PREPARATION OF PROTEIN – POLYELECTROLYTE COMPLEX BY SELF-ASSEMBLING

Loredana E. NITA,[a,*] Aurica P. CHIRIAC, Alina DIACONU and Mihai ASANDULESA

"Petru Poni" Institute of Macromolecular Chemistry, 41-A, Grigore Ghica Voda Alley, 700487 Iaşi, Roumania

The complexation of globular proteins with both natural and synthetic flexible polyelectrolytes is of considerable technological importance. In the study the critical conditions for the formation of complex systems between bovine serum albumin (BSA) and polyelectrolytes was examined. Poly(aspartic acid) (PAS), belonging to the family of synthetic polypeptides, was chosen as polyelectrolyte, as it is a typical biocompatible, biodegradable, and water-soluble polymer. Different molar ratio protein-polyelectrolyte complexes obtained by mixing PAS and BSA solutions were investigated by various characterization techniques such as: DLS studies, chemical imaging on near infrared region, ESEM studies and dielectric spectroscopy.

The optimum conditions for the BSA/PAS complex formation were established. Our results showed that the best proportion for the formation of interpolymer complex was the equimolecular molar ratio (1/1).
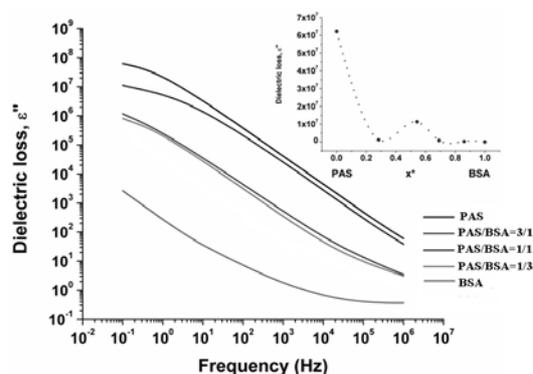
INTRODUCTION

During recent decades, self-organization phenomena in macromolecular systems based on synthetic polyelectrolytes, as well as in complex systems containing natural macromolecules, have been extensively and successfully investigated. First of all, the studies have been aimed at gaining deeper understanding of self-organization mechanisms in biological systems. An example is the phenomenon of DNA compaction, which was revealed upon examination of the conformational behavior of DNA double-helix complexed with cationic surfactants in nonpolar solvents such as chloroform.[1-3] The results opened unique opportunities for the design and fabrication of new polymeric materials and structures of various scales and dimensions, ranging from nano-to macroscopic, and with variable characteristics determined by the environmental conditions. Such materials and structures are referred to as functional, whereas the co-assemblies themselves are regarded as "smart".[4]

The complexation of proteins with natural and synthetic polyelectrolytes is interesting at least from two points of view. The first concerns the way in which the polymers interact with nonflexible protein molecules, an understanding of which could provide a better explanation of the interaction mechanism of polyelectrolytes with ionic colloidal particles. The second refers to the extent of biochemical activity maintained in the resulting complexes, the answer to which is central for the molecular design of composite protein-polymer systems, such as immobilized enzymes, as well as to the design of protein separation processes using water-soluble polymers.[5]

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[*] Corresponding author: e-mail: lnazarie@yahoo.co.uk; tel.: +40232217454; fax: +40232211299

The complexation of globular proteins with either natural or synthetic flexible polyelectrolytes is also of considerable technological importance. Systematic studies of Dubin and co-workers on the complexation of globular proteins with synthetic polyelectrolytes of high linear charge density have revealed two critical pH values for complexation.[6]

Proteins and polyelectrolytes interact, primarily via electrostatics, to form complexes, which can have widely varied stoichiometry, architectures and phase states. To a considerable extent, these represent equilibrium systems and are thus amenable to numerous techniques of investigation. The biofunctionality of the proteins, apparently unperturbed under most conditions, has resulted in growing interest in the behavior of these complexes under laboratory and in vivo conditions. A broad range of experimental techniques makes it possible to establish the boundaries that define the existence of multiple states that result from protein–polyelectrolyte interactions. These techniques include but are not limited to: turbidimetry and light scattering (both static and dynamic); small angle neutron scattering; calorimetry (primarily isothermal titration); microscopies – AFM, EM (including SEM and cryo) and confocal; surface plasmon resonance and quartz crystal microbalance; electrospray mass spectrometry; and capillary electrophoresis.[7]

