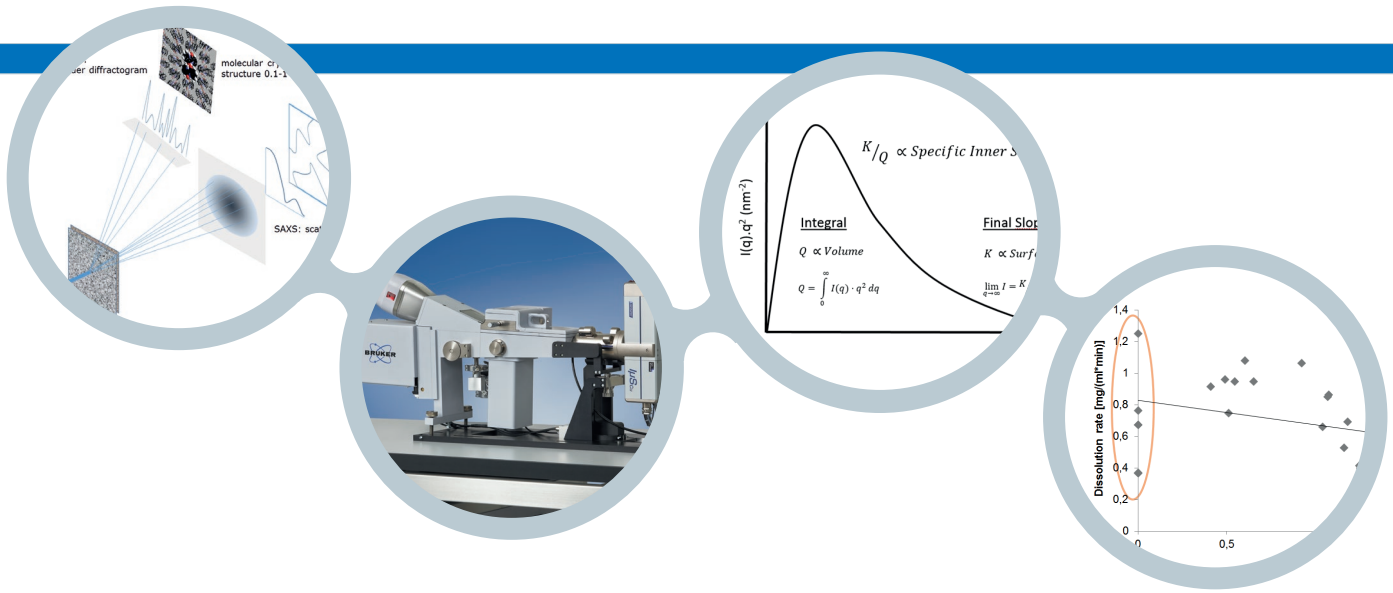


Application Note XRD 601

SWAXS in Protein Drug Development

Specific Surface, Crystallinity and Dissolution of Freeze-Dried Fibrinogen[1]

In the development of protein therapeutics, one of the most demanding challenges is the search for optimal lyophilized powder qualities [1]–[3] which can be readily dissolved and reconstituted into their active form. Such protein powders are the preferred form for storage and distribution. However, they may have strongly varying dissolution properties, depending on how they are produced [4]. Residual moisture, crystallinity and/or additives are determining factors [5]–[7]. Analytical methods to predict the dissolution properties are needed to efficiently identify the optimum candidates for further development without wasting precious material.

Dissolution proceeds at surfaces [8]. Crevasses and capillaries are dominating factors in wetting and solvent penetration [9]. Hence, the *specific surface* (surface per unit volume) is a powerful indicator for the dissolution propensity. Small Angle X-ray Scattering (SAXS) is the key analytical technique to determine this quantity quickly.

The second important factor in the dissolution process is crystallinity, which frequently increases with aging and/or humidity [11], and therefore, needs to be critically assessed. Crystallinity is measured by Wide-Angle X-ray Scattering (WAXS, conventionally powder diffraction). Performing SAXS and WAXS measurements (SWAXS) on the same sample simultaneously is essential to avoid uncertainties arising from different histories, especially with amorphous or close-to-amorphous powders, which are likely to undergo aging upon storage.

