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Pyrrolidinium Octanoate Carboxylate as PIL Agent in the Growth Mechanism of Lysozyme Spherulites

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Abstract

In this research the impact of pyrrolidinium octanoate carboxylate (PyO) on Lysozyme (Ly) spherulite forms using the method of vapour diffusion with hanging drops (HDVD) was investigated. Two different stock solutions at low alkaline pH were tested: 0.1 M NaAc (the first one) and 0.1 M TRIS hydrochloride contained 0.2 M ammonium sulfate and 25 % wt. PEG 3450 as crystallant agents (the second one). The experiments were performed at 18°C using two PyO concentrations (0.4 M and 1.6 M) in each stock solution. PyO of booth concentrations lead to the formation of Ly - SNLC (Ly single needle-like crystals), observed by optical microscopy one day after the droplets deposition, excepted the stock solution of 0.1 NaAc based on 0.4 M PyO where Ly microspheres were identified by electron scanning microscopy. The growth mechanism of the Ly spherulites of type I obtained using 0.4 M PyO in 0.1 M TRIS/crystallant agent could be summarised as follows: Ly-SNLC → Ly-like axialites → Ly spherulites of type I. The growth mechanism of the Ly spherulites of type II using 1.6 M PyO in 0.1 M TRIS/crystallant agent can be summarised as follows: Ly-SNLC → Ly-like axialites → Ly spherulites of type II.

Keywords: Pyrrolidinium Octanoat, Lysozyme, Crystallisation Agent, Spherulite, Stock Solutions

Introduction

The protein crystallization plays an important role in many biotechnological fields, such as pharmaceutical research and design and food industry. The protein crystallization has become of considerable interest not only to pharmaceutical, biotechnological, food and chemical industries, but as promising tools in the protein engineering, drug design and other applications to biological systems. One of the major challenges today, in the biological research is to understand biological processes, to create new therapeutic proteins and new drugs for the treatment of illnesses and injuries, to elucidate their 3D structure. In addition, a better purification by crystallization and the long term conservation of the protein crystals, avoiding their chemical or thermal degradation and the reducing of their activity are very important points of interest. These involve understanding of the structure, functions and interactions proteins with their environment.

Depending on the physico-chemical conditions and their chemical structure, the proteins form various soft-materials, such as: microspheres, aggregates, fibres, nanotubes, spherulites etc. (LI & CHANG. [1]). Finding optimal conditions for the protein crystallization experiments are generally very difficult and this requires the optimization of all parameters involved in the

process (pH, temperature, buffer solution, concentration of the protein, precipitants, stabilizer, additives etc.) (LI & al. [2]).

The most important goal of the fundamental studies of the protein crystal growth is to identify and to reduce *the disorder* present in crystallized proteins. Before being crystallized, the proteins have to be very well solubilised in a donned solvent. The protein solution thus obtained will be brought into a supersaturated state by the evaporation of the solvent. Water isn't a good solvent, because their evaporation leads to the structural degradation of the proteins.

During the last years scientists have turned their attention to a new class of solvents used as green solvents for a wide range of both inorganic and organic materials, comprising the proteins: the ionic liquids (ILs). They have understood that these green solvents constitute attractive materials for the biotechnology applications and desirable agents to improve the protein crystallization [GARLITZ & al. [3]; PUSEY & al. [4]; HEKMAT & al. [5]; JUDGE & al. [6]).

As a subclass of ILs, the protic ionic liquids (PILs) present interesting physical-chemical properties such as: a chemical structure consisting entirely of ions involving a great ionic conductivity, melting points at or below room temperature, a great thermal and chemical stability, a high solubility power, and an extremely low vapour pressure making them non-flammable solvents (KENNEDY & al. [7]). PILs have demonstrated their ability to increase the crystallization rates and produce larger protein crystals (PUSEY & al. [4]). One of the special interest attributed to ILs consist in their use as green solvents to preserving the bimolecular media, protecting the proteins against thermal degradation (BAKER & al. [8]; FUJITA & al. [9]), aggregation or hydrolysis (BYRNE & al. [10]), unfolding proteins (SUMMER & al. [11]; LANGE & al. [12]).

