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Microporous Hydrophobic Membranes for Crystallization of Biomolecules

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Membrane crystallization is today recognized as an innovative and efficient method for enhancing crystallization of biomolecules, allowing the production of crystals with controlled shape, size distribution, and polymorphism. Membranes are used both to promote an efficient mass transfer of solvent for a better control of the supersaturation, and to activate heterogeneous nucleation for a reduced induction time. The development of membranes having ordered structures and tailored surface properties is, therefore, a key issue in order to fully exploit the potential of membrane crystallization. In this work, theoretical and experimental correlations between the physico-chemical properties of the membranes, kinetics of nucleation and the characteristics of the final product are presented.

1. Introduction

Crystallization is an excellent technique for the purification of chemical species by solidification from liquid mixtures. In pharmaceutical industry, crystallization is extensively applied to polymorphism selection; in biochemistry, crystallization is used for detailed description of proteins structure at the atomic level, which is mostly achieved by X-ray diffraction analysis of single biomolecular crystals. The use of nanostructured hydrophobic membranes in order to promote the crystallization of biomolecules has been investigated in recent years (Di Profio et al., 2010). According to this innovative methodology, membranes are used in order to: (i) generate supersaturation by transferring the vaporized solvent from the mother solution to an hypertonic salt solution, under a partial pressure gradient; (ii) promote heterogeneous nucleation, so decreasing the energetic barrier required for the aggregation of critical nuclei and, ultimately, reducing the induction time of the crystallization process even at low supersaturation (Drioli et al., 2015).
Membrane crystallization systems make use of hydrophobic membranes; their hydro-repellent character avoids the passage of solvent in liquid state, while sustaining a liquid/vapour interface at pore mouths. The resulting gradient of partial pressure between the two sides of the membrane, activated by a concentration difference to avoid thermal degradation of biomolecules, is the driving force to the evaporation of the solvent that generates a supersaturated solution (figure 1).
According to the Dusty Gas Model theory, the flux of the i-th volatile component (J_i) through a porous medium driven by a partial pressure gradient (∇p_i) between both sides is (Curcio et al., 2005):

$$J_i = -\frac{D_e}{RT}\nabla p_i \tag{1}$$

where D_e is the effective diffusion coefficient, R is the gas constant, and T is the absolute temperature. The effective diffusion coefficient is calculated as a function of the Knudsen diffusion coefficient and molecular diffusion coefficient:

$$D_e = \frac{\varepsilon}{\tau}\left(\frac{3}{2r}\sqrt{\frac{\pi M_i}{8RT}} + \frac{1-y_{air}}{D^0_{i/air}}\right)^{-1} \tag{2}$$

where ε, r and τ are the porosity, average pore radius and tortuosity of the membrane, respectively, and M is the molecular weight and y_{air} the mole fraction of air in the membrane pores.

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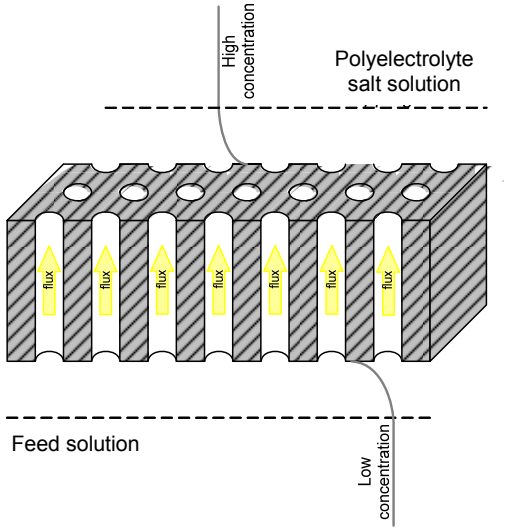

Figure 1: Basic operational principle of a membrane crystallization system: an aqueous solution of polyelectrolyte salt (stripping solution) contacting the hydrophobic membranes is used to generate a partial pressure gradient that drives the transport of solvent from the solution of biomolecules (retentate solution). The progressive removal of solvent induced supersaturation.

2. Heterogeneous nucleation on porous membranes

According to the Classical Nucleation Theory (CNT), crystallization can be considered an activated process: a energy barrier ΔG^* must be crossed in order to induce the formation of critical nuclei having, by definition, equal probability (50%) to grow or to dissolve.