Soluble protein–polyelectrolyte complexes can be examined from several points of view. From the polymer perspective, the complex might be described as a type of macromolecule with measurable dimensions and persistence length; in some circumstances this complex appears to be free-draining. Polyelectrolytes would not interact electrostatically with proteins of the same net charge unless they had anisotropic charge distribution. The general concept of a protein region with a local charge density differing from or – more significantly – opposite to the global charge appears to have first arisen from protein retention in ion-exchange chromatography at the "wrong side of pI" (or equivalently on the "wrong" type of column).[7,11,12]

The aim of this study was to assess the critical conditions needed in order to obtain protein – polyelectrolyte complex systems (PPC) between bovine serum albumin (BSA) and a synthetic polyelectrolyte. The interactions leading to complex formation were found to occur on a local level, between a sequence of charges of the protein surface and a complementary set of charges along a short segment of the polyelectrolyte. BSA is a relatively large globular protein, with well-known structure and physicochemical properties, which possess nonhomogeneous charge distributions. Poly(aspartic acid) (PAS) was chosen as polyelectrolyte. It belongs to the family of synthetic polypeptides, and it is a typical biocompatible, biodegradable, water-soluble polymer with dispersing activity.[8]

RESULTS AND DISCUSSION

Dynamic light scattering (DLS)

DLS is a primary tool of investigation, reported in 35% of the papers published in the last 5 years.[7] The multiple modes typically observed have usually been attributed to the unbound protein, intrapolymer and interpolymer complexes (soluble aggregates). In the case of protein–polyelectrolyte coacervates, DLS yielded the first evidence of what appeared to be an anomalously fast diffusional mode, subsequently linked *inter alia* with equilibrium mesophase organization.[13,14] From our knowledge, in literature are not present extensive studies concerning the critical micellar concentration of PAS. Thus, in the first part the influence of the PAS concentration upon the particles size and zeta potential evolution it was investigated.

In Fig. 1a the hydrodynamic diameter behavior as function of PAS concentration is presented. Thus, a first considerable increase in the diameter appears at low concentration (below to 0.2%) and a first peak with maximum hydrodynamic diameter is registered at about 0.5% concentration of PAS. This behavior suggests that the maximum association occurs at this concentration. On increasing the PAS concentration (up to 2% of PAS) a decrease in the BSA intramolecular associations was observed.

At this point the increasing of PAS content has a positive influence upon intermolecular association concretized in growth of PPC hydrodynamic diameter. This assumption is sustained also by the zeta potential values, presented in Figure 1b. The zeta potential shows smaller modulus for the concentrations which corresponds to the intramolecular associations. This is logical because if the intramolecular association occurs the number of free functional groups decreases and implicit the modulus of zeta potential decreases.
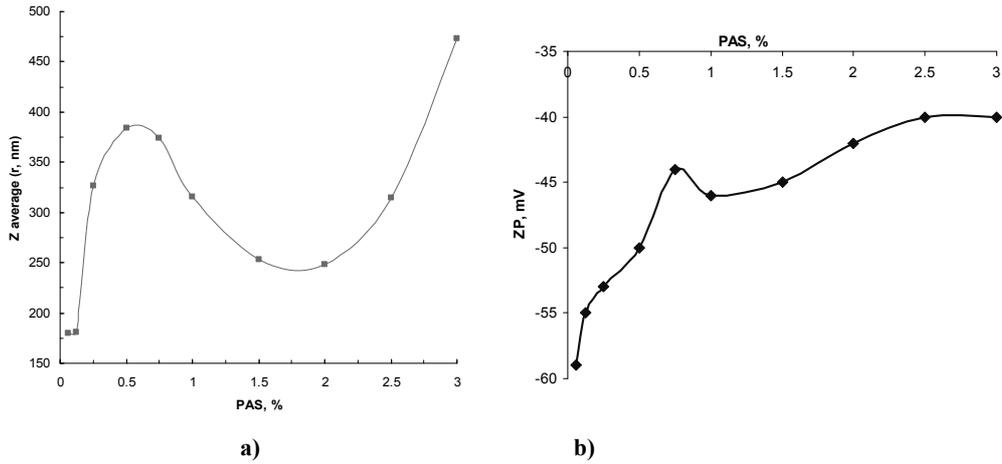
a)   b)

