



Title of the thesis: “Novel crystallization platforms for drugs and biomolecules: self-assembled surface functionalization and gels”

Ph.D. candidate: Fiora Artusio

Supervisor: prof. Roberto Pisano

SUMMARY

The production of crystalline materials represents a key point in the pharmaceutical field. The majority of drug manufacturing processes take advantage of crystallization to isolate or purify an active ingredient. Moreover, crystals of complex macromolecules, such as proteins, are vital for the determination of their molecular structure. In this scenario, crystallization can be carried out with the help of heteronucleants, minimizing the required amounts of drug. The present thesis investigates the design and application of bidimensional, i.e., flat surfaces, and tridimensional systems, i.e., gels, to the crystallization of target molecules, ranging from small-molecule drugs to biomacromolecules.

As a first step, the functionalization of glass with different chemical groups was investigated. Self-assembled monolayers immobilized on glass were selected since they provided an ideal platform for studying the heterogeneous nucleation of small-molecule drugs. Thanks to their sub-nm roughness, such surfaces offered the possibility to physically deconvolute the effect of the exposed chemistry on the crystallization outcome from the competitive action of surface topography. Trimethoxysilanes were selected as functionalizing agents thanks to their high reactivity towards hydroxylated glass. A general and adaptive protocol for the synthesis of self-assembled monolayers was identified thanks to the thorough physico-chemical characterization of surface attributes. The preliminary surface activation was obtained by means of incubation in piranha solution. Then, the self-assembly of 3-mercaptopropyltrimethoxysilane, 3-aminopropyltrimethoxysilane, 3-glycidyoxypropyltrimethoxysilane, and 3-(trimethoxysilyl)propylmethacrylate was investigated. The presence of specific chemical groups, the thickness and the uniformity of the functionalized layer, the absence of defects, the surface wettability, the zeta potential, and the topography were evaluated according to different sets of synthesis conditions. An optimized formulation was designed in terms of reaction medium and silane concentration. The impact of reaction time and temperature on surface properties was also evaluated. The tuning of the reaction time was identified as the key parameter for adapting the proposed synthesis protocol to the four silane chemistries. As a result, a portfolio of surfaces carrying thiol, amino, glycidyoxy, and methacrylate groups, which were able to offer different kinds of interaction with a target molecule in solution, along with sub-nm roughness, was obtained.

Self-assembled monolayers were first employed as supports for the heterogeneous nucleation of a model small-molecule drug, namely aspirin. The batch crystallization of aspirin in ethanol/water mixtures was studied with and without monolayers, thanks to an *ad hoc* designed apparatus for the automatic acquisition of microscopy images and temperature control. The experiments pointed out that the heteronucleant chemistry had a strong impact on the nucleation time of aspirin. Methacrylate and amino groups acted as nucleation promoters by boosting the nucleation kinetics and maximizing the probability of finding crystals in a defined timeframe. Conversely, thiol groups acted as nucleation inhibitors. To identify the dominant mechanism of



interaction, the investigation focused on the interface between monolayers and aspirin. Thin films of crystallized drug were prepared with the help of spin-coating techniques and were analyzed by X-rays diffraction. The confinement of crystallization within a limited volume revealed the ability of surface chemistry to promote the oriented growth of crystals, as confirmed by scanning electron microscopy analyses of the films. A matching between the acid surface tension component of the monolayers and the preferential nucleation of certain crystal facets was identified. Furthermore, the preferential interaction between aspirin molecules and specific chemical groups was investigated *via* force spectroscopy. The adhesion force between aspirin (100) crystal plane and different monolayers strongly depended on the exposed groups.

Monolayers were also employed to modify the nucleation step of much more complex molecules, like proteins, by altering the time needed to observe the first crystals in solution. A finer tuning of the nucleation time was obtained for proteins with wider metastability zones. Also, the effect of pH on the protein crystallization was studied, and both the crystal habit and the nucleation time were successfully tuned by establishing pH gradients. The surface charge of the protein had a major role in governing the aspect ratio of the crystals at different pH. Nevertheless, in order to gain a robust control over the protein crystallization and get monodispersed and reproducible crystal populations, tridimensional systems were introduced.

The last part of the thesis focused on the gelification of the medium of crystallization to establish convection-free environments. The action of silica and agarose gels was first compared: the former acted as nucleation inhibitor, whereas the latter was able to strongly induce the crystallization of proteins, even at very low supersaturation levels. The crystallization of proteinase K, insulin, and lysozyme was studied inside agarose gels. The agarose concentration gradient was identified as the key parameter for the tuning of the nucleation step. Crystal size and nucleation density displayed opposite dependencies on the agarose content. Interestingly, the action of agarose gels resulted to be independent both of the protein nature and of the average crystal size, denoting a predominant physical interaction with the gel fibers and allowing the mathematical modeling of the process. Agarose was employed to study protein crystallization in batch and in counter-diffusion environments to demonstrate its applicability to a wide range of proteins, precipitants, and crystallization techniques. Crystals populations with average crystal size ranging from 10 μm to hundreds of μm were prepared, thus potentially covering the full range of application from neutron diffraction to serial crystallography.

In conclusion, the crystallization pathway of small-molecule drugs and complex biomacromolecules was successfully modified thanks to the fine-tuning of chemical and physical interactions between the heteronucleants and the target molecules. By carefully matching the heteronucleant and the solute features, the nucleation time was significantly shortened, the crystallization success was enhanced, or, conversely, the crystallization phenomena were strongly delayed or inhibited. Self-assembled surface functionalization and gels served as an effective tool to guide crystallization and increase its reproducibility.