Imidazolium Octanoate Carboxylate as New Branching Agent in Lysozyme Crystallization

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A new protic ionic liquid (PIL), imidazolium octanoate carboxylate (ImO), was investigated as agent for advanced crystallization of Lysozyme (Ly) protein, through the method of hanging-drop vapour-diffusion (HDVD). ImO was tested at two concentrations in 0.1 M NaAc solutions at 20°C: 0.4 M and 1.6 M. The Ly morphology was investigated by optical microscopy analysis, two days and one week after the droplets deposition. ImO concentration has a clear effect on Ly-spherulites formation at low alkaline pH. One week after the droplets deposition, Ly-spherulites of type I are formed using 0.4 M ImO and Ly-spherulites of type II, using 1.6 M ImO. Two days after the droplets deposition, by using 0.4 M ImO in crystallization experiments, some Ly-crystals like thick branches rich in short and fine needles, are observed. The splitting mechanism of the growth-bifurcation cycling process, are based on the adsorption of the ImO molecules to the needle tip, acting as a template. ImO acts as Ly branching agent, leading to obtain different microstructural Ly-spherulitic architectures in crystallization experiments.

Keywords: Protic Ionic Liquid, Lysozyme, Crystallisation, Spherulitic Growth

The protein molecules play an important role in all biological process involving the living cells. The knowledge of the protein structures has a crucial importance in a wide range of diseases (e.g. cancer disease), and researchers propose original methods in order to elucidate their structures by X-ray crystallography, solution-state NMR spectroscopy, Monte Carlo simulation etc [1,2]. In the last decades the crystallization proteins have been intensively studied due to their major impact over the field of protein engineering and medicine deign [3]. The proteins are able to form various soft-materials structures, such as: crystals, aggregates, fibres, nanotubes, spherulites etc., depending on their structure and the physico-chemical conditions [4]. In order to control the morphology and the particles size in crystallization industrial processes, it is very important to clearly make a difference between crystal growth and aggregation phenomena. Literature does not provide clear explanations concerning the mechanism of the formation of polycrystalline particles obtained in some crystallization situations. Finding optimal conditions for crystallization protein continue to be currently a bottleneck in structural genomics, because it requires the optimization of the main factors that affect directly the protein crystallization process, such as: *p*H, buffer concentration, ionic strength, concentration of protein macromolecules, temperature, additives and their concentration, presence of the substrates, precipitants, inhibitors or specific stabilizer etc. [5].

Lysozyme (Ly) is a small globular protein isolated from hen egg-white having a molecular weight of 14 KDaltons, often used as model to understand the protein crystal growth and design, by varying the crystallization conditions or the type of the crystallization additives. Some studies have involved a protic subgroup in the class of ambient temperature fluid systems, now referred to as "protic ionic liquids" (PILs) formed by a proton transfer between Brönsted acid and Brönsted base [6-8]. They are ambient liquid salts with melting points at or below room temperature, and present a high thermal and chemical stability. PILs are non-flammable due to their negligible vapour pressure, have a good electrochemical stability and great ionic conductivity [6]. They are miscible and capable of dissolving a wide range of inorganic and some organic molecules to high concentrations, and are desired preserved media for bimolecular compounds, protecting them against degradation determined by aggregation or hydrolysis [9], increasing of temperature [10,11] or unfolding proteins [9,12,13]. These properties make these green solvents attractive materials for the macromolecules applications and desirable additives for the biomolecules crystallization [14,15-17, 18-21].

Using different ILs as crystallization additives, Ly protein has been shown previously to form not only large crystals, but aggregates into amyloid fibrils at low *p*H [22,23], micro-granular precipitate, needle crystals or 3D protein crystals, amorphous, spherulites [14,16, 24].

There is insufficient knowledge of PILs—protein interactions in various experimental conditions of Ly crystallization, and thereby it remains a huge interest in better understanding the formation and the growth of the different protein morphologies, by varying PILs concentration and structure. In this purpose, our work aims to explore the impact of a new PIL still unstudied until now (ImO) in Ly crystallisation at low alkaline *p*H, through the method of hanging-drop vapor-diffusion (HDVD). In order to elucidate the effect of ImO chemical structure on the Ly-crystallization experiments, the results will be finally compared with those obtained in the case of another PIL, already studied by our group in previous studies: pyrrolidinium octanoate carboxylate, PyO [24].