In biological sciences, spherulites are like a common 'failure mode' of the protein crystallization process. They are also known as 'sea urchin' poly-crystalline entities, due to the radial morphology determined by the solidification of the microcrystal patterns (COLEMAN *&* al. [13]; TANAKA *&* al. [14]; MUSCHOL *&* al. [15]; HEIJNA *&* al.[16]; HEIJNA *&* al.[17];GRANASY *&* al. [18]). Spherulite forms are always constructed around a nucleation centre by assembly of the multiple lamellas that span out within a spherical envelope keeping constant the nanocrystalographic angles. This growth mode was often met in polymers (PHILIPS & al. [19]; SPERLING & al. [20]), elementary selenium (BISAULT & al. [21]), simple organic liquids (MAGILL & al. [22]), biological molecules (PHILIPS & al. [19]), etc. In the medicine field the protein spherulite structures have been found in several types of neurodegenerative diseases, such as Alzheimer and Parkinson (CASTRO & al. [23]).

According to their nucleation mechanism described in literature, spherulites are divided into two morphologic categories (COLEMAN *&* al. [13]; GRANASY *&* al. [18]; HEIJNA *&* al. [16]): spherulites of type I being the result of a heterogeneous nucleation and the spherulites of type II being the result of a homogeneous nucleation (Figure 1).

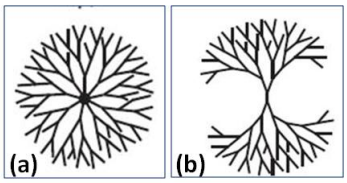


Figure 1: Schematic representation of spherulites type [Coleman *& al.,* 1960 and Heijna *& al.,* 2007]:
a) spherulite morphology of type I formed by heterogeneous nucleation on a foreign particle;
b) spherulite morphology of type II formed by the homogeneous nucleation of a single crystalline needle.

The complexity of the spherulites formation phenomena is often mentioned in literature. For example, at low pH the Lyzozyme (Ly) protein has been shown previously to form not only needle crystals and 3D protein crystals, but also amyloid fibrils, amorphous, micro-granular precipitate and spherulite forms (BYRNE & ANGELL. [24]; YAGI & al. [25]). The protein spherulite forms were already obtained using different ILs (PUSEY & al. [4]; HEKMAT & al. [5]), but there are still many gaps to completely understand the spherulite growth process. This bottleneck involves many major obstacles for the pharmaceutical field and pharmaceutical design.

Recently in literature was demonstrated the ability of protic ionic liquid media to protect Ly against thermal degradation by aggregation or hydrolysis. Taking into account these premises, we have chosen Ly as target protein and two PILs as crystallization agents unstudied until now in literature.

In order to contribute to understanding the spherulites formation and growth phenomena, this research aims to explore the impact of a new PIL as the pyrrolidinium octanoate carboxylate (PyO), on the Lyzozyme (Ly) protein crystallization at low alkaline pH. Ly is used as target model by varying the crystallization conditions (stock solutions, concentration) and the concentration of crystallization agent (0.4 M and 1.6 M PyO). The Ly morphologies will be investigated by optical microscopy after being trials maintained two days and one week after the droplets deposition, in various growth periods. SEM-EDX analysis will be use also, to put in evidence some Ly morphologies and it elementary chemical composition.

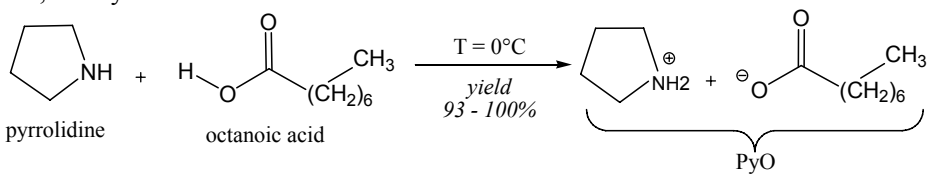
Materials and methods

Materials for protic ionic liquid synthesis

The precursors for PyO synthesized in our lab were imidazole commercially available from Fluka, pyrrolidine (redistilled, 99.5 %) and octanoic acid (98 %) from Aldrich. Both precursors were used without further purification.

Synthesis of PyO

The PyO are synthesized through a neutralization reaction of the pyrrolidine (Brönsted bases) with the octanoic acid (Brönsted acid) into molar ratio of amine/acid 1:1, according to procedure described in Scheme 1 (ANOUTI *&* al. [26]; ANOUTI *&* al. [27]). The octanoic acid was added slowly to amine with stirring in a three-necked round-bottom flask immersed into the ice bath and equipped with a dropping funnel. The compositions were stirred during four hours keeping a constant temperature of 0°C. Transparent slightly coloured PyO were obtained, finally.