Fig. 1 – The influence of PAS concentration upon the hydrodynamic diameter (**a**) and zeta potential (**b**).
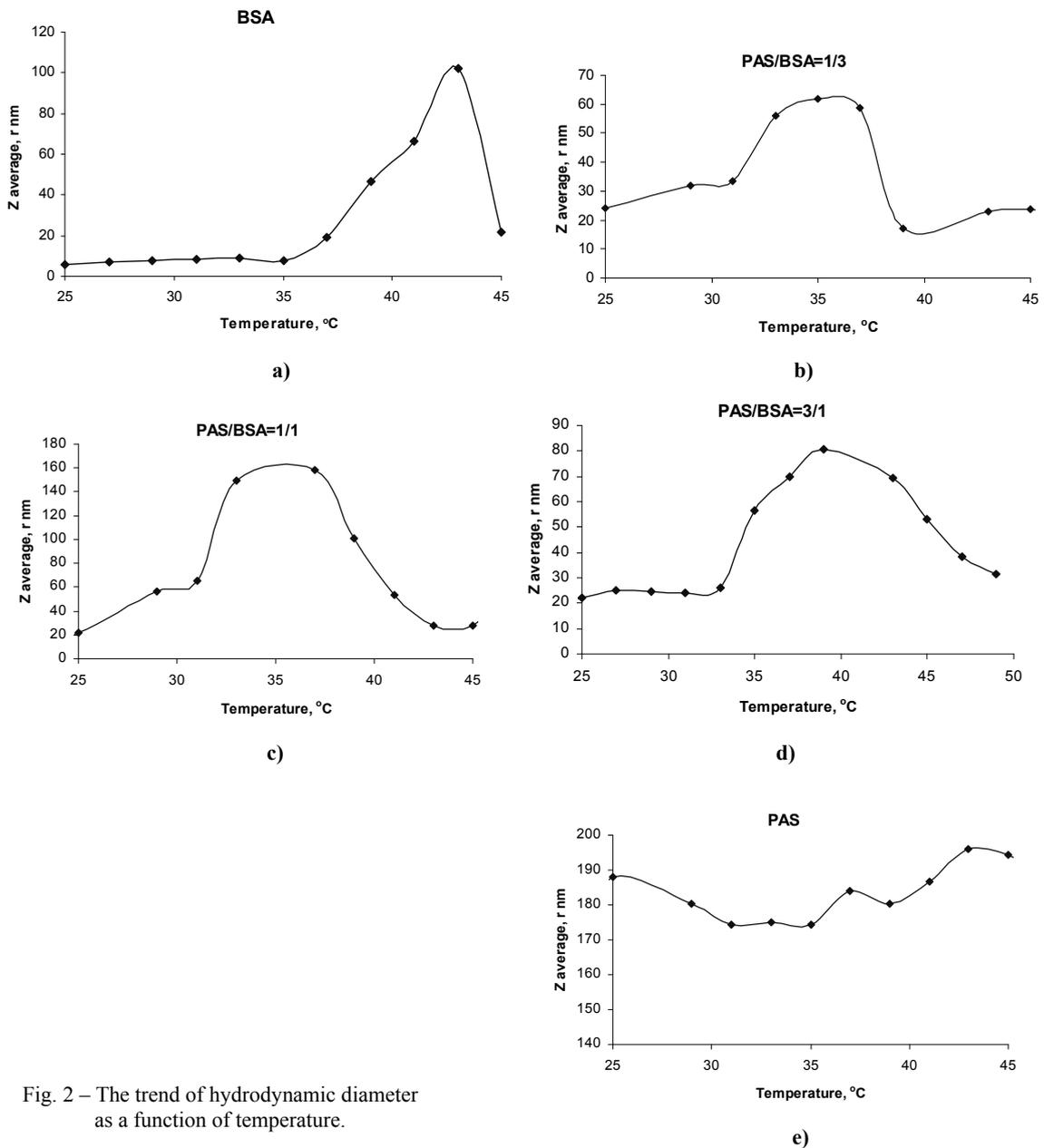

a)

b)

c)

d)

e)

Fig. 2 – The trend of hydrodynamic diameter
as a function of temperature.

