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600d - SURFACE-INDUCED NUCLEATION STRATEGIES: SEEKING SYMMETRIES BETWEEN SELF-ASSEMBLY OF HETERONUCLEANTS AND CRYSTALS

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Biography

Fiora Artusio is a Ph.D. candidate in Chemical Engineering at Politecnico di Torino. She is working at the Molecular Engineering (MolE) Lab under the supervision of Prof. Pisano. She got her MSc Degree in 2016 studying photo-activated polymerization for the synthesis of nanocapsules for drug delivery. Her research is now focused on the development of experimental methodologies for the control of pharmaceuticals and biopharmaceuticals crystallization. She has carried out research activity in Paris (LSI, Ecole Polytechnique) and at JRC – European Commission, concentrating on electron microscopy and surface analysis techniques respectively. She is currently working on protein crystallization at Laboratorio de Estudios Cristalograficòs (LEC) in Granada.

Giacomo Ceccone is a Staff Scientist at Joint Research Centre (JRC) – European Commission in Ispra. He received his Degree in Physics from University of Pavia (Italy) in 1984. After post-doctoral fellow at the University of Minnesota (USA) and TASC Laboratory, (Trieste, ITALY) he joined the JRC in 1990 where he is working in the Directorate F – Health, Consumers and Reference Materials. Dr Ceccone's main research interests concern the application of surface analysis techniques (e.g. XPS, ToF-SIMS, Synchrotron Radiation-based spectro-microscopies) to the physico-chemical characterization of surfaces with particular emphasis on nanomaterials and micro/nano-plastics. Dr Ceccone has published more than 150 papers in peer-reviewed journals, 4 book chapters and delivered more than 80 presentations at international conferences.

Roberto Pisano is a Professor of Chemical Engineering at Politecnico di Torino (Italy), where he received his Ph.D. in 2009. Professor Pisano's research focuses on the application of both computational and experimental methods to engineering chemical products and processes, with particular emphasis to pharmaceutical processing and formulation of both small molecules and biologics. He has worked with many pharmaceutical companies in research or consulting. He has published more than 80 papers, 11 book chapters, 1 edited book on freeze-drying of pharmaceuticals and currently has 4 patents issued or pending.

Abstract

The present paper presents the application of surfaces having defined and controlled attributes as heteronucleants for the crystallization of a model pharmaceutical molecule. The synthesis of the substrate was optimized in order to relate surface features to the crystallization outcome. Extremely flat and topographically uniform glass supports bearing amino and thiol head groups were successfully synthesized and characterized by means of contact angle, AFM and XPS analyses. Such surfaces were then used as supports for aspirin (ASA) crystallization in order to investigate their influence on nucleation kinetics. Compared to untreated glass, amino-functionalized glass was dramatically nucleation-active, whereas thiol-functionalized supports strongly repressed ASA heterogeneous nucleation. The promoting or inhibiting action towards the stabilization of ASA nuclei on a functionalized surface and their successive growth into crystals was therefore related to the chemistry of exposed head groups.

Introduction

Nucleation represents the core of a variety of processes occurring in nature, such as ice or cloud formation [1], but it is also responsible of amyloid aggregation leading to Alzhemeir's disease [2] or even creation of virus capsids [3]. The formation of new phase in defined environment as a consequence of self-assembly and ordering processes represents a *fil-rouge* for all these phenomena. More specifically, nucleation can occur homogeneously or heterogeneously, depending on whether aggregation mechanism involves the presence of single or multiple phases. In the latter case, stable nuclei are formed taking advantage of favorable interactions with foreign surfaces which lower the energetic cost for new interface area formation. However, heterogeneous nucleation often occurs in uncontrolled manner because of impurities in the system, leading to significantly different crystallization outcomes. This aspect is pivotal to pharmaceutical and biopharmaceutical industries as nucleation not only affects process kinetics, but also crystalline form, habit and size selection, as well as downstream processes [4].