Scheme 1: The synthesis of PyO by neutralization reaction of pyrrolidine with octanoic acid

Physicochemical properties of synthesized PyO at ambient temperature

Density measurement. The PyO density was determined by using an Anton Paar densimeter (DMA 4500M). At temperature of 18°C the density of pure PIL synthesized was 0.9469(± 0.1%) $g \cdot cm^{-3}$.

Conductivity measurement. The ionic conductivity was performed by using a Consort (C862) digital multifrequency conductimeter calibrated with KCl standard solutions ($1,10^{-1}$, 10^{-2} mol/dm^3). At temperature of 18°C the ionic conductivity of pure PyO was 940 (± 2%) $\mu S \cdot cm^{-1}$.

Rheological_measurement. The dynamic viscosities were measured using a TA instrument rheometer (AR 1000) with conical geometry at various temperatures (from 15 to 50°C). At temperature of 18°C the viscosity of pure PyO is was 46.26 (± 0.1%) $mPa \cdot s^{-1}$.
Refractive index. The refractive index was measured at temperature of 20°C using an ABBE instrument, calibrated with deionized water. At 18°C the refractive index of PyO was 1.4605.
Coulometric Karl-Fischer titration. The water content was checked using coulorimetric Karl-Fischer titration prior to any crystallisation experiments. The water content of the final PyO product before crystallisation experiments was of 3650 ppm. This result is in agreement with the hydrophilic nature of pyrrolidinium PILs, as shown in literature (ANOUTI *&* al. [26]).
The pH of pure PyO synthesised is low alkaline of pH 8.0 value.

Materials for protein crystallization
Sodium acetate (NaAc): $CH_3\text{-}COO^-Na^+ \cdot 3H_2O$, TRIS hydrochloride: $C_4H_{11}NO_3 \cdot HCl$, ammonium sulfate ($(NH_4)_2SO_4$) and PEG 3450 were purchased from SigmaAldrich.
Deionised purified water with a Mili-Q MX (>15 MΩcm) water system.
Hen egg white Ly BioUltra, lyophilized powder, 98 % (SDS-PAGE) commercially available from SigmaAldrich.

Method of preparation of the crystallization solutions
Stock solutions
Two stock solutions were prepared: 0.1 M NaAc ($CH_3\text{-}COO^-Na^+ \cdot 3H_2O$) of pH 7.9 and 0.1 M TRIS hydrochloride ($C_4H_{11}NO_3 \cdot HCl$) at pH 8 using deionised water (>15 MΩcm).
Preparation of the crystallant solution
A crystallant solution was prepared using 0.1 M TRIS hydrochloride stock solution with the addition of 0.2 M ammonium sulphate and 25 % wt. PEG 3450 as precipitate agents.
Protein solutions
Ly was used as source material for crystallization experiments after dissolving of 50 mg protein into 1 mL of each stock solution: 0.1 M NaAc and 0.1 M TRIS hydrochloride.
Mother liquor
PyO was used as crystallization agent for two concentrations in both stock solutions: 0.4 M and 1.6 M respectively. Thus, four mother solutions (liquors) were finally obtained.
Droplets deposition by HDVD method
All crystallisation experiments were made at ambient temperature by vapour diffusion with hanging drops method (HDVD). Ten droplets were made from each tested solution. The crystallization droplets, consisting from 5 μL of protein solution and 5 μL of mother solution containing PyO, were prepared. First, the 5 μL droplets from the protein solution have been placed on a glass slide or Ni metal plates and was covered by the second droplet of 5 μL mother liquor. The assembly consisting of the glass slide with the overlapping droplets was placed face down over a tank containing 0.5 mL mother solution. The trials were analyzed by optical microscopy and SEM, after one day, two days and one week after the droplets deposition.
Optical microscopy investigations
The Ly morphologies growth in aqueous solutions by using PyO as PILs crystallization agents were investigated through optical microscopy (Olympus BX41) and SEM.
Electronic microscopy investigations
The surface morphology of the droplets deposited on Ni metallic plates was characterized by scanning electron microscopy (SEM) coupled with EDX analysis (Philips XL-30FEG).

Results and discussions

Ly morphologies using different stock solutions and variable PyO concentrations

The Ly crystallization was tested by using two concentrations of PyO 0.4 M and respectively 1.6 M.