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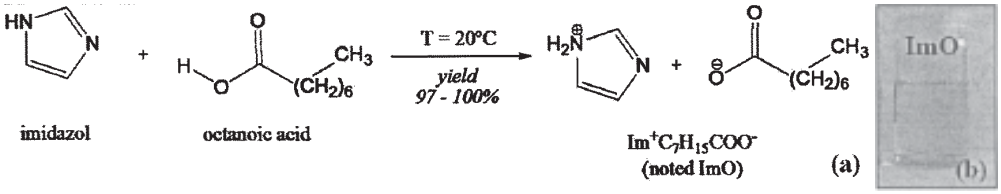

Scheme 1. (a) Synthesis of ImO; (b) transparent slightly coloured ImO obtained

Table 1
PHYSICO-CHEMICAL CHARACTERISTICS AT 20°C OF SYNTHESIZED ImO ARE AS FOLLOWS: DENSITY (ρ), IONIC CONDUCTIVITY (σ), VISCOSITY (η), REFRACTION INDEX (nr) AND RESIDUAL WATER CONTENT

Physicochemical parameters	ρ (g.cm^{-3}) ±0.1%	σ (μS.cm^{-1}) ±2%	η (mPa·s) ±0.1%	n_r	residual water (ppm)
ImO	0.99	736	62.43	1.47	980

Experimental part

Materials and methods

Materials for PILs synthesis

ImO precursors synthesized were imidazole commercially available from Fluka, and octanoic acid (98 %) from Aldrich. Precursors were used without further purification.

Synthesis of imidazolium octanoate carboxylate, ImO

ImO was synthesized through a neutralization reaction of the imidazole (Brönsted base) with the octanoic acid (Brönsted acid), according to procedure described elsewhere (scheme 1) [7]. The molar ratio of amine/acid is 1:1. The carboxylic acid was added slowly to amine with stirring in a three-necked round-bottom flask immersed into ice bath and equipped with a dropping funnel. The composition was stirred during 4 h keeping a constant temperature of 20°C. Transparent slightly coloured ImO was obtained.

HPLC analysis

The qualitative analysis and certification of the ImO synthesis reaction was verified through HLPC method (High Performance Liquid Chromatography), using a Chromatograf Finnigan Surveyor (Thermo Scientific) equipped with diode detector and autosampler. The analyses were performed using an analytical column BDS HYPERSIL C18 (150x4.6 mm, 5 μm porosity), acetonitril 100% as internal standard and solvent for each precursor and synthesized PILs. The chromatograms show that the octanoic acid and imidazol have different retention times in comparison with the final products synthesized (fig. 1). These results confirm that the neutralisation reaction took place and ImO was synthesized. Any precursor traces in the final product could be observed.

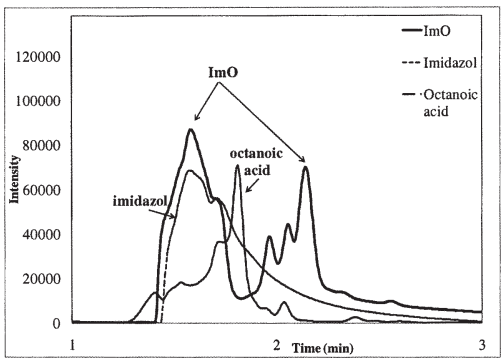

Fig. 1. HPLC spectra of ImO and their precursors: imidazol and octanic acid

Physicochemical properties of synthesized ImO

In table 1 are grouped some physico-chemical parameters of ImO measured at 20°C, such as: density (ρ), ionic conductivity (σ, viscosity (η), refraction index (n_r) and residual water content.

ImO density was determined by using an Anton Paar densimeter (DMA 4500M).

The conductivity measurements was performed using a Consort (C862) digital multifrequency calibrated with KCl standard solutions (1, 10^{-1}, 10^{-2} mol/dm^3).

The viscosity was measured using a TA instrument rheometer (AR 1000) with conical geometry at 20°C.

Refractive index was measured at 20°C using an ABBE refractive index instrument, calibrated with deionized water.

ImO was analyzed for their water content using coulometric Karl-Fischer titration, prior to any crystallisation experiments. The result is in agreement with the hydrophobic nature of PILs based on imidazolium cation (980 ppm for ImO). [8]

Materials for protein crystallization

Sodium acetate (NaAc): CH_3-$COO^-Na^+ \cdot 3H_2O$ (M=138.069g/mol) (from SigmaAldrich), deionised purified water with a Mili-Q MX (>15 MΩcm) water system, Hen egg white Ly BioUltra, lyophilized powder, 98 % (SDS-PAGE) from SigmaAldrich.