The trend of particles size as function of temperature for the pure polymers and their corresponding PPCs are presented in Fig. 2 a-e. It can be observe that for BSA an agglomeration arises at temperature up to 37°C which corresponds to the beginning of denaturation process. The maximum of intermolecular associations occurs at 43°C. For PPCs the temperature at which associations occurring decreases at: 35°C for PAS/BSA=1/3, 37°C for PAS/BSA=1/1 and takes place around of 40°C for PAS/BSA=3/1. The fact that the temperatures at which the PPCs form intermolecular associations are different and in relation with the PPC composition, evidence the intra-molecular physical bonds of BSA, but as well strong intermolecular links, which appears between PAS and BSA. Fig. 2e shows that the PAS associations are not influenced by the temperature. The trend is measured for all samples at 1% concentration (concentration at which PAS is partially associated, see Fig. 1a).

The near infrared chemical imagistic (NIR-CI) is used to provide qualitative and quantitative analysis of localized and space-averaged chemical compositions. NIR-CI technique makes the fusion between near-infrared spectroscopy and image analysis and it is useful for pharmaceutical applications to reveal the extent of the ingredient blending, particle size distribution, the presence of polymorphs, and the trace of contaminants. The method is also used to visualize the spatial distribution of the chemical compounds in a sample provided by a chemical image.[15-18]

Red-R, green-G and blue-B (RGB) images were created from the data that originated from the image pixels of the studied structures (pure polymers and the mixture between them at different ratio), as can be seen in Fig. 3. The only matrix that was not rescaled is the original data for imported RGB image files. Samples with higher amount of PAS are more homogeneous.

Evince program uses PLS-DA (Partial Least Squares Discriminant Analysis) model for classification of observations into different classes. Thus, the multidimensional variable space was reduced to a more manageable size and a more easily overviewed. Analyzing data by PLS-DA we are able to conclude upon homogeneity of the prepared PPCs, as well as on the ratio between the co-partners of the PPC. Through PLS-DA the spectral data are decomposed into a set of a small number of classification scores. The scores of PLS-DA include the information of both the spectral variable and the response variable (as for example blending composition or ratio) [18]. Fig. 4 shows the PLS-DA model for PPC having PAS/BSA ratio of 1/1. PLS-DA model assigns color code for the pure components. Thus, light gray was assigned to BSA, while black color to PAS. For PPC the color is obtained by soft analysis. The dark gray color of PPC complex, having the color between those of BSA and PAS, evidences and confirms the presence of both compounds (PAS and BSA) in PPC as well as homogeneity.

The PLS-DA model also allows the obtainment of predicted values of the spatially averaged contents of the two components in the PPCs complex (Table 1). These predicted values are very close to those obtained by **EDAX analysis** (Table 1). The resulting EDAX maps are illustrated in Fig. 5. The average contents of the two components calculated from EDAX study was based on the sulfur content of BSA.

The predicted values obtained from NIR-CI analysis are very close from the BSA content obtained from EDAX analysis (calculated from the sulfur value).
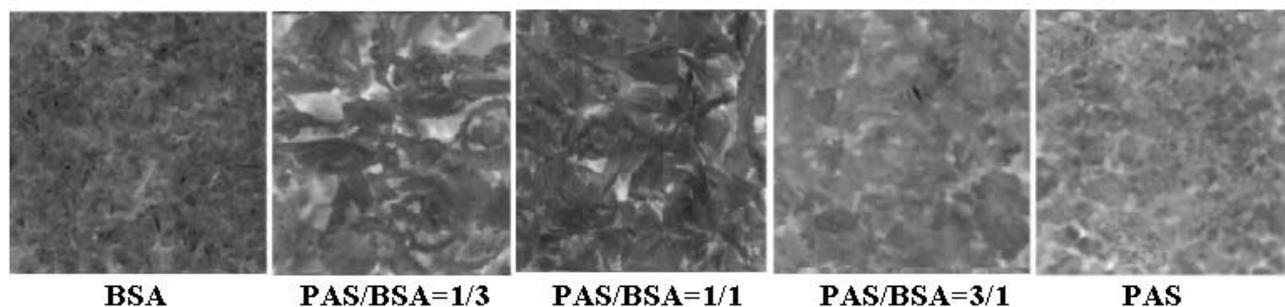