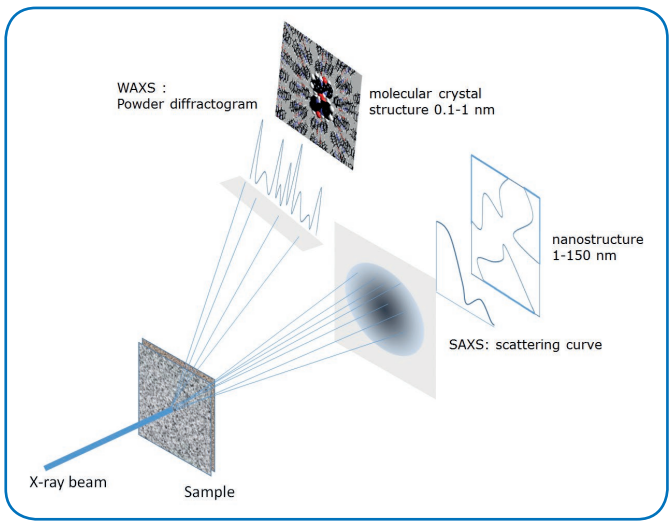
SWAXS is essentially non-invasive and requires no pre-treatment such as extensive drying – baking, as e.g. in BET techniques. With state-of-the-art instruments the analysis is very fast: significant data are typically obtained within few minutes. Hence, SWAXS is an analytical tool that complements other techniques, such as particle sizing, gas sorption analysis, calorimetry (also integrated in Bruker MICROCALIX®) etc. in the pharmaceutical solid state analytics laboratory.

[1] A detailed account of this work has been recently published in: Wahl V, Saurugger EM, Khinast JG, Laggner P. Eur J Pharm Biopharm. 2015;89:374–82 (Ref.10).

SWAXS Instrumentation

A SWAXS instrument typically consists of an X-ray source with beam-shaping optics, a sample cell, and two detectors, one for small-angle, and a second one for wide-angle recording (scheme in Fig 1). The use of two separate detectors has the advantage over e.g. curved linear detectors or large image plates, that the counting times can be individually adapted to different signal strengths in SAXS and WAXS, resp.

(a)



(b)

Fig. 1: (a) Scheme of a typical SWAXS Small- and Wide-Angle scattering system; (b) the Bruker MICRO® system platform – a compact table-top laboratory system, with the option of an integrated micro-calorimeter to study structural transitions.

Why bother with SAXS, why not use a diffractometer?

Two factors are essential for quality in SAXS: firstly, high angular precision and resolution at lowest scattering angles (typically <=1 mrad) is required, which is the main distinction to conventional diffractometers. And secondly, minimal instrument background, provided by an evacuated beam-path and by a high-precision collimation system, typically a Kratky-type collimator. Detection at the very lowest angles is required to resolve nanostructure up to 100 nm and above (fig. 2). Low background is essential to optimize signal statistics, since the scattering curve decays by 3-4 orders of magnitude in the analytically interesting angular window (1-100 mrad , i.e. down to dimensions of ~1 nm). This is of paramount importance for the measurement of surface area by final-slope (Porod) analysis, as described below. While diffractometers are designed to work optimally in the wide-angle range for crystallographic (X-ray powder diffraction) purposes, they cannot be applied for high-precision SAXS measurements.