Method of preparation of the crystallization solutions

Stock solution

A stock solution of NaAc (CH_3-$COO^-Na^+ \cdot 3H_2O$) is prepared in deionised water (>15 MΩcm) to make a final concentration of 0.1 M NaAc, *p*H of 7.8.

Protein solution

Ly is used as source material for crystallization experiments by dissolving of 50 mg protein into 1 mL 0.1 M NaAc.

Mother liquor

ImO was used as crystallization agent in two concentrations in 0.1 M NaAc stock solution: 0.4 M and 1.6 M. Thus, four mother solutions (liquors) were finally obtained.

Droplets deposition method

All experiments were performed at ambient temperature. Ten droplets were placed on the glass slides for each experiment. The crystallization droplets, consisting in 5 μL of protein solution and 5 μL of mother liquor containing ImO, were obtained. First, the 5μL droplet from the protein solution is placed on a glass slide and it is

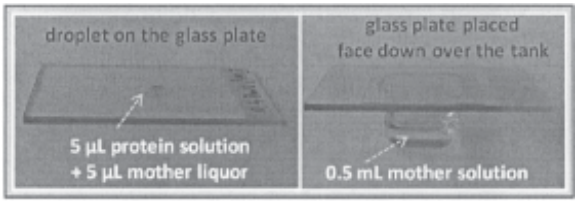

Fig. 2. The hanging-drop vapour diffusion method (HDVD)

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 (fig. 2). The trials were analyzed by optical microscopy, two days and one week after the droplets deposition.

Optical microscopy investigations

The Ly morphologies grown in aqueous solutions containing ImO were investigated by optical microscopy (Olympus BX41).

Results and discussions

ImO concentration effects in time on Ly-spherulitic morphology

The Ly crystallization using ImO in two concentrations of 0.4 M and 1.6 M respectively, will be discussed bellow.

Ly crystallization using 0.4 M ImO

Figure 3 shows the Ly morphologies obtained two days after the droplets deposition by addition of 0.4 M ImO as crystallization agent in the stock solution. The presence of some Ly morphologies with thick branches rich in fine needles could be observed on the optical images. The branching angles are approximately 30 ± 5°. A repeated splitting process, similar to that described in the literature by Liu [25], is observed. The observed splitting mechanism is also known in literature as *crystallographic mismatch branching* (CMB) and it was reported on the growing mode of spherulites [5, 20, 25, 26]. ImO acts as an additive and plays the role of a 'branching promoter' in Ly crystallization experiments. The splitting mechanism is based on the adsorption of the ImO molecules to the needle tip acting as a template. A cyclic mechanism can be observed from optical image, which starts by a primary nucleation stage and it continues with a growth-bifurcation cycling process: primary nucleation $\rightarrow$ (growth $\rightarrow$ CMB)$_n$ $\rightarrow$ ..., (fig. 3b). If the branches cross over each other, they can be finally blocked in their bifurcation process, and the phenomenon seems to stop the Ly morphology growth.

In figure 3b is shown the growth of a branched spherulitic form without fine needles. It is a typical arboresque growth which starts as a single needle (parent fibre) that splits into two new needles (daughter fibres) at each end, mentioned in literature as "Cayley-like tree" structure [27]. According to authors, the main parameters of this specific structure are the bifurcation number n, the length of an individual needle and the bifurcation angle θ. Each branch could be the source of a new front of nucleation and crystal-like growth. Thereafter it will be finally covered by fine needles as seen in figure 3c. We can conclude that CMB mechanism in this case is induced by the adsorption of the ImO molecules used as additive on the spherulites needles surface, thus increasing both the bifurcation number and the length of the needles.