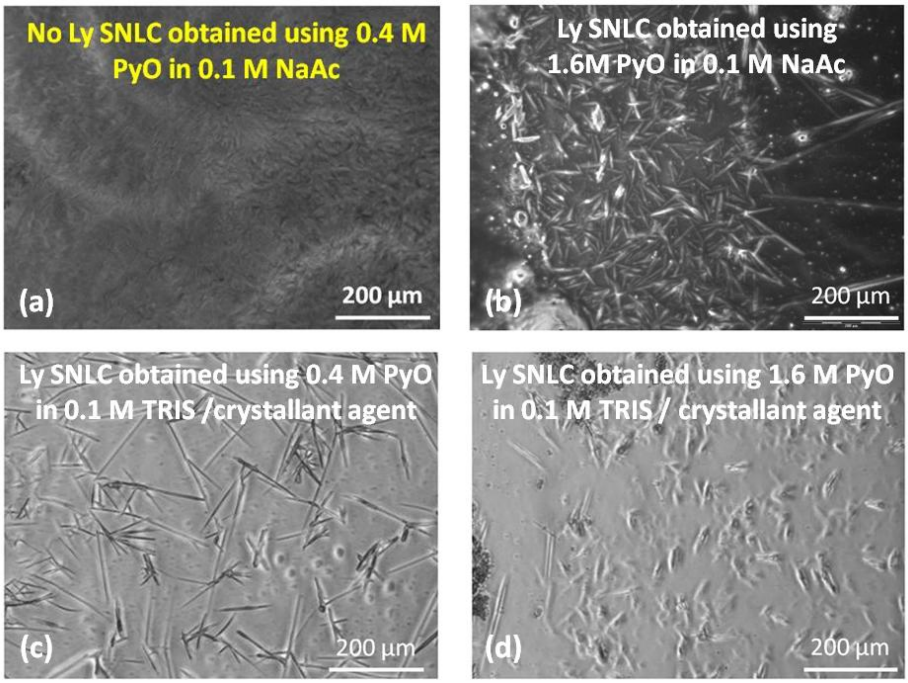


Figure 2. Optical images of the initial Ly morphology formed one day after the droplets deposition, using: (a) 0.4 M PyO in 0.1 M NaAc; (b)1.6 M PyO in 0.1 M NaAc;
(c) 0.4 M PyO in 0.1 M TRIS/crystallant agent; (d) 1.6 M PyO in 0.1M TRIS/crystallant agent.

Figure 2 shows the Ly crystallization results obtained by HDVD experiments with the addition of PyO as crystallization agent. Firstly was tested the growth of Ly protein in 0.1 M NaAc stock solution, and second in 0.1 M TRIS with crystallant agent (0.2 M ammonium sulfate and 25 % wt. PEG 3450). All samples were analysed by optical microscopy one day after the droplets deposition.

As shown in Figure 2b-d, the Ly morphologies formed in all droplets, one day after their deposition on the glass plates, presented the aspect of *single needle-like crystals* (SNLC), except on 0.4 M PyO in 0.1 M NaAc where no Ly - SNLC were observed (Figure 2a). The appearance (length and morphology) of the Ly - SNLC obtained in each case was different, depending on the type of the stock solution and of the PyO concentration used. For example, using 1.6 M PyO in NaAc stock solution leads to the formation of elongated Ly - SNLC whose length and thickness was between those obtained using 0.4 M PyO and 1.6 M PyO in TRIS/crystallant agent. The Ly SNLC formed using 0.4 M PyO in TRIS/crystallant agent were thin and long, while those obtained using 1.6 M PyO in TRIS/crystallant agent were short and thick. From the optical images is evident that the PIL concentration and the stock solution nature presented a high influence on the growth of Ly morphologies obtained by HDLD.

Growth mechanism of Ly spherulites using 0.4 M PyO in 0.1 M TRIS/crystallant agent

The obtaining of the Ly - SNLC forms only one day after the droplets deposition is a very interesting aspect of the growth mechanism of Ly spherulite forms using this PIL, PyO. In our previous work we reported results concerning the Ly spherulite formation with a nearly spherical shape leading by the ramifications enriched in a radial direction, using PyO in TRIS/crystallant solution, two days and one week after the droplets deposition (STEFAN & al. [28]). One week after the droplets deposition, the formation of two types of spherulites were observed: spherulite forms of type I were started to form using PyO at lower concentration (0.4 M), while spherulite forms of type II were obtained using PyO at higher concentration (1.6 M).