Gibbs free energy of the crystalline phase is sum of the contributions from bulk and surface energy, and involves terms such as the surface tensions of the nucleus-liquid (γ_L), nucleus-membrane (γ_i) and liquid-membrane (γ_s) interfaces. Young equation for surface tensions is not applicable since it is strictly valid for ideal and non-porous surfaces. For a porous membrane, a modified form of the Young equation correlating the surface porosity (ε) to the measured and equilibrium contact angle (θ) is used:

$$\left(\gamma_S - \gamma_i\right) = \gamma_L\left[\cos\theta + \frac{4\varepsilon\left(1+\cos\theta\right)}{\left(1-\varepsilon\right)\left(1-\cos\theta\right)}\right] \tag{3}$$

The resulting energetic barrier to heterogeneous nucleation occurring on a porous membrane ΔG^*_{het} is:

$$\frac{\Delta G^*_{het}}{\Delta G^*_{hom}} = \frac{1}{4}\left(2+\cos\theta\right)\left(1-\cos\theta\right)^2\left[1-\varepsilon\frac{\left(1+\cos\theta\right)^2}{\left(1-\cos\theta\right)^2}\right]^3 \tag{4.a}$$

where

$$\Delta G^*_{homogeneous} = \frac{16}{3}\pi\gamma_L^3\left(\frac{\Omega}{\Delta\mu}\right)^2 \tag{4.b}$$

In equation (4.b), Ω is the molar volume, and $\Delta\mu$ the chemical potential gradient between the crystalline phase and the mother solution. If ε = 0, equation (4.a) reduces to the classical CNT form describing the heterogeneous nucleation on solid surfaces. The function expressed by equation 4.a is plotted in figure 2 as a function of the membrane porosity of home-made PVDF membranes and measured contact angle for lysozyme solution (40 mg/ml, 2% w/v NaCl).

In general, using polymeric films as heteronucleants offers a promising alternative to biomolecular crystallization, since their structure and chemistry are easily tunable over a wide range by a variety of established fabrication methods conventionally used for membranes preparation. Combining the function of tailored polymeric surface with crystallization technology might open interesting opportunities in the field of crystal engineering (Di Profio et al., 2014).

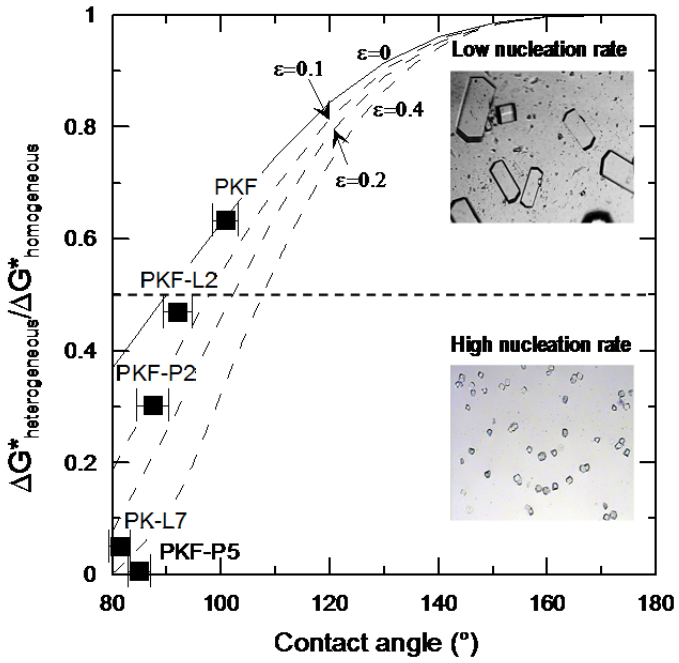

Figure 2: Ratio of Gibbs energy barrier for heterogeneous and homogeneous nucleation as a function of the membrane porosity and the contact angle of lysozyme solution (protein: 40 mg/ml; NaCl as precipitant: 2% w/v). Lines are from equation (2). Abbreviations: PKF - Kynarflex 2800 (polymer); PK- Kynar 460 (polymer); L – LiCl (additive); P- PVP (additive)

A well documented advantage of membrane crystallization techniques over conventional methods is represented by the accelerated rate of the crystallization process, as demonstrated by induction time analysis and nucleation/ growth rate measurements. Membrane crystallization experiments carried out on various hydro-soluble proteins showed the possibility to obtain a crystalline product at lower induction times with respect to those measured when using conventional vapour diffusion techniques (figure 3). Moreover, porous membrane surfaces activate specific molecules-membrane interactions that favourably affect the mechanisms of nucleation thus allowing molecules to aggregate in conditions of supersaturation that would not be adequate for the spontaneous nucleation.