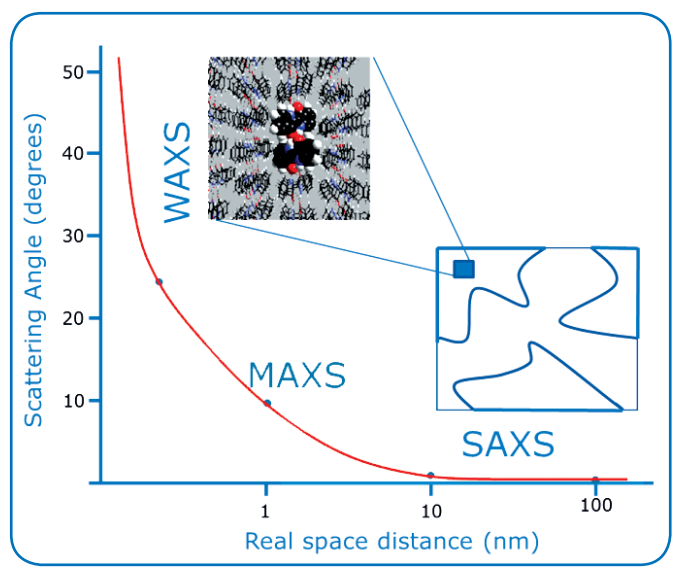


Fig.2 : Relationship between diffraction angle and real space dimensions. The angular range for nanostructure is progressively smaller as the structures grow, and vice versa. WAXS (XRPD), MAXS (middle-angle scattering), and SAXS domains, respectively, require quite different detection strategies (accumulation times, angular range) and therefore the use of separate detectors, allowing free choice of different accumulation times in a time-multiplexed way, is the method of choice. In the Bruker MICRO®, MAXS can be measured by reducing the sample-to-detector distance.

High brilliance X-ray sources (Incoatec IµS microfocus source and point-focusing optics) and efficient detectors have reduced SWAXS measurement times in the laboratory down to minutes, in favorable cases even to seconds. The Bruker MICRO® SWAXS platform (Fig. 1b) is designed for use in laboratory screening alongside other preparative and analytical standards.

Specific surface – Porod Analysis

SAXS is sensitive to (electron) density contrast within the sample at the nano-scale, typically between 1 and 100 nm. In randomly oriented two-phase systems, SAXS analysis allows to infer important quantities relating to the inner structure of the particles within the powder. In the following, a brief summary of SAXS shall be given.

The integral intensity, the so-called 'Invariant' Q,

$$Invariant\ Q \equiv \int_0^\infty I(q)q^2 \cdot dq = V.(\Delta\rho)^2 \cdot \varphi_1\varphi_2 \cdot 2\pi^2 \qquad Eq.1$$

where I(q) is the scattering intensity, and q is the angular argument ($q = 4\pi\frac{sin\theta}{\lambda}$, with λ…wavelength, 2θ……scattering angle) is proportional to the total scattering volume V, the electron density contrast Δρ, and the volume fractions φ_1,φ_2.

The second important value measured from the scattering curve is the 'final slope', the intensity decay towards large angles, according to Porod's law [12], [13]

$$\lim_{q\to\infty} I(q) = S \cdot (\Delta\rho)^2 \cdot {}^{2\pi}/_{q^4} \qquad Eq.2a$$

which is proportional to the total system surface, including closed pores and heterogeneities within the solid matrix, and the contrast. In practice, the scattering data are fitted by eq. 2b

$$I(q) = K_1 + {}^{K_2}/_{q^4} \qquad Eq.2b$$

where K_1 is a constant background. This equation is used to extrapolate the integral in equation 1 from the largest experimental q-region to q→∞ .

Combining equations 1 and 2 leads to the specific surface. The ratio between the decay coefficient K_2 and the Invariant Q is proportional to the specific surface S/V (S_i), since

$$S_i = K_2/Q \cdot \varphi_1\varphi_2 \cdot \pi /d \qquad Eq.3$$

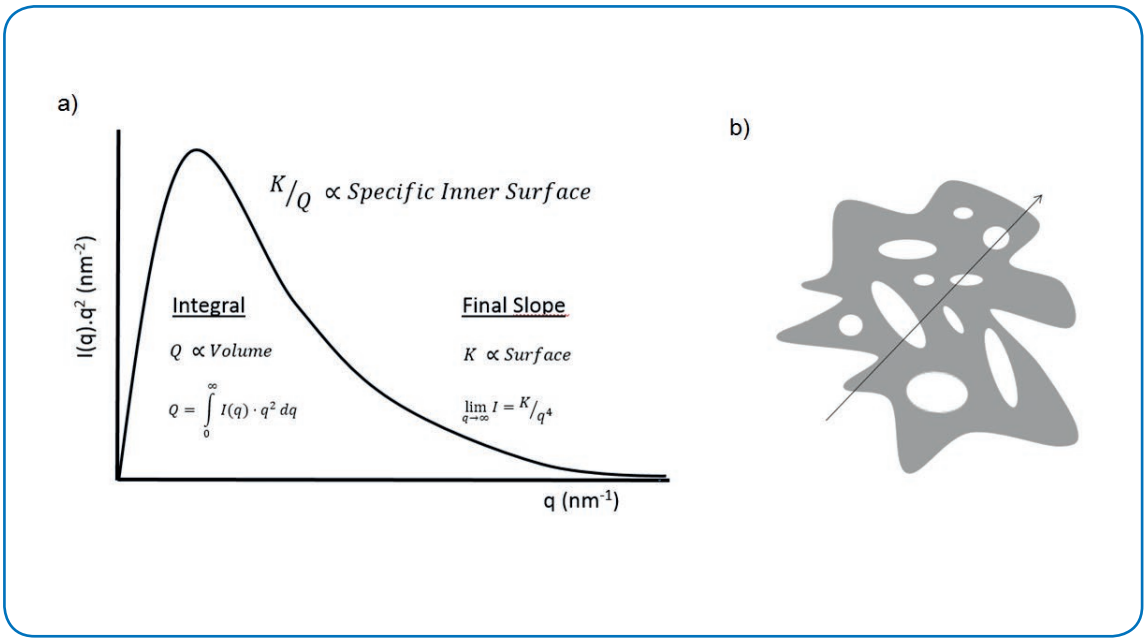
where d is the density of the solid phase. The volume fraction can be determined separately by either gravimetric, or X-ray absorption measurement.