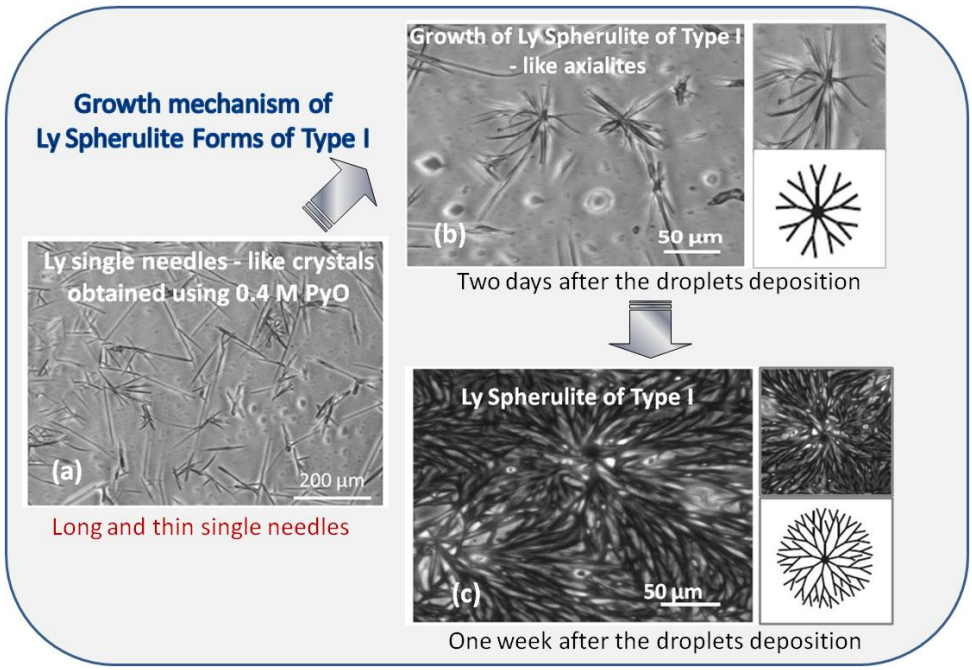


Figure 3. Growth mechanism of the Ly spherulites using 0.4 M PyO as protic ionic liquid agent in 0.1M TRIS/crystallant agent.

Figure 3 shown the images obtained using 0.4 M PyO in TRIS/crystallant agent, one day, two days and respectively one week after the droplets deposition.

The Ly growth mechanism that we consider can be described in three phases. An initial phase concern the nucleation and the occurrence of thin and long Ly - SNLC forms in one day after the droplets deposition (Figure 3a). As can been observed, this phase continues with a second one by the transformation of Ly-SNLC into the Ly - axialites (two days after the droplets deposition) (Figure 3b). This process allows understanding that the transformation is continuous in time. The growth of Ly - axialites leads finally to the formation of the spherulitic type I morphologies (Figure 2c), according to a mechanism that could be summarized as follows: *SNLC → axialites → spherulites type I.* These remarks are in agreement with those found in literature (GRANASY & al. [18]; CASTRO & al. [23]) that shown similar stages on the protein spherulite formation. The originality of our work consists in using of PyO as protic ionic liquid agent to improve the formation of Ly spherulite morphologies type I at a low alkaline pH.

Growth mechanism of Ly spherulites using 1.6 M PyO in 1.6 M TRIS/crystallant agent
In Figure 4 a similar mechanism in three steps will be proposed concerning the growth of Ly spherulites of type II using 1.6 M PyO of higher concentration, in 1.6 M TRIS/crystallant agent. In our previous work we reported only the final morphology of Ly spherulites type II formed using 1.6 M PyO in TRIS/crystallant agent, one week after the droplets deposition. Without knowing the initial morphology of the Ly forms, one day after the droplets deposition, the growth mechanism was unknown (STEFAN & al. [28]). From these experiments, the initial formation of the Ly - SNLC forms one day after the droplets deposition allows us to understand the continuity of the process and complete the growth mechanism of Ly spherulites of type II formation.