BSA   PAS/BSA=1/3   PAS/BSA=1/1   PAS/BSA=3/1   PAS

Fig. 3 – RGB image for the pure polymers and their mixtures.

Fig. 4 – The PLS-DA model of 1/1 molar
ratio PAS/BSA complex.

PLS-DA model

BSA

PAS/BSA=1/1

PAS

Element	Wt%
CK	66.44
NK	13.80
OK	18.28
SK	01.63

BSA

Element	Wt%
CK	59.49
NK	12.78
OK	22.55
NaK	03.24
SK	01.28
ClK	00.63

PAS/BSA=1/3

Element	Wt%
CK	54.36
NK	10.52
OK	23.45
NaK	06.53
SK	0.95
ClK	04.04

PAS/BSA=1/1

Element	Wt%
CK	42.84
NK	07.73
OK	29.99
NaK	12.36
SK	00.44
ClK	06.54

PAS/BSA=3/1

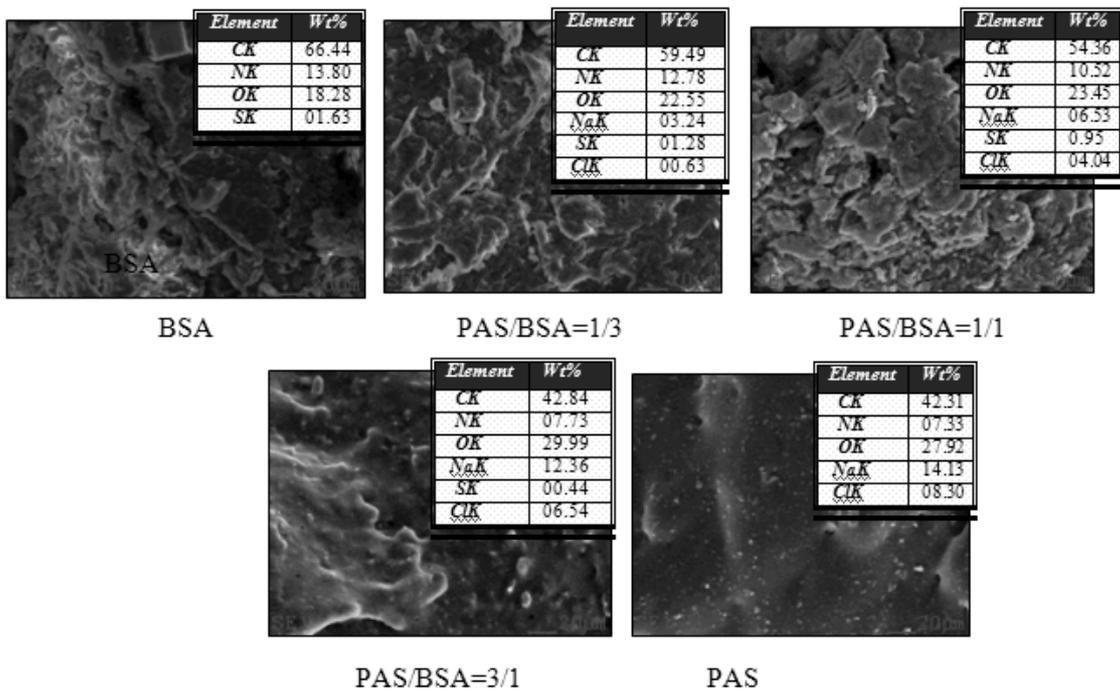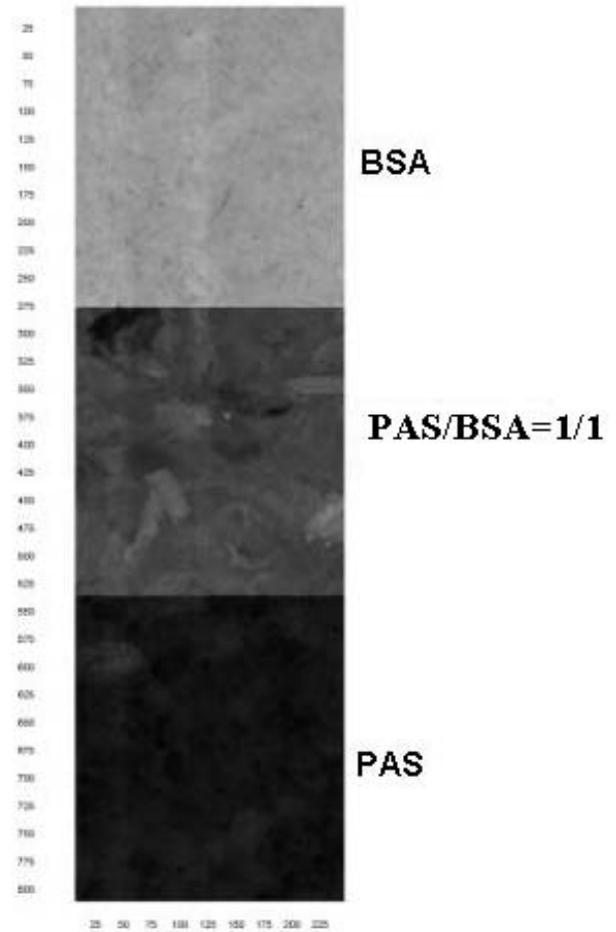| Element	Wt%
CK	42.31
NK	07.33
OK	27.92
NaK	14.13
ClK	08.30