The Ly morphology becomes different one week after the droplets deposition. Figure 4 presents the Ly crystallization results by HDVD method using 0.4 M ImO in 0.1 M NaAc. The spherulite forms have the aspect of some

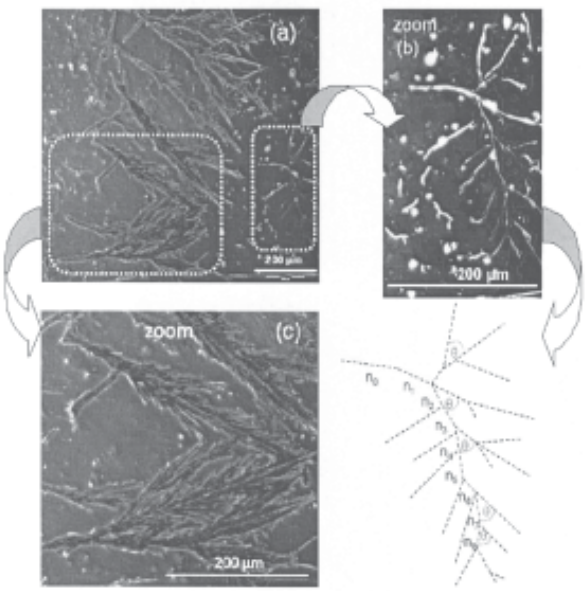

Fig. 3. Optical images of the initial Ly morphology formed two days after the droplets deposition, using 0.4 M ImO in 0.1 M NaAc: (a) crystal growth as branched forms like thick branches rich in fine needles; (b) crystal growth as "Cayley-like tree" structure [24]; (c) zoom from (a) image

bundles with fine and long fibres that grow up in a radial crossing direction (fig. 4a). The growth mode observed in figure 4b takes place according to the theory developed by Gránásy [28] concerning the "*growth front nucleation*" (*GFN*) of the spherulitic crystals, that are formed by the interface propagation of the front of nucleation. In figures 4(a-d) we can observe long and fine Ly-fibres that grow in a preferred direction. A single spherulite form (fig. 4e) shows the radial distribution of the axialite *arms* (needle-like crystals) of the spherulite which grow and branch out. The form and distribution of its needle-like crystals oriented in a radial direction confirms it to be spherulitic form type I, according to the literature [27, 28].

Also, on the droplets edge of some samples there were captured images of single Ly needle-like crystals (SNLC) with an elongated aspect (fig. 4f). The initial phase of the spherulite formation seems to be due to the axial growth pattern of SNLC which leads finally to the formation of axialites, according to the theory of Castro et al. [29]. There is no doubt, in Ly crystallization process ImO has a influence on growth of axialites and it leads to a final spherulitic type I morphology, according to the mechanism that we have summarized in a previous paper as follows: *SNLC*$\rightarrow$ *axialites* $\rightarrow$ *spherulites* [24]. The spherulite form type I captured on the optical image presented in figure 4e, branches out forming sheaves ('*axialite arms*') [26] and showing the axial growth mode.

Ly crystallization using 1.6 M ImO

When the ImO concentration increases from 0.4 M up to 1.6 M in 0.1 M NaAc, the Ly morphology has a different aspect. Figure 5 shows the new Ly morphology obtained one week after the droplets deposition. On the droplets surface it can be observed the presence of Ly spherulite forms with arboresque morphology. Ly-Spherulite structures have 'flower' shapes and the architecture of the interconnecting Ly-spherulitic forms has a rather homogeneous distribution on the droplets surface (fig. 5, on the left), compared to that of the spherulites formed using 0.4 M ImO in crystallization experiments (fig. 4a-c). The diameter of one spherulite is around 100 µm (fig. 5, on

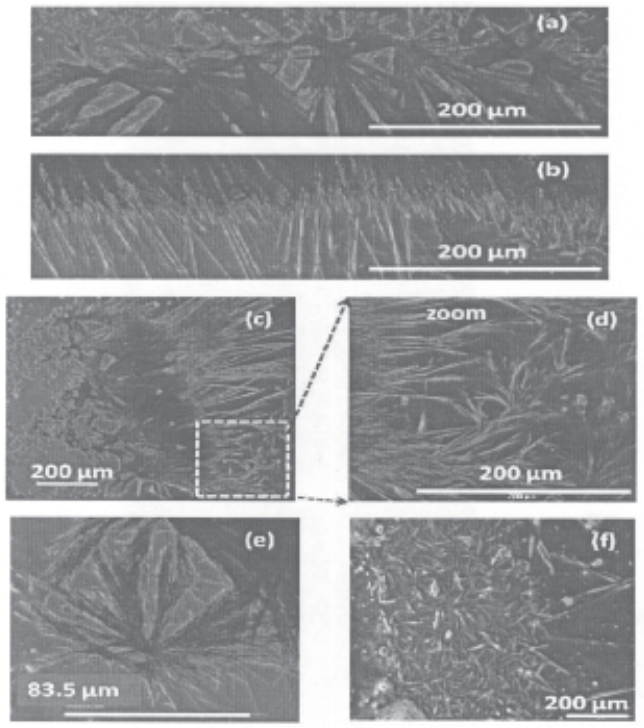

Fig. 4. Optical images of the Ly morphology formed using 0.4 M ImO in 0.1 M NaAc, one week after the droplet deposition: (a) bundles with fine and long Ly fibres in radial crossing directions; (b) the *growth front* of the long Ly fibres observed in (a); (c) axialites arms of the bundles with long Ly fibres that grow up in a preferred direction; (d) zoom from (c) on the Ly fibres growth; (e) single spherulitic form; (f) single Ly needle-like crystals (SNLC)