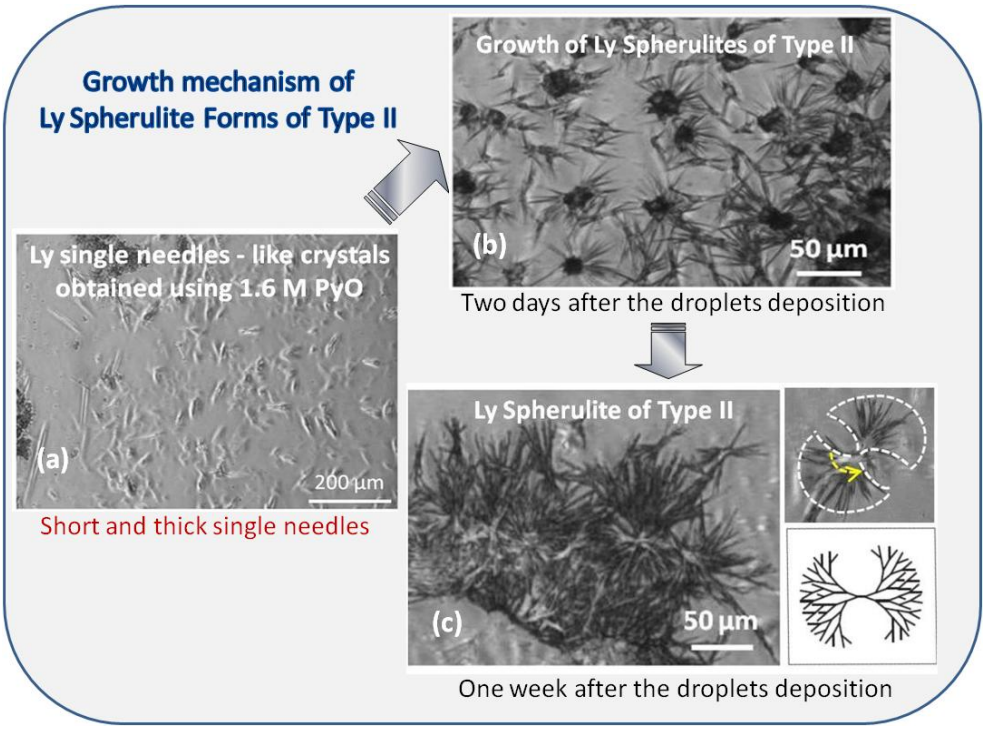


Figure 4. Growth mechanism of Ly spherulites using 1.6 M PyO
as protic ionic liquid agent in 0.1 M TRIS/crystallant agent.

As can been observed in Figure 4, the mechanism of the Ly growing started with the occurrence of some short and thick Ly - SNLC forms homogeneously distributed on the samples surface (Figure 4a) and it continued with the development of Ly - like axialites (Figure 4b). Similar to the previously mechanism, at lower PyO concentration, the growth of Ly axialites in a radial direction leads to the spherulitic type II morphologies (Figure 4c) with the formation of two uncrystallized holes - like eyes, inaccessible to crystals branching (HUTTER& BECHHOEFER [29]; GRANASY & al. [18]). In this case, the mechanism could be summarized in a similar manner as follows: *SNLC → axialites → spherulites type II,* being in agreement with the results reported by CASTRO & al. [23] and GRANASY & al. [18]). Another aspect of our work originality consists in using for first time of PyO of higher

concentration to improve the formation of Ly spherulite morphologies type II. This data is unreported until now in literature.

Study of the crystallization droplets using 0.4 M PyO in 0.1 M NaAc by depositing on Ni metallic plates

The first crystallization experiment using 0.4 M PyO in 0.1 M NaAc leads on a new question: are these a failure of the crystallization experiments or is because of the too lower concentration used for this type of stock solution? This can be the reason do not obtain Ly - SNLC and spherulite forms. In order to answer of these questions, four samples in same identical conditions by depositing the droplets on Ni metallic plates instead the glass plates have been prepared. The samples were investigated by scanning electronic microscopy (SEM) one week after the droplets deposition. Their surfaces have been characterized by elementary analysis (EDX). In Figure 5 is presented the SEM micrographs obtained for the droplets which have been deposited on Ni metallic plates.

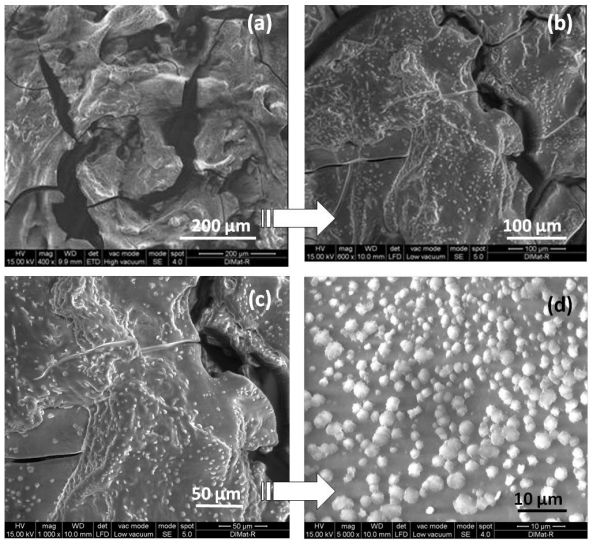


Figure 5. SEM micrographs for the Ly crystallization phenomena using 0.4 M PyO as crystallization agent in 0.1 M NaAc stock solution with different magnifications: (a) micrograph of a sample with a scale bar = 200 µm; (b) zoom of the first image, scale bar = 100 µm; (c) zoom of the second image, scale bar = 50 µm; (d) visualisation of Ly microspheres, scale bar = 10 µm.