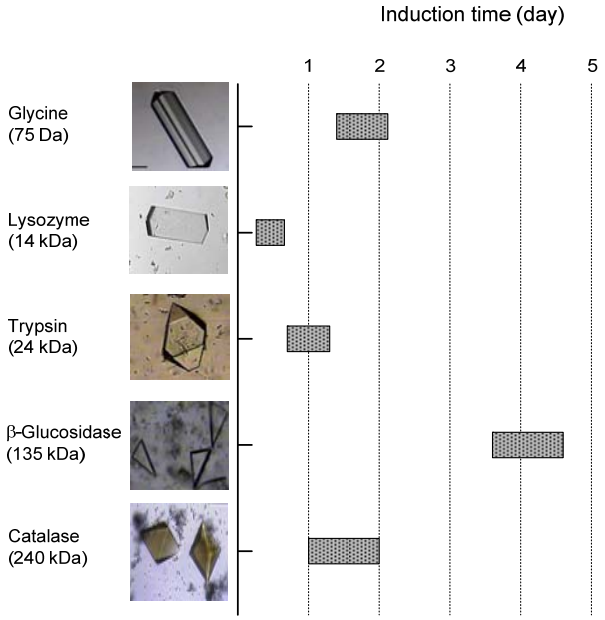

Figure 3: Typical ranges of induction times for biomolecules crystallized by hydrophobic membranes.

3. Surface chemistry and polymorph selection

Membrane surfaces can promote heterogeneous nucleation merely by lowering the surface energy of aggregating molecules (non-specific adsorption), but also by structural matching driven by specific polymer-molecule interfacial interactions according to a mechanism analogous to epitaxial growth. Capability of polymorph selection, kinetically driven by the preferential aggregation of molecules along specific crystalline facets, corroborates these assumptions. Curcio et al. 2014 investigated the influence of specific intermolecular interactions between acetaminophen (ACM) and polyimide (PI) during nucleation, using PXRD to identify preferred orientation of crystal facets on the surface.

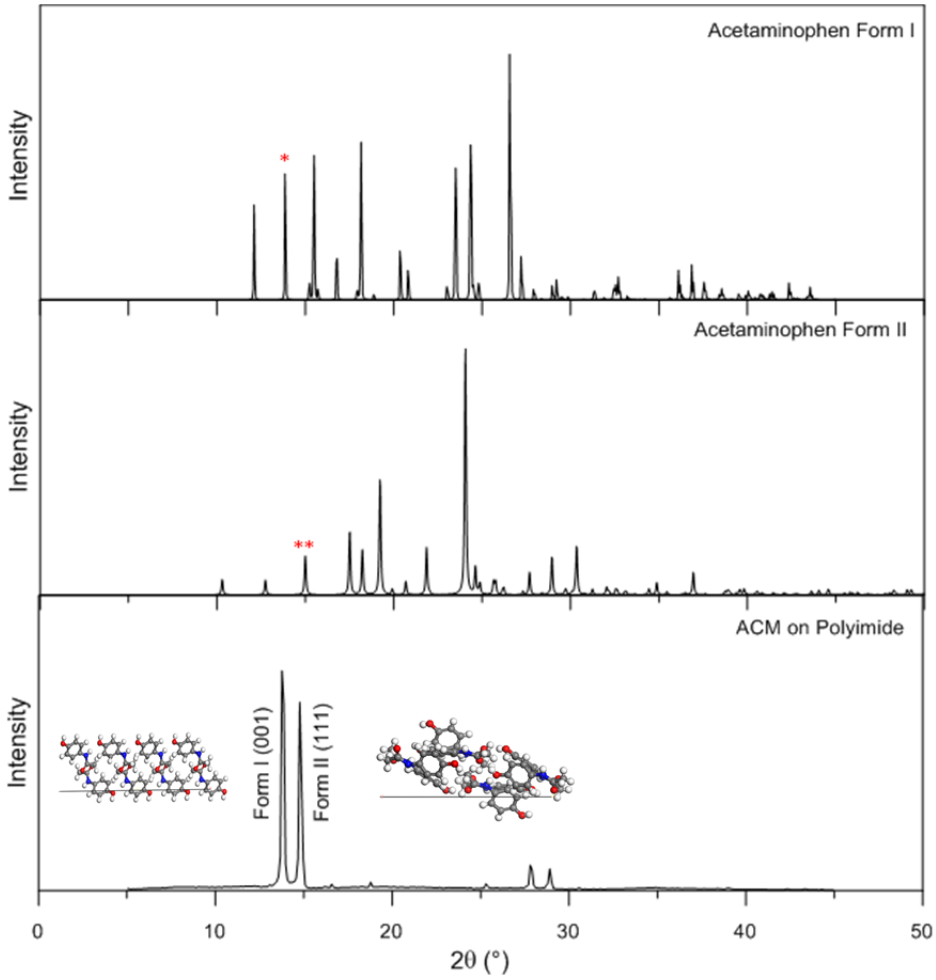

Figure 4: PXRD diffractograms showing concomitant polymorphism in acetaminophen (ACM) crystals grown on polyimide (PI) surface. The experimental pattern is compared to the calculated patterns for ACM Form I (top) and Form II (center) obtained from the crystallographic information files.