An additional parameter, the average chord length $\bar{l}$ through solid and pore space in any direction, can be obtained from the reciprocal specific surface, according to

$$\bar{l} = Q/K_2 \cdot 4/\pi \qquad Eq.4$$

No knowledge of the volume fraction is required for the determination of $\bar{l}$.

Since one single scattering curve contains all necessary information, the determination of specific surface by SAXS is a very simple and rapid process. The theoretical scheme together with a practical example is shown in Fig. 3.



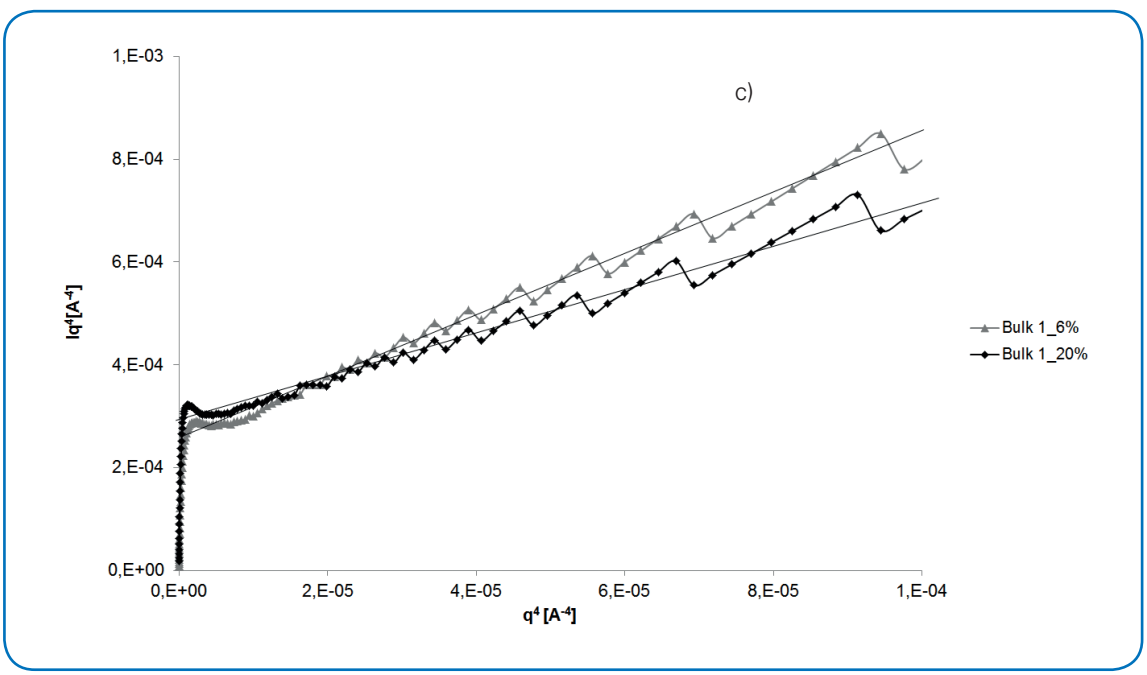


Fig 3: (a) The theoretical formalism for specific surface analysis from SAXS; (b) scheme of outer and inner surface in a porous particle system; (c) 'Porod plot' $I.q^4$ vs. q^4, for two selected samples; the ordinate intersection is proportional to the surface and the slope is the constant background (from Ref. 7).