the right), close to that observed in the single spherulitic form type I using 0.4 M ImO (fig. 4e).

ImO has a clear influence on the growth mode of Ly-spherulite morphologies, playing the role of a *branching agent* in Ly crystallization experiments (fig. 3) and leading to the formation of two types of Ly-spherulitic forms: of type I, using 0.4 M ImO and of type II, using 1.6 M ImO. Both spherulitic structures, present a radial growth (see fig. 6), with almost the same diameter, but an intrinsic symmetry of the crystalline spherical shapes is observed only in the case of Ly-spherulites of type II formed using a higher ImO concentration.

The viscosity parameter determines the molecular mobility of the particles and has a direct control of the manner that molecules participate on the spherulitic growth. Higher the ImO concentration into the stock solution, more viscous the drops. Consequently, molecules are less mobile, having much longer time to establish physical interactions between them in order to form nucleation centres and to start the growth of the nucleation front. This observation is in accord with the Granasy theory about the influence of the viscosity parameter on the molecular mobility into the drops in the spherulitic growth phenomena [28].

The viscosity parameter is not the single factor which has a direct influence on the spherulites formation and growth. In absence of ImO molecules, water dipoles maintain the hydratation state of the protein. In the presence of the ImO molecules, the charged groups on the surface of the Ly-protein exhibit a Coulombic attraction, between opposite charges on ImO molecules (cations or anions), shielding the charged groups of the protein molecule. When the water vapour molecules diffuse from the drop into the reservoir solution, the hydratation state of the protein

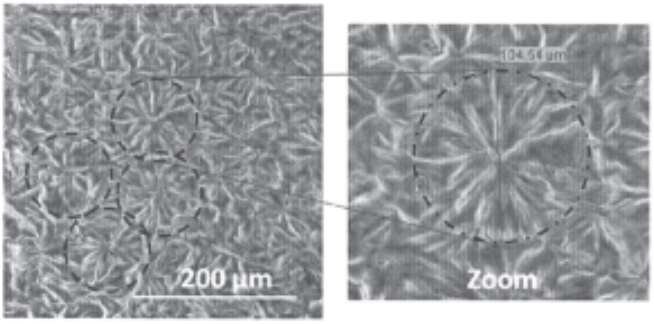

Fig. 5. Optical images of the Ly spherulitic morphology formed using 1.6 M ImO in 0.1 M NaAc: the architecture of interconnecting Ly spherulitic forms, one week after the droplets deposition (on the left); one single spherulitic form type II with a diameter around 100µm (on the right)

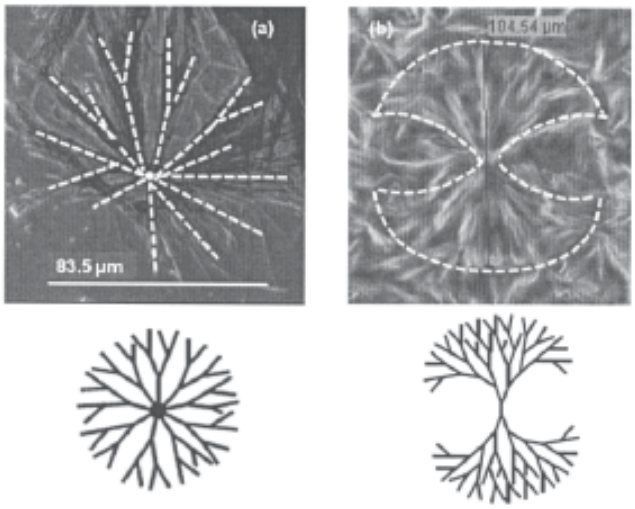

Fig. 6. Ly spherulitic morphologies formed one week after the droplets deposition using ImO: (a) spherulitic form of type I obtained using 0.4 M ImO; (b) spherulitic form of type II obtained using 1.6 M ImO

reduces. The protein and ImO concentrations in the drop increase, leading to more interactions of the protein surface with the environment and thereby with ImO molecules. When all water molecules were evaporated, the available ImO molecules were completely attracted in the Coulombic attractions. In order to satisfy the electrostatic requirements, the protein molecules begin to self-associate, and thereby, the primary nucleation phenomenon starts, in the presence of ImO molecules. From this point, ImO begin to act as a *'branching promoter'* in the splitting mechanism of Ly-spherulites, by mediating of the *growth-bifurcation* cycling process.