Diffractograms revealed the concomitant presence of both the metastable polymorph II (orthorhombic) and the stable form I (monoclinic) of ACM. In agreement with the hydrophilic character of PI, the major reflection at 13.9° is corresponding to the preferential orientation of form I crystals along {001} with hydroxyl groups of ACM oriented towards the polymeric surface. The major reflection at 15.0° is related to the preferential growth of form II crystals along the plane (111) with hydroxyl groups of ACM obliquely oriented towards the substrate. Interfacial interactions are likely to involve both imide functionality and carbonyl groups in polyimide. ACM form II was observed as the first crystallizing polymorph, then progressively rearranging into the most thermodynamically stable form I.

The crystallization of polymorphs is affected by competitive nucleation, growth, and transformation from a metastable to a stable form. In general, the Ostwald's step rule (or rule of stages states) that the first form to crystallize is the one with the largest Gibbs free energy (the most soluble), which then transforms to the next most soluble form (involving the smallest loss of free energy) through a process of dissolution and re-crystallization (Ostwald, 1897).

Polymorphism is a relevant phenomenon in pharmaceutical industry, since the different structures of polymorphs result in different physicochemical properties, like melting point, solubility, dissolution rate, compressibility, which can have great influence on the bioavailability, filtration, tableting etc. The interesting potentialities of membrane crystallization in this field are highlighted in the case-studies briefly reported below.

Glycine exhibits at least three polymorphic forms: α, β and γ; β-glycine is the least stable form crystallizing from water/alcohol mixtures. Polymorphs α and γ typically grow from pure aqueous solutions at different ranges of pH: α-glycine appears for $4 < pH < 8$, while the γ-form at pH < 4 or pH > 8. Different strategies have attempted to obtain the selective production of the γ-form from almost neutral aqueous solutions. Crystallization of glycine on microporous hydrophobic polypropylene membranes at pH 6.2 resulted in the preferential crystallization of a specific polymorph depending on the extent of the transmembrane flux of solvent (J). For $J < 1.4 \cdot 10^{-2}$ mL/h, the only form obtained was γ-glycine, while for $J > 1.8 \cdot 10^{-2}$ mL/h only α-glycine appeared (Di Profio et al., 2007a).

Paracetamol (acetaminophen) is a widely used antipyretic and analgesic drug. The three known polymorphs are: the monoclinic form I (space group $P2_1/n$) - thermodynamically stable at room temperature; the orthorhombic form II (space group P_{cab}) - metastable at ambient condition; the form III, obtained by crystallization from the melt. Membrane-assisted crystallization allowed the selective production of a specific paracetamol polymorph by a careful control of the solvent evaporation rate (J) through the membrane pores. Experimental evidences proved that, for $J \geq 7.9 \cdot 10^{-2}$ mL/h the polymorph obtained was the metastable form II, while for $4.3 \cdot 10^{-2} \leq J \leq 6.6 \cdot 10^{-2}$ mL/h only the thermodynamic monoclinic product appeared (Di Profio et al., 2007b).

In addition, experimental investigations allowed at identifying different nucleation mechanism for different ranges of supersaturation: at high supersaturation, nucleation was prevalently homogeneous, whereas at low supersaturation heterogeneous nucleation mechanism prevailed. Therefore, at very low values of J ($< 2 \cdot 10^{-2}$ mL/h), polypropylene membrane surface provided sites for heterogeneous nucleation, thus enhancing the probability of nucleation for the orthorhombic form II.

Carbamazepine (CBZ) is a water-insoluble drug requiring high dosage (> 100 mg/day) in therapeutic treatment. Since the solubility and dissolution rate of the most thermodynamically stable forms are lower than that of the metastable forms, the ability to produce higher-energy polymorphs is desirable. CBZ exists in a dihydrate form and, at least, in five anhydrous forms: primitive monoclinic (CBZ III), triclinic (CBZ I), C-centered monoclinic (CBZ IV) and trigonal (CBZ II), sorted by thermodynamic stability in decreasing order, and a orthorhombic form (CBZ V). Membrane crystallization tests revealed that, at increasing transmembrane flux, the amount of CBZ I in the precipitate solid phase decreased, while the amount of CBZ IV increased. Data are coherent with the assumption that the formation of metastable forms is promoted at higher supersaturation rate (Caridi et al., 2012).

4. Conclusions

The ability to promote and control heterogeneous nucleation kinetics by modulating the physico-chemical parameters of polymeric membranes (i.e. surface chemistry, porosity, roughness, hydrophobic/hydrophilic character etc.) represents a unique feature of membrane-based crystallization techniques. Major benefits are evident in the crystallization of biomolecules, nucleating on the membrane surface at higher rate with respect to conventional crystallization techniques, while maintaining an excellent structural order. Moreover, the proven ability to select a specific polymorphic form through the fine control of supersaturation rate and the promotion of specific chemical interactions, is of huge interest in the pharmaceutical industry.

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