It has to be noted that Porod's law relates to ideal hetero-phase systems with sharp boundaries. Real systems (fractals etc.) may deviate from this ideal model. However, for practical purposes, and in view of the difficulties of dealing with non-Euclidean geometries or complex boundary layers, it is often justifiable to analyze the data in conventional way, as if they were from ideal systems.

Wide-angle Scattering / Crystallinity

The WAXS patterns show strong variations in crystallinity (Figure 4). At the lowest water contents, no discrete Bragg peaks could be observed and hence, the systems are crystallographically amorphous. With increasing water content three sharp peaks at 2θ of 19°, 21.3° and 24°, resp. appear. As there is no reference record of fully-crystallized fibrinogen powder diffraction available, it is not possible to calibrate for the true percentage of crystallinity. As an operative parameter, therefore, a crystalline/amorphous ratio (crystallinity) was determined from the strongest peak at 21.3° relative to the continuous background between this and the 24°-peak, as indicated Fig. 4.

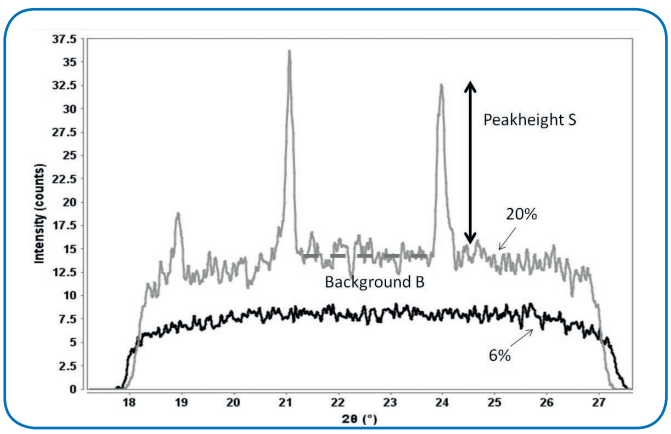


Fig. 4: Typical WAXS patterns of the lyophilized fibrinogen powders. The arrows indicate the measurement of the crystalline/amorphous ratio S/(S-B). For better visibility the two curves are differently magnified (from Ref.10).

Practical Example:

Fibrinogen: Hydration – Inner Surface – Crystallinity - Dissolution

Lyophilized preparations of fibrinogen, the principal protein of blood coagulation and an important factor of wound healing by a complex aggregation cascade, are increasingly used in surgery. It is well known that both, surface area and crystallinity [6] of fibrinogen powders are sensitive to hydration, i.e. residual water content. To systematically study these phenomena, and to search for the most significant analytical parameter correlated to dissolution propensity, 24 samples of lyophilized fibrinogen (six different lots, courtesy of Baxter AG, Vienna), were exposed to controlled humidity to yield water contents of 6, 9, 13, and 20 %, resp. SWAXS, initial dissolution rate, as well as BET surface measurements were performed.

Result No. 1: The specific surface diverges at the limit of amorphicity

Figure 5 shows that at water contents above ~ 9% the specific surface area and crystallinity follow a reciprocal relationship, i.e. the surface decreases with increasing crystallinity. At the lowest water contents, however, where the WAXS patterns show zero crystallinity, the S_i values are found to vary unsystematically (red-rimmed areas in Fig.5). This indicates high structural variability in the amorphous state.