PAS

Fig. 5 – Maps from EDAX study for pure polymers and their PPCs.

Table 1

Predicted values for albumin content in the bioactive compounds obtained from NIR – CI and EDAX analyses

Sample	Theoretical ratio (% BSA)	Calculated ratio from EDAX (% BSA)	Calculated ratio from PLS-DA (% BSA)
PAS/BSA=1/3	75	78.5	76
PAS/BSA=1/1	50	58.5	48
PAS/BSA=3/1	25	27	22

Dielectric spectroscopy analysis

As it is well known the dielectric properties of a material depend on its chemical structure. As a consequence, it would be expected as a polyelectrolyte or a protein as well their mixture to polarize in an electric field to give information's about the self-assembling ability and functional groups orientation. The dielectric loss, ε", of PAS, BSA and their PPC have been recorded at 37°C and between 0.1 Hz to 1 MHz frequency range.

The energy dissipated by the electric dipolar moments, which orient themselves in the direction of an external electric field, is expressed by the dielectric loss, ε". It includes the energy required to align molecular dipoles and energy dissipated due to ionic conductivity in the presence of an alternative field.[19] Fig. 6 exhibits the frequency dependence of the dielectric loss in a double logarithmic scale. At low frequencies, ε" possesses high values (*e.g.* $10^3 - 10^8$, at 0.1 Hz) and it

gradually decays attaining minima (*e.g.* $10^{-1} - 10^2$, at 10^6 Hz). The dependence of dielectric loss on various molar ratios between PSA and BSA, at a selected frequency of 0.1 Hz, is presented in the inset of Fig. 6.

The dielectric loss evolution for different PAS/BSA contents shows also that optimum "ε" value corresponds for 1/1 ratio between PAS and BSA.