Recently, the role of external surface features on crystallization of Active Pharmaceutical Ingredients (APIs) is being investigated by relating surface chemistry and morphology to nucleation kinetics and polymorph selection [5–7]. For example, surfaces carrying pores were found to strongly accelerate nucleation kinetics, provided that these were not spherical [8]. Selecting one specific crystalline form of a given substance can also be achieved by simply acting on the heteronucleants, keeping constant all the other crystallization conditions [9–11]. However, it is difficult to isolate and distinguish between chemical and morphological interactions between a surface and a to-be-crystallized molecule.

Spatial confinement of chemical reactions represents a useful strategy for the synthesis of a variety of devices, such as nanocapsules and nanohydrogels [12,13]. Here, by confining the functionalization reaction to an active solid-liquid interface, Self-Assembled Monolayers (SAMs) on glass were synthesized. We present an organic approach to the study of heteronucleants quality attributes in order to relate them to crystallization of small molecule drugs. The complete and diversified characterization of surface properties is vital, as the interplay of complex and different aspects of a surface is a key-aspect when promoting or

inhibiting nucleation. By carefully tailoring synthesis conditions, we synthesized high-quality SAMs on amorphous substrates and successively applied them to the crystallization of a pharmaceutical model molecule.

Materials & Methods

Chemicals

Glass coverslips were purchased from Neuvitro (Vancouver, USA). Hydrogen peroxide (30 wt % in water, ACS reagent), sulfuric acid (ACS reagent, 95.0 – 98.9%), anhydrous toluene (99.8%), toluene (ACS reagent, ≥ 99.5%), ethanol (puriss. p.a., ≥ 99.8%), 3-aminopropyltrimethoxysilane (APTMS, 97%), 3-mercaptopropyltrimethoxysilane (MPTMS, 95%) and water (HPLC grade) were purchased from Sigma-Aldrich (Cesano Maderno, Italy).

Synthesis of self-assembled monolayers

Glass coverslips were pre-activated and cleaned in 3:1 *piranha* solution for 1 h and thoroughly rinsed with water afterwards. Then, they were incubated in 5.4 x 10^{-2} M solution of silane in anhydrous toluene. Reaction time was 30 min for APTMS and 15 h for MPMTS. Final rinsing with toluene, toluene-ethanol mixture and ethanol served as last synthesis step.

Surface characterization

Contact angle (CA) analyses were firstly carried out in order to assess the effectiveness of functionalization step and surface uniformity. A MSE DigiDrop (GBX, France) was used to dispense 3 µL HPLC water drops on substrates and calculate sessile drop contact angles. Surface topography was evaluated by means of Atomic Force Microscopy (AFM, Solver NANO, NT-MDT Spectrum Instruments, Russia). Cantilever frequency was 1 Hz, scanned area was 1 x 1 µm and 256 lines per scan were acquired. Finally, chemical analyses were performed with X-Ray Photoelectron Spectroscopy (XPS, AXIS ULTRA, DLD Kratos Analytical, UK). Pass energy was set at 80 keV. Wide spectra were collected from 1100 to 0 eV values of binding energy. The angle between sample surface and analyzer was set at 90°. Calibration for all the spectra involved setting hydrocarbon C1s at 285.0 eV. Data were processed with CasaXPS software (version 2.3.20).

Crystallization studies

We designed and implemented a high-throughput crystallization platform in order to perform a large number of crystallization experiments simultaneously. Aspirin was dissolved in 38% ethanol in water and crystallization was carried out in wells containing SAMs. Supersaturation was set at 1.8 and crystallization temperature was 15 °C. Crystallization outcome was studied according to kinetic and thermodynamic aspects, involving nucleation induction time and crystalline form and habit selection. The first aspect was studied by means of optical microscopy coupled to motorized X-Y stage (M125C, Leica Microsystems, Germany) and *in-house* cooling

system, enabling time-lapse multiple-positions acquisition and ensuring maintenance of precise supersaturation conditions until the nuclei formation. Nucleation kinetics was evaluated by calculation of detectable induction time, corresponding to the time when detectable crystals could be evinced inside each crystallizer.