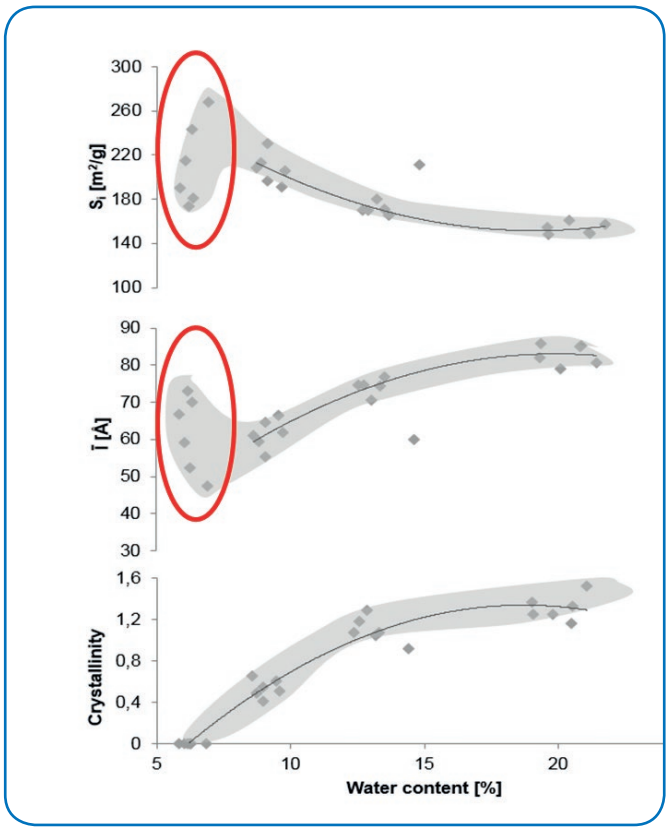


Fig. 5: Specific surface S_i , average chord length, and crystallinity, as functions of water content (from Ref.10).

Result No. 2: Dissolution rate decreases with increasing specific surface

The initial hypothesis was that surface area should positively correlate to dissolution, as follows from classical thermodynamics (Noyes-Whitney, Ref.9). SAXS data have shown that the specific surface decreases significantly with increasing water contents, indicating a compaction or sintering as crystallinity grows (reminiscent of the aging of snow crystals). Figure 6 shows the dissolution rates as functions of the SAXS specific surface for different samples at three different water contents. As it turns out, contrary to what would be expected from the initial hypothesis, the dissolution rates *decrease* as the surface increases. This is most pronounced in the amorphous condition, at 6% water, but also at higher hydrations this trend is observable, yet within narrower ranges of surface area.

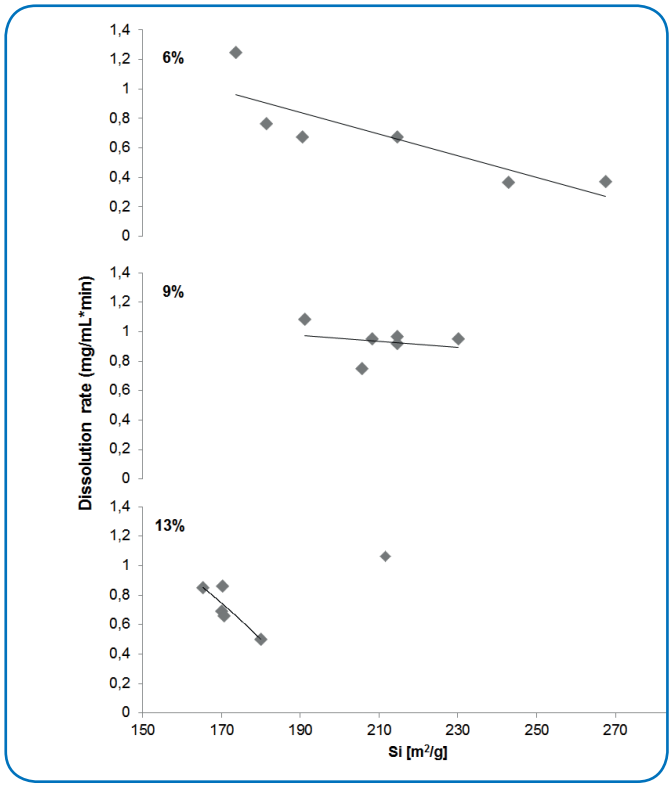


Fig.6: Dissolution rates of freeze-dried fibrinogen as function of specific surface at different degrees of hydration (from Ref.10).

Without further information, any explanation for this observation must remain speculative. However, there are indications that the amount and nature of additives, among others detergents such as Polysorbate-80 (Tween 80), present in the lyophilization process might play a role. A possible reason for the inverse correlation between inner surface and dissolution rate might also be a hydrophobization through protein conformational changes. Depleting the protein of the minimal water layer that stabilizes the 'native' conformation may lead to partial or complete unfolding, thus increasing the specific surface and at the same time leading to hindered approach of water (wetting) to exposed hydrophobic surfaces, which in turn slows down dissolution.