Our performed investigation confirms the interplay between the hydrogen bonds, the hydrophobic and electrostatic forces for the final preparation of the self-assembled structure between BSA and PAS, whose behavior sustains the formation of these complexes. At the same time, these weak forces, respectively the electrostatic forces, hydrogen-bonding forces and the hydrophobic interactions, which generating the self-associate systems, it is difficult to ravel their relative contributions, dependent on the environment.
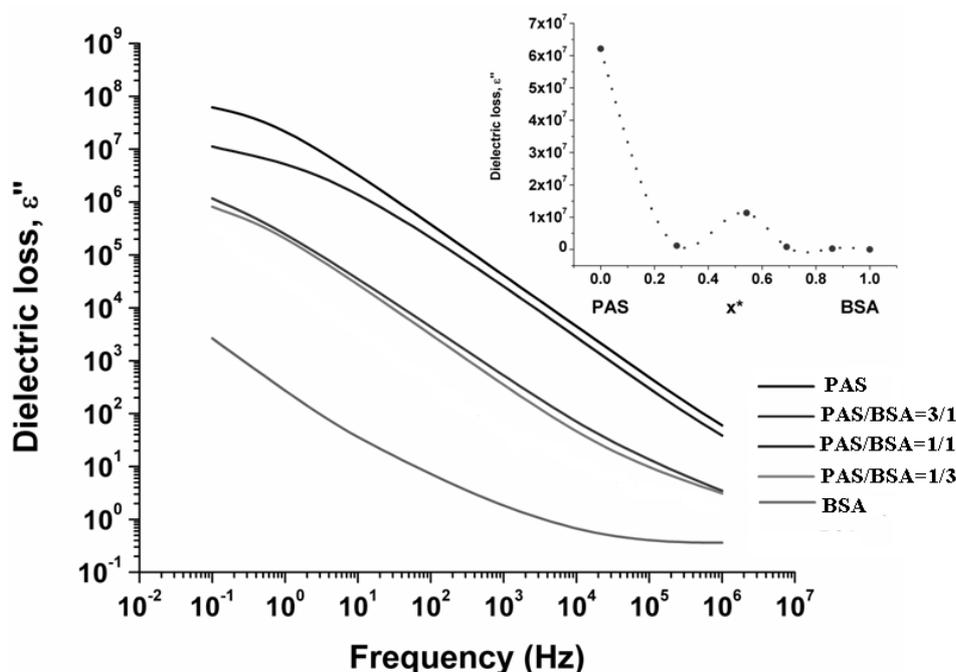

Fig. 6 – Dependencies of dielectric constant vs. frequency for PAS, BSA and their mixtures.

EXPERIMENTAL

Materials and samples preparation

Poly(aspartic acid) (PAS) was synthesized in our laboratory by a reaction in two steps as described in detail before.[9,10] In brief firstly its precursor - poly(succinimide) (PSI) - it was prepared by thermal polycondensation of L-aspartic acid (Fluka Chemical provenience), in the presence of o-phosphoric acid (analytical reagent of 85%, Chemical Co. provenience) as catalyst, in mesitylene / sulfolane solvents mixture (both from Fluka Chemical provenience), at 180°C, during 6 h. In the second step PSI was hydrolyzed in alkaline medium at –5°C for 1 h. The prepared polymer had the molecular weight of about 15,110 g/mole and the polydispersity index of 1.317.

Bovine serum albumin (BSA) from Sigma (with purities ranging from 95-99%, and molecular weight of about ~66,000 Da) was also used. BSA was applied in numerous biochemical applications due to its stability and lack of interference within biological reactions.

Samples preparation

Aqueous solutions containing 1 g/dL of PAS and BSA, respectively, were prepared by dissolution of each polymer sample in Millipore water under magnetically stirring for 60 min and then the homogeneous solutions were kept at rest in refrigerator at 5°C for 24 h. The PAS/BSA mixture was prepared by direct mixing of different ratios of the polymers as aqueous solutions of 1 g/dl concentration, for 60 min. Thus, different PAS /BSA molar ratios were obtained, namely 0, 1/3, 1/1, 3/1, and 1, respectively. The pH of solutions was kept at 7.4. The mixtures were allowed to equilibrate for 24 h and kept at the temperature till further experimental investigations were carried out. During each experiment the total polymer concentration in the mixture was above their CMC and maintained constant. The prepared mixtures remained clear for all studied ratios.

Characterization

DLS analysis

Dynamic Light Scattering (DLS), also known as Photon Correlation Spectroscopy (PCS), was the technique used for tracking the formation in solution of the nanoparticles between the macromolecular compounds. The technique takes advantage of the Brownian motion of particles in solution, which is a stochastic process due to collisions with surrounding molecules.