Results

SAMs were built starting from methoxy-silane molecules carrying a propyl chain with a terminal functionality. The latter represented the only difference among our monolayers, being thiol or amino-terminated. The portfolio of elected end groups permits the deep investigation of secondary interactions between surface and solute molecules and their impact on nuclei formation. As a matter of fact, our final goal is to design and develop a versatile benchmark for crystallization studies assessing the extent of specific and non-specific interactions. This is achieved by only changing substrate, while all crystallization conditions, *e.g.* supersaturation, solvent, temperature, are not to be modified.

Proving surface functionalization

Trimethoxysilanes were chosen because of their high reactivity and degree of packing when organized in monolayers. These molecules have been anchored to pre-activated glass substrates by means of condensation reactions between the surface hydroxyl groups and the hydrolyzed methoxy groups of silanes. The synthesis conditions have been optimized in terms of reaction solvent, functionalizing agent, reaction time and temperature in order to ensure the formation of reproducible single layers of organic matter on glass. As a matter of fact, for crystallization studies devoted to the mechanistic understanding of surface effects on nucleation, it is crucial to deal with extremely uniform substrates both as concerns the sample itself, but also *intra* and *inter*-batch homogeneity. This need arises from the intrinsic stochastic nature of nucleation, thus requiring a high number of experiments involving the same experimental conditions to get statistically significant data.

Thorough characterization of the crystallization substrates has been carried out in order to confirm functionalization effectiveness and gain a structured overview of surface properties. First, contact angle analyses confirmed surface functionalization: as regards MPTMS, glass surface turned out to be more hydrophobic after monolayer assembly, whereas, for APTMS, a more hydrophilic surface was obtained, as reported in Table 1. Absence of local deposits of polymerized silanes was confirmed by AFM, which also confirmed the achievement of an extremely regular functionalization. Moreover, some interesting insights into surface roughness were collected. It turned out that the presence of a monolayer on the surface did not alter surface mean roughness, whereas, when uncontrolled functionalization was carried out, major effects on this parameter were detected. More specifically, untreated glass represented an extremely flat surface, having R_q well below 1 nm, *i.e.* 130 pm. Then, covalent bonding of an organic monolayer on top led to comparable average roughness values for all the functionalities. This feature represents a particular appealing aspect of the application of high-quality SAMs to heterogeneous nucleation studies since, as topography is not altered by the

functionalization process, the effect of different superficial chemistries may be isolated from morphological interactions between substrate and solute molecules.

The effectivity of functionalization was corroborated by means of XPS. Analyses were carried out for different purposes: the main goal was the confirmation of the presence of characteristic elements of SAMs on glass. Compared to untreated glass, MPTMS and APTMS-functionalized supports led to sulfur and nitrogen characteristic peaks in the XPS spectra, respectively. Further confirmation derived from the comparison of characteristic elements ratios between the ideal and real case. C/S or C/N theoretical ratios for SAMs are equal to 3. We measured 6.6 and 4.4 respectively. Slightly higher elemental ratios were attributed to superficial contamination of the samples, but also denoted that functionalization was likely to occur in monolayers.

The characterization of SAMs led us to confirm the suitability of these surfaces to be used as heteronucleants in crystallization studies.

Table 1. Contact angle, roughness and elemental composition of untreated glass, thiol- and amino-terminated SAMs.

Sample	**CA, °**	**%O**	**%Si**	**%C**	**%S**	**%N**
Untreated glass	49.1 (+/- 1.9)	54.6	21.6	20.4	-	-
Thiol-SAM	65.7 (+/- 1.7)	55.3	21.3	19.1	2.9	-
Amino-SAM	37.1 (+/- 0.6)	30.3	15.0	43.1	-	9.8

Addressing the effect of surface chemistry on crystallization of pharmaceuticals

In this study, self-assembled monolayers (SAMs) have been selected as ideal candidates to probe superficial chemical effects on nuclei formation and successive crystal growth. An amorphous substrate was selected for supporting monolayer assembly in order to exclude epitaxial interaction between the final heteronucleant and solute molecules. In this way, we were able to exclude complex ordering phenomena rising from lattice matching between surface and growing nuclei. In addition, such an approach enables the development of crystallization supports which are structurally inert, but chemically active towards nucleation. Extremely flat surfaces were selected in order to deconvolute, as much as physically possible, the contribution of surface chemistry and topography to nucleation phenomena.

