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Time evolution of amyloid fibril length distribution described by a population balance model

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HIGHLIGHTS

- A population balance model is used to simulate beta-lactoglobulin fibril formation.
- The complete length distribution is considered together with monomer conversion.
- Shaking-induced fragmentation alone cannot justify the absence of the lag phase.
- Shaking affects also primary nucleation rate and morphology of nuclei.

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ABSTRACT

The formation of toxic protein aggregates is thought to be the key event in several neurodegenerative human diseases. In the last years, the combination of experimental characterization and kinetics models based on mass action laws or molecular dynamics gained important insights into the mechanistic description of the aggregation process. In this work, we investigate the fibrillation of β-lactoglobulin, a common model protein for amyloid aggregation studies with relevant applications in the food industry. In addition to the determination of the fibrillation kinetics by Thioflavin-T, we measure the time evolution of the fibril length distribution during the aggregation process, in both stagnant and shaking conditions. A population balance equation model is used to simulate the experimental data. The model describes successfully the kinetics and the complete fibril length distribution in both conditions. We show that the description of the length distribution is fundamental in discriminating the correct mechanistic picture of the aggregation process. In particular, secondary nucleation due to length-dependent breakage is found to occur in both conditions, with larger extent under shaking conditions. However, it is found that, at least for the system under investigation, fragmentation by breakage alone cannot justify the absence of the lag phase when shaking is applied. This is also related to the effect of shaking on the primary nucleation rate and on the morphology of nuclei and fibrils formed in the early stages of aggregation. This effect is possibly to be attributed to the presence of hydrophobic air interfaces created through shaking.

1. Introduction

Non-native aggregation of amyloidogenic proteins is thought to be the key event in more than 20 human neurodegenerative disorders such as Alzheimer's, Parkinson's and systemic amyloidosis (Lansbury and Lashuel, 2006). The formation of amyloid fibrils from native or (partially) unfolded monomers is the consequence of a complex multi-steps process including the formation of metastable oligomers (Chiti and Dobson, 2006). It is widely believed that such oligomers represent the most pathological species (Walsh and Selkoe, 2004), although the exact mechanism of action and their role in the aggregation process and cytotoxicity are still not understood. Many biophysical studies investigate protein aggregation in vitro to get fundamental understanding about the complex processes occurring in biological systems (Serio et al., 2000; Souillac et al., 2003). Despite the differences in structure and amino acid composition of the aggregating proteins and the broad range of conditions in which amyloid fibrils can be obtained in vitro, experimental evidences suggest the existence of a common mechanism for fibril formation (Wu and Shea, 2011). Indeed, in the majority of the systems the aggregation process can be described as a nucleated polymerization followed by fibril fragmentation (Knowles et al., 2009). However, alternative

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mechanism such as template assembly, monomer-directed conversion and nucleated conformational conversion have also been proposed (Serio et al., 2000).

Since pioneering work of Oosawa et al. on the actin system (Oosawa and Kasai, 1962), deterministic kinetic models have represented a very useful tool in attempting a mechanistic description of the aggregation process. Different approaches have been followed in the literature (Morris et al., 2009). Most of the kinetic models are based on mass action laws (Andrews and Roberts, 2007; Ferrone, 1999). From the comparison between model simulations and experimental data meaningful lumped, kinetic parameters can be quantitatively estimated and important information about the process can be derived. This includes identification of key steps of the process (Lee et al., 2007), presence of alternative path-ways (Powers and Powers, 2008), presence of a critical concentration (Lomakin et al., 1996), size of the critical nucleus (Xue et al., 2008). Particularly, population balance models have been recently shown to be appropriate and effective for a quantitative analysis of the single steps constituting the overall kinetics of fibril formation (Knowles et al., 2009; Pallitto and Murphy, 2001).

The mass-action based models can well represent the global behavior of a system, although they lack basic information at a microscopic level. When questions on the microscopic level are addressed, models based on molecular dynamics are more suitable (Pellarin and Caflisch, 2006). With this technique both the kinetics (Pellarin et al., 2010) and the thermodynamics (Schmit et al., 2011) of the system can be investigated at a microscopic level.

The comparison of the mentioned models to experimental data is crucial for the validation of the proposed mechanism. Many techniques have been developed to follow the aggregation kinetics, with absorbance and fluorescence measurements being the most commonly employed (Nilsson, 2004). Most of these techniques can quantitatively measure only the total amount of monomer units converted to fibrils. Therefore, most of the models in the literature are validated only versus monomer conversion. Other works (Lomakin et al., 1997; Modler et al., 2003; Pallitto and Murphy, 2001) took additionally into account the time evolution of fibril size measured by light scattering techniques, although in these cases only average quantities were considered. The continuous improvement of experimental techniques allows a better characterization of the system. Recently, fibril length distributions obtained by atomic force microscopy (AFM) (Adamcik et al., 2010; Xue et al., 2009b) have been considered in model development (Schmit et al., 2011; van Raaij et al., 2008). However, a model trying to describe the time evolution of the entire fibril length distribution is still lacking.