Result No 3: Crystallinity correlates poorly with dissolution

Wide-angle X-ray scattering (WAXS) of dry fibrinogen powder also showed significant variations with water content (Fig. 7). While at lowest humidity no discrete Bragg peaks could be observed – the system is 'X-ray amorphous' –, three peaks in the range between 19° and 25° (2θ) could be observed at higher water contents showing increasing crystalline domains within the powder particles. It is important to note that these results were obtained in transmission geometry, simultaneously to the SAXS measurements, and that the sample capillaries were continuously rotated during exposure (by the 'SpinCap' accessory) in order to avoid preferential orientation artifacts.

As a semi-quantitative parameter, the intensities of the 21-Å peak (2θ) have been taken. In the absence of a 100% crystalline powder, no information can be given about the true percentage of crystallinity. However, the 21-Å-peak intensities can be correlated to used as relative parameters (Fig. 7)

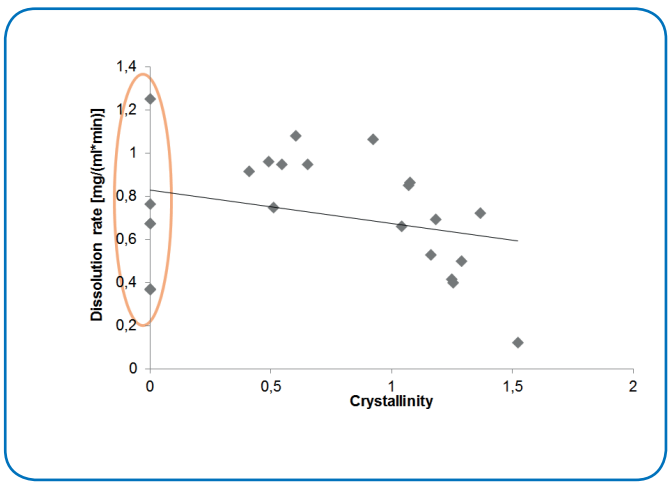


Fig 7: Dissolution rate versus crystallinity from WAXS.

Figure 7 shows that there might be a general trend that dissolution rate decreases with increasing crystallinity (10), however, it is clearly impossible to establish a correlation of any predictive potential. This is most clearly evident at zero crystallinity, where the dissolution rates span a range almost wider than over the whole set of humidities investigated.

Result No 4: Surface from SAXS is larger than the BET surface

BET measurements performed on the same set of samples showed a generally similar trend, i.e. decreasing BET surface with increasing humidity, but at about hundred-fold lower surface values (around 1 m^2/g). The correlation plot of SAXS and BET data is shown in Fig. 8.

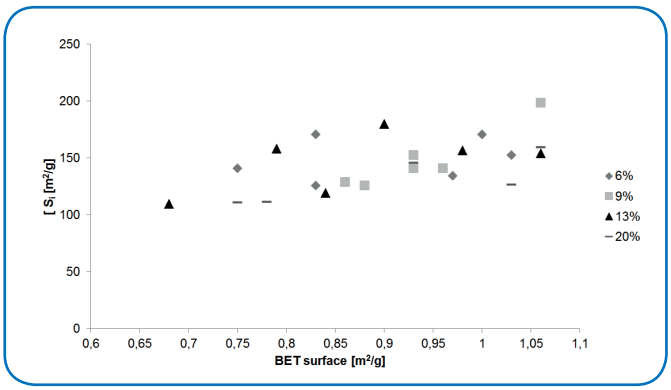
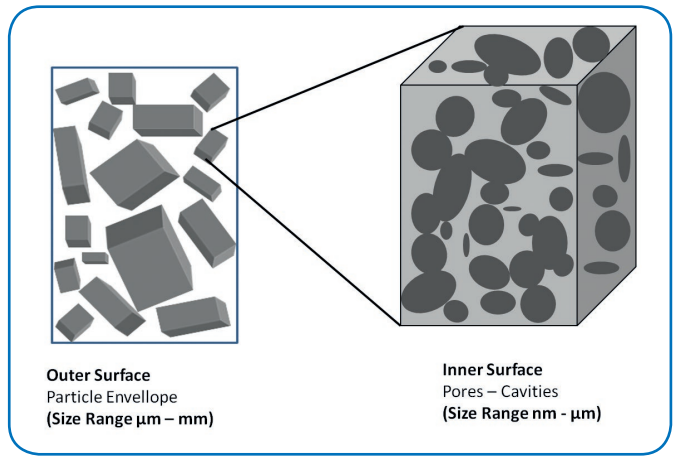


Figure 8: Correlation between SAXS specific and BET surface for all samples studied. Original water contents are indicated by the different symbols (from Ref.10).