Starting from experimental observation, cumulative probability distribution curves for ASA crystallization were calculated and reported in Figure 1a. Untreated glass was considered as reference for comparing drug nucleation kinetics.

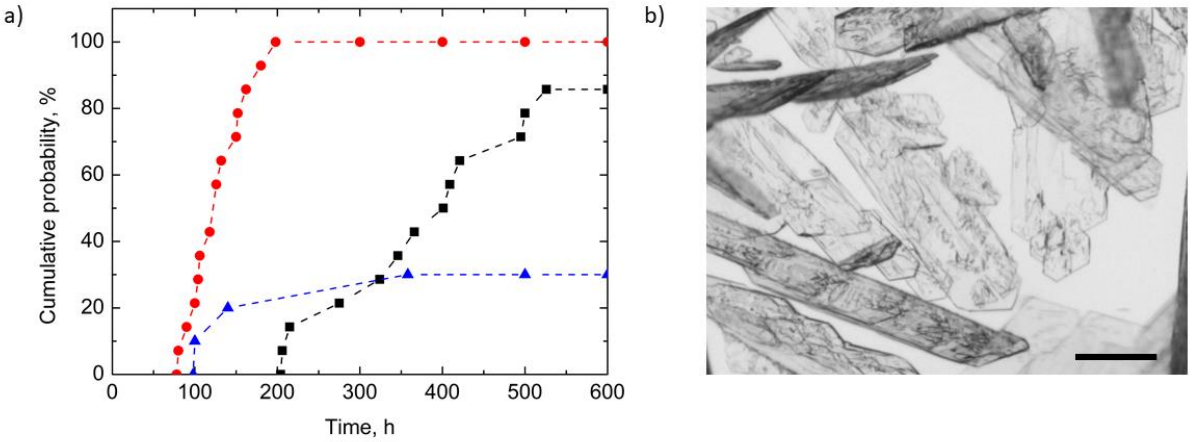

Figure 1. a) Cumulative probability of observing nucleation phenomena for untreated (square, black curve), thiol-SAMs (triangle, blue curve) and amino-SAMs (circle, red curve). b) Optical microscope image of ASA crystals grown on SAMs, scale bare is 100 µm.

Both SAMs were responsible for halving nucleation onset time compared to the untreated substrates. However, the amino-SAMs led to a dramatic boost in nucleation kinetics, as well as the concentration of nucleation events in a shorter time interval. These observations corroborated the nucleation activity of amino surface groups towards ASA. Moreover, crystals were observed in all the wells during the test time, leading to 100% probability of observing nucleation in the system. We related this ability to favorable acid-base interactions between surface and ASA. Conversely, the thiol-SAMs strongly inhibited ASA nucleation, leading to only 30% crystallized wells. This property was attributed to electrostatic repulsion between solute molecules and surface, impeding stable cluster formation in contact with the surface. ASA crystallized according to a plate-like configuration on all the investigated surfaces, as depicted in Figure 1b.

Conclusion

An optimized method for the synthesis of APTMS and MPTMS self-assembled monolayers is here presented, along with a set of characterization techniques for the evaluation of surface quality and features. Such a reference platform is to be linked to the crystallization behavior of heteronucleants. Self-assembly phenomena were therefore exploited at two different levels: firstly, for the synthesis of heteronucleants with defined properties and, then, for the study of the interplay between surfaces and self-assembly of solute molecules in solution leading to crystals. Such surfaces could act as ASA nucleation promotors or inhibitors, thus enabling the tuning of nucleation kinetics.

Abbreviations

AFM, Atomic Force Microscopy; API, Active Pharmaceutical Ingredient; APTMS, 3-aminopropyltrimethoxysilane; ASA; Aspirin; CA, Contact Angle; MPMTS, 3-mercaptopropyltrimethoxysilane; SAM, Self-Assembled Monolayer; XPS, X-Ray Photoelectron Spectroscopy.

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