In this work, we apply a population balance equation model to describe the aggregation process not only in terms of monomer conversion but also of the time evolution of the entire fibril length distribution. It is clear that describing the entire length distribution and not only some averages constitutes a greater challenge for the model and therefore provides a better validation of the adopted kinetic model. In particular, we consider the fibrillation of β-lactoglobulin (β-LAC) at acidic pH. β-LAC is the major whey protein in cow's milk commonly used as a model protein for fibril formation (Arnaudov et al., 2003). Moreover, both the monomeric protein and its aggregates are found in relevant applications in the food industry (Kulozik et al., 2003). It is well known that β-LAC undergoes fragmentation under the examined operating conditions and this superimposes with the aggregation process (Akkermans et al., 2008). Nevertheless, this remains an interesting model-system to apply the population balance approach to describe the evolution of the fibril length distribution in time. In order to account for the fragmentation process an effective fraction of aggregating fragments has been used in the kinetic model as suggested by Kroes-Niboer et al. (Kroes-Nijboer et al., 2011).

We characterize the aggregation process in two different conditions, namely in stagnant condition and under vigorous shaking. The aggregation kinetics is measured by Thioflavin (ThT) binding and fibril length distributions are obtained by AFM pictures.

2. Experimental results

The in vitro aggregation of β-LAC has been extensively investigated in the literature (Ako et al., 2010; Arnaudov et al., 2003; Bromley et al., 2005; Mahmoudi et al., 2007; Navarra et al., 2007). The morphology of β-LAC aggregates was found to be strongly affected by environmental factors, such as temperature, pH and salt concentration. Both globular aggregates and worm-like or straight fibrils have been observed (Carrotta et al., 2001; Loveday et al., 2010).

In this work, the kinetics of fibril formation was investigated at high temperature (80 °C) and low pH (2.0) in absence of salt and at a protein concentration of 10 g L^{-1}. In order to discriminate the fibrillation mechanism, the aggregation was performed under shaking and not shaking (stagnant) conditions. The description of the experimental procedure is reported in the Supplementary Material.

We measure the kinetics of fibril formation by an indirect technique (ThT binding) and the change of the monomer concentration by size exclusion chromatography (SEC). However, under the conditions used in this work, the β-LAC monomer is hydrolyzed into smaller fragments, which are the actually aggregating units (Kroes-Nijboer et al., 2011). As it can be seen in Fig. 1, the decrease in intensity of the SEC chromatograms as well as the right-shift and the broadening of the main peak during incubation show the fragmentation of the protein. This was confirmed by MALDI-TOF measurements at the end of the aggregation kinetics, which indeed detected a variety of fragments and showed the absence of the intact β-LAC (data reported in the Supplementary Material). During incubation, fragments are simultaneously produced by hydrolization and consumed by aggregation; as a consequence, it is not possible to evaluate the fragments conversion to fibrils by SEC only. It is worth noticing that, after the aggregation is finished protein fragments are still visible in the SEC spectra (Fig. 1), indicating that only a fraction of the produced fragments is capable to form fibrils.

Fibril formation under quiescent condition monitored by ThT shows a lag-phase of approximately 4 h (Fig. 2(A)), while under shaking no lag phase is observed (Fig. 2(B)). As expected, due to the stochastic nature of the nucleation process (Fodera et al., 2009), a larger scattering of the experimental data is observed under stagnant conditions (Fig. 2(A)). It can be seen that the rate of fibril formation is higher in shaking conditions, as indicated by the steeper slope of the ThT signal. Finally, the aggregation process reaches a plateau after roughly 30 h or 20 h for the experiments carried out at 0 rpm and 600 rpm, respectively.

In Fig. 3 the histograms of fibril length distribution and the corresponding AFM pictures at different incubation times and conditions (undisturbed and shaking) are shown. Most of the fibrils formed by β-LAC are straight and unbranched. A marked difference is observed in the length of the fibrils. At 600 rpm a short fibril population characterized by a narrow size distribution prevails throughout the experiment. Nevertheless, few long fibrils are visible as well, which grow in size until the end of the process. In non-shaking conditions the fibril length distribution is broader in the early stages of the process compared to the end of the experiment, indicating that longer fibrils get shorter at later time points. This is