As can been observed in Figure 5, above 100 µm scale bar, there is no formation of Ly – SNLC (Figure 2b-d). At the same time, in Figure 5c at 50 µm as scale bar a homogeneous distribution of some punctually white structures can been seen. In Figure 5d these forms appear surprising as microspheres with the diameters of average around of 5 µm as it can be seen in Figure 6b. Their elementary composition have been analysed by EDX and the results are presented in Figure 6c.

The EDX analysis indicates that in elementary microspheres composition are presented only the atoms corresponding of the Ly protein chemical structure as follows: C, O, N and S respectively. The few Ni traces present in EDX analysis come only from the metallic Ni plates used as support for the deposited crystallization droplets. SEM and EDX analysis confirms clearly that the founded microstructures are the Ly protein microspheres, and their

smaller dimensions around 5 μm explain why it is not possible to observe them by optical microscopy (Figure 2a).

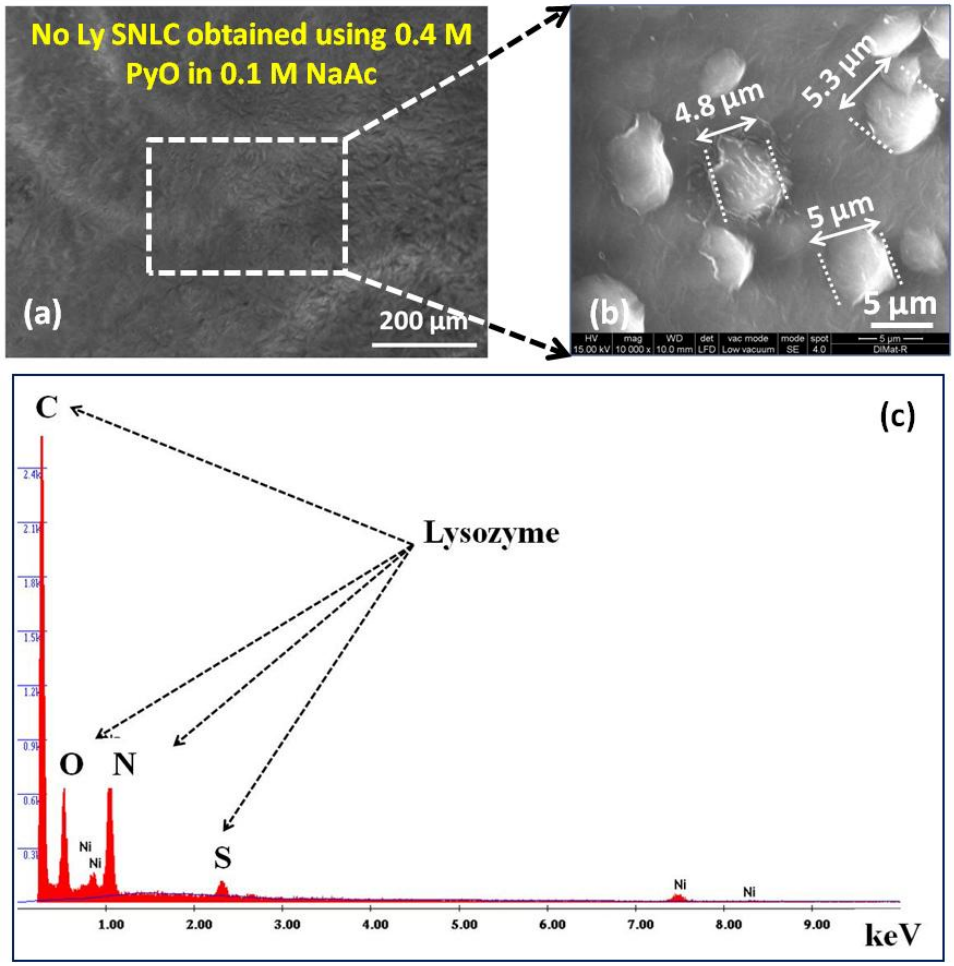


Figure 6. SEM micrographs and EDX analysis of the Ly crystallization phenomena using 0.4 M PyO as crystallization agent in 0.1 M NaAc stock solution.

