1})	**Ole_1-BBI (mg ml^{-1})**	**Average diameter (nm)**	**Ole_2-BBI (mg ml^{-1})**	**Average diameter (nm)**
0.02	0	No particles	0	No particles
	1.0	No particles	1.0	70
	2.0	90	2.0	50
0.5	0.5	230	0.5	190
	1.0	180	1.0	90
	2.0	120	2.0	60
PVP-OD6500 (mg ml^{-1})	**Ole_1-BBI (mg ml^{-1})**	**Average diameter (nm)**	**Ole_2-BBI (mg ml^{-1})**	**Average diameter (nm)**
0.02	0	No particles	0	No particles
	1.0	No particles	1.0	80
	2.0	110	2.0	70
0.5	0.5	270	0.5	230
	1.0	200	1.0	90
	2.0	130	2.0	80

3.4. Protein release kinetics

It is well known that solubilization of proteins using polymeric systems often leads to irreversible conformation changes in the structure of the protein globule and to the full loss of protein biological activity. In this work the BBI release kinetics from the polymeric nanoparticles on the basis amphiphilic PVP derivatives was studied and the biological activity of released protein was determined. The BBI release kinetics from PVP-OD 2600 nanoparticles (figure 5) shows that after 48 h of incubation in phosphate (pH 7.4) or acetate (pH 5.5) buffer practically complete protein release is observed. Further incubation of nanoparticles in buffer is accompanied by very slow accumulation of BBI in solution. The BBI activity against trypsin was determined after 48 h incubation of polymeric nanoparticles. The activity was about 85–90%. This points to the stability of the protein to the processes of encapsulation.

4. Conclusions

Polymeric aggregates of PVP-OD with and without encapsulated native or hydrophobized soybean proteinase inhibitor were prepared and characterized. The difference in affinity of proteins with different oleoylation degree to amphiphilic PVP was determined, which becomes apparent when Ole_2-BBI interacts with polymer of concentration lower than the aggregation concentration. It was shown that the inclusion of hydrophobized proteins in PVP-

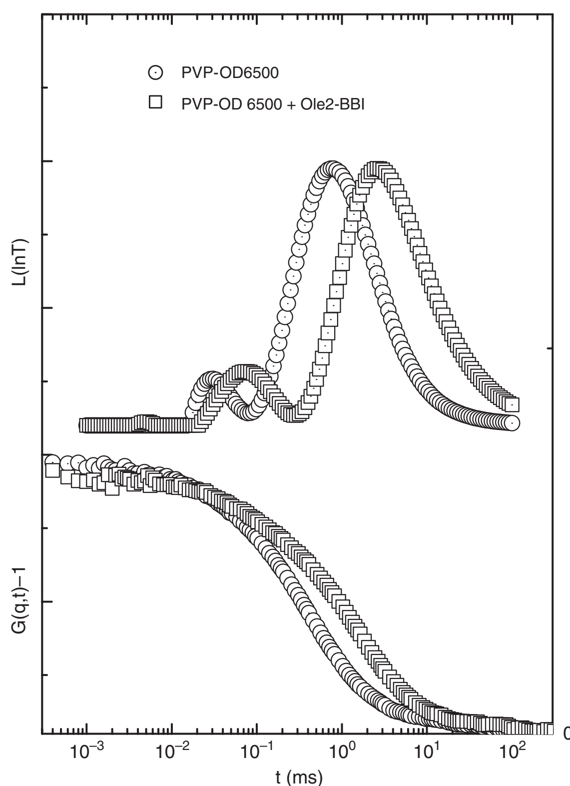

Figure 4. Experimental correlation functions of PVP-OD 6500 (0.5 mg ml^{-1}), and PVP-OD 6500 (0.5 mg ml^{-1}) + Ole_2-BBI (2.0 mg ml^{-1}) at a scattering angle of 90° along with their normalized distributions of relaxation times.

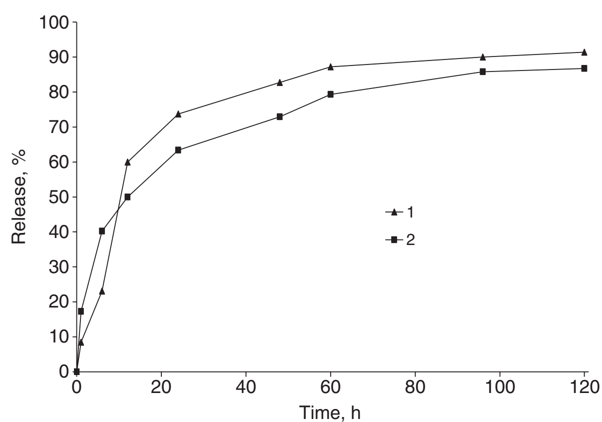

Figure 5. BBI release from PVP-OD 2600 nanoparticles in 0.05 M phosphate (pH 7.4; 37 °C) (1) or acetate (pH 5.5; 37 °C) (2) buffer.

OD aggregates in aqueous medium can provide small micelle-like particles (50 nm), which fully solubilize the protein and prevent it from inactivation.

Acknowledgments

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