ImO chemical structure effect on Ly crystallization experiments

In a previous study we reported some results concerning the formation of spherulites type I and II by using another protic ionic liquid as crystallization agent: PyO (pyrrolidinium octanoate carboxylate) [24]. An interesting aspect concerning the formation and the growth mode of Ly-spherulites, by using ImO and PyO as crystallization agents should be discussed in this paper. In both cases, the crystallization experiments occurred by the same HDVD technique for the droplets deposition on the glass plates at low alkaline *p*H and ambient temperature. Ly-protein concentration was 50 mg/mL in both experiment sets. In our previous study, the crystallization experiments using 0.4 M PyO in 0.1 M NaAc did not lead to obtain Ly-

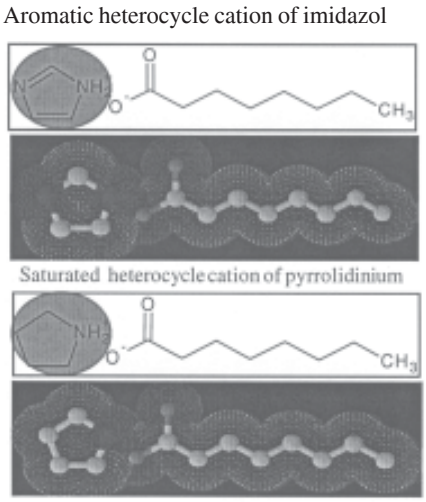

Fig. 7. PIL chemical structure of ImO and of PyO, obtained by using the ChemSketch programme (2 D and 3 D viewers)

spherulitic forms or Ly-SNLC (single needles like crystals). But, Ly-spherulitic forms of type I and II were finally obtained by addition of TRIS/crystallant agent in 0.1 M NaAc solution. These observations lead to a new conclusion: the chemical structure of the PILs has got a clear effect on the Ly-crystallization experiments.

The explanation for different Ly-spherulite morphologies formed in both studies is attributed especially to the structural differences between the PILs used as branching agents. PyO and ImO chemical structures have a common anion with an identical alkyl length (n = 8 for the octanoic carboxylate -$C_nH_{2n+1}COO^-$), but completely different cations (fig. 7). The imidazolium cation of ImO is an unsaturated heterocyclic amine and the pyrrolidinium cation of PyO has a saturated heterocyclic one. Their distinct cation structures involve differences between the physico-chemical properties of PILs, like hygroscopicity and viscosity parameter. PyO is more hygroscopic than ImO and the residual water content is 3.7 times higher in pyrrolidinium than in imidazolium PILs (3650 ppm in PyO vs. 980 ppm in ImO), but ImO is more visqueous at ambient temperature (62 mPa.s for ImO vs. 42 mPa.s for PyO). The electrons delocalisation of the unsaturated heterocycle of ImO cation is responsible for ImO behaviour in Ly-spherulites formation mechanism, by reinforcing the physical interactions with the Ly-molecules in time.

Conclusions

Ly-spherulitic forms are obtained by HDVD method, using two PIL concentrations in 0.1 M NaAc: 0.4 M ImO and 1.6 M ImO. The Ly morphologies were investigated by optical microscopy means, two days and one week after the droplets deposition. The viscosity parameter and the chemical structure of ImO molecules are involved in the mechanism formation of two Ly spherulitic forms, one week after the droplets deposition: Ly-spherulites type I are obtained using 0.4 M ImO and Ly-spherulites type II using 1.6 M ImO. Two days after the droplets deposition, by using 0.4 M ImO in Ly-crystallization experiments, Ly-crystals like thick branches rich in short and fine needles, are observed in optical images. ImO acts as protein *branching agent* in the splitting mechanism of Ly-crystals. The growth-bifurcation cycling process is based on the adsorption of the ImO molecules to the needle tip, ImO acting as a template in Ly crystallization experiments.

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