Effect of the PyO viscosity on the Ly crystallization experiments

One of the most important transport properties involved in the spherulitic Ly growth process, is the low mobility of the fluids that compose the droplets. According to the theory of the transport properties of the fluids in the crystallization process (GRANASY& al. [18]; HEIJNA & al. [16]; ANDREASSEN & al. [30]), a lower mobility of the molecules that compose the crystallization droplets involves a long life of the dynamic heterogeneities and favour the formation and the growth of the spherulite morphologies. The viscosity parameter provides data of the molecular mobility and it has directly control of the manner that molecules participate on the growing crystal.

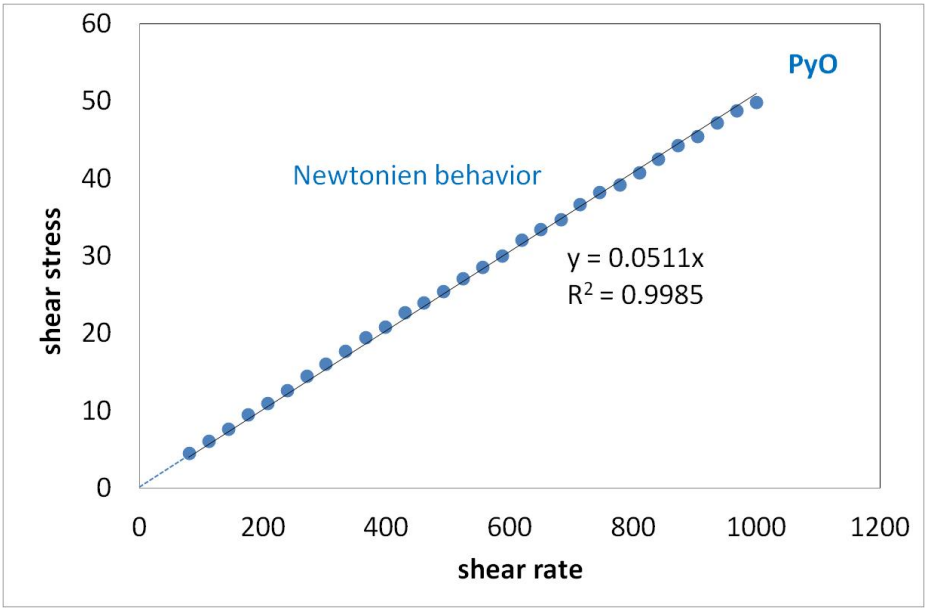


Figure 7. Investigation of the Newtonian behavior of pure PyO as protic ionic liquid.

As can be seen in Figure 7, at temperature of 18°C the PyO viscosity remains constant even if the shear rate was modified. This shown that PyO had a Newtonian behaviour and the phenomena is also known in literature as *‚elasticity of the liquid'*. This property is a very important aspect in the crystallization process and explains why there are no modifications of the PyO structure involved by the diffusion gradients that occur inside the droplets. The high viscosity of PyO (46.26 ± 0.1% mPa·s^{-}1) involves a lower mobility of the molecules and leads to different Ly spherulite morphologies with an intrinsic symmetry of the crystalline spherical shapes.

Conclusions

The originality of this research consists in the study of using PyO, unreported until now in literature, as crystallization agent of Ly protein. PyO capacity to form Ly spherulitic morphologies of type I and II through the increasing of PIL concentration in different stock solutions were tested. This work represents not only a fundamental aspect in understanding how could be better manage the protein crystallization, but it also a practical challenge for biotechnology field. Novel Ly-morphological forms obtained by HDVD, such as Ly-SNLC were identified. On the other hand, the occurrence of these initial Ly - SNLC morphologies allowed establishing the growth mechanism of the spherulites of type I (using 0.4 M PyO in TRIS/crystallant agent) and of the spherulites of type II respectively (using 1.6 M PyO in TRIS/crystallant agent and have been observed by optical microscopy. In case of using of 0.4 M PyO in 0.1 M NaAc doesn't lead to Ly-SNLC or Ly-axialites by optical microscopy, but the protein microspheres on the droplets surfaces were observed by electronic scanning microscopy.

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