The effect of temperature upon the prepared PPC was evaluated in the field of 25-45°C using a Peltier device ±0.1°C.

Zeta potential and size estimation was determined during the heating processes by using a Zetasizer ZS apparatus.

Zeta Potential (ζ) – was determined by using Smoluchowski relationship:

$$\xi = \eta\mu / \varepsilon, \text{ and } k\alpha \gg 1 \qquad (1)$$

where: μ - electrophoretic mobility, η - viscosity, ε – dielectric constant, k and α - Debye-Hűckel parameter and particle radius respectively.

The *electric conductivity* was determined concomitantly with zeta potential measurements.

The evaluation of the PPC particle size was made also on the same Zetasizer ZS apparatus. The Mie method is applied over the whole measuring range from 0.6 nm to 6 μm for which is capable for measuring the device. The hydrodynamic diameter (D_H) of the prepared PPC aggregates was determined by using the following equation:

$$D_H = \frac{kT}{3\pi\eta D} \qquad (2)$$

where D_H is the hydrodynamic diameter, k – Boltzmann constant, T – temperature, η – viscosity, D – diffusion coefficient. The hydrodynamic diameter, often expressed by symbol Z or z-average, is related to the intensity means, and not a mass or number means, being calculated from the signal intensity. DLS experiments present high reproducibility with the deviation of the average diameter within 5%. The polydispersity index of samples was not higher than 0.4.

PPC samples were investigated by *chemical imaging* on *near infrared region* with statistical analysis methods. Acquisition of optical and spectral data was carried with an integrated Chemical Imaging Workstation, provide by SPECIM Spectral Imaging Ltd (Finland). The optical data have been collected with an ImSpector N17E imaging spectrograph for a resolution of each image of 320 X 640 pixels. The chemical images were taken with a NIR spectral camera, respectively an imaging spectrograph type ImSpector N17E at a rate of 60 – 350 Hz. The original image was recorded at a spatial resolution of 320x640 pixels and they has been collected as a cube of data with two pixels variable (X and Y axis) and the third as absorbance variable (Z axis). The data was processed with EVINCE Chemometric software package in order to explore the spectral and spatial information and classified and quantified image content.

ESEM studies were performed on the PPC samples fixed by means of colloidal copper supports. The samples were covered by sputtering with gold thin layers (EMITECH K 550x). The coated surface was examined by using an Environmental Scanning Electron Microscope (ESEM) type Quanta 200 operating at 30 kV with secondary electrons in high vacuum mode.

Dielectric spectroscopy

Dielectric permittivity measurements were performed using the Novocontrol Dielectric Spectrometer (GmbH Germany), CONCEPT 40. The dielectric response, expressed by the dielectric loss *"ε"*, has been recorded at 37°C in the frequency range of 0.1 Hz to 1 MHz. A Novocontrol Quatro Cryosystem device was used in order to control the temperature with 0.1°C stability. The samples with different PAS/BSA molar ratios, respectively 1, 1/3, 1/1, 3/1, and 1 were prepared as pellets with 0.6 mm thick and then sandwiched between two gold coated plate electrodes.

CONCLUSIONS

The study evidences the possibility of polyelectrolyte complex formation between poly(aspartic acid) and bovine serum albumin. The preparation of self – assembled structures was evidenced by DLS studies, chemical imaging on near infrared region, ESEM studies and dielectric spectroscopy.

The evolution of hydrodynamic diameter and zeta potential of the PPC as function of temperature

shows the formation of intermolecular association for the PPCs as: 35°C for PAS/BSA=1/3, 37°C for PAS/BSA=1/1 and around 40°C for PAS/BSA=3/1. Equimolar ratio seems to be most useful in biomedical applications being close to physiological temperature. The NIR-CI analysis evidences and confirms the presence of both protein and polyelectrolyte – BSA and PAS – in the formed PPC as well the homogeneity of the complex. The predicted values concerning the PPC composition obtained from NIR-CI analysis are near of those resulted from EDAX analysis (calculated from the sulfur value). The dielectric loss behavior for different PAS/BSA ratios shows also the optimum "ε" value at PPC content of 1/1 PAS/BSA ratio.

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