The reasons for this large difference to the surface seen by SAXS are not completely understood, but it can be argued that SAXS measures the open *and* closed crevasses or pores, while BET only sees the open ones. Perhaps even more important might be the prolonged degassing *in vacuo,* necessary for BET, which is likely to lead to collapse of loose fibrinogen network structures.

It is illustrating to consider the principal difference between outer surface area as determined by BET, and specific surface as measured by SAXS. The outer surface is the envelope surface of the particles delimiting their space occupied mechanically, as measured by optical particle sizing and the surface of the pores accessible to the probing gas atmosphere (Fig. 9). For spheres or polyhedral geometric bodies there are simple scaling laws relating the specific surface to the particle size, e.g. for spheres, it holds that $S/m = 6/(r.\Phi.\rho)$, where r is the radius, Φ is the packing volume fraction, and ρ is the solid matrix density). A model calculation for random, close packing of spheres, as shown in Figure 8, demonstrates that in typical powders of particle sizes in the range of 1-100 µm the outer surface is in the range between 0.1 and 10 m^2/g, in agreement to what is generally found by BET. The specific surface from SAXS, can be much larger, as it refers to the outer surface *plus* closed pores, cavities and fractures within the particles. The typical pore dimensions are a fraction of the envelope size, i.e. in the nm to µm range.

(a)



(b)

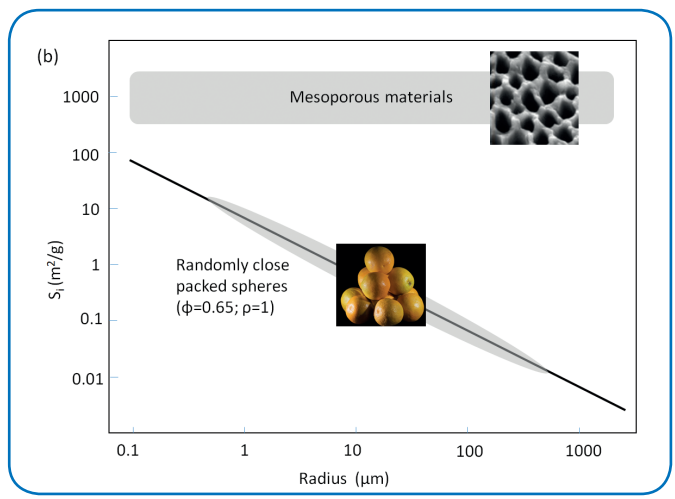


Fig. 9: a) Scheme of outer and inner surface; (b) specific outer surface calculated for randomly close packed spheres with a solid volume fraction of 0.65 and a matrix density of 1; the hatched area indicates the typical range for the specific inner surface of mesoporous materials (from Ref.7).

SUMMARY

SAXS presents a practical tool to characterize the compactness and density fluctuations of protein powders at the nanoscale. The specific surface of lyophilized fibrinogen powders, measured by SAXS, has predictive power in the assessment of protein solubility as it correlates to dissolution rate. Its potential is highest at low crystallinities - high amorphicity - where crystallographic techniques fail, and where different preparations show strong variations in surface area . As compared to BET, surface measurement by SAXS has three major advantages: (a), it measures both, the outer envelope surface of particles and the inner pores, even the initially closed ones, (b), it can be performed on hydrated samples without the danger of destroying sensitive structures by prolonged drying at elevated temperatures ('baking'), and (c), it is fast and simple. In the technologically most interesting amorphous state, this study has shown that dissolution rates as well as inner surface can vary widely, indicating that amorphous preparations of fibrinogen can attain widely different forms and stabilities. From the correlations between S_1 and dissolution rates for different batches at the same degree of hydration, dissolution properties can be predicted from SAXS, without the need for lengthy dissolution tests.

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