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Sup35NMp morphology evaluation on Au, Si, formvar and mica surfaces using AFM, SEM and TEM

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ABSTRACT

Prion and some other incurable human neurodegenerative diseases are associated with misfolding of specific proteins, followed by the formation of amyloids. Despite the widespread usage of the transmission electron and of the atomic force microscopy for studing such amyloids, many related methodological issues still have not been studied until now. Here, we consider one of the first amyloids found in *Saccharomyces cerevisiae* yeast, i.e. Sup35NMp, to study the adsorption of monomeric protein and its fibrils on the surface of mica, silica, gold and on formvar film. Comparison of linear characteristics of these units calculated by processing of images obtained by the atomic force, transmission and scanning electron microscopy was carried out. The minimal number of measurements of fibril diameters to obtain the values in a given confidence interval were determined. We investigated the film formed by monomeric protein on mica surface, which veiled some morphology features of fibrils. Besides, we revealed that parts of the Sup35NMp excluded from the fibril core can form a wide "coat". The length of the protein forming the core of the fibrils was estimated.

1. Introduction

Misfolding of some proteins and formation of amyloids (the protein aggregates enriched with β-structures) result in some human incurable diseases such as the Alzheimer's, the Parkinson's, the Huntington's (Breydo and Uversky, 2015) and the transmissible spongiform encephalopathies (TSE: Kuru, Creutzfeld-Jacob, Fatal familial insomnia) (Prusiner, 2013). Differences in pathogenesis of associated diseases are linked mostly with the specific properties of the amyloid fibrils such as their stability or their linear characteristics (Baxa et al., 2006; Peelaerts et al., 2015; Peelaerts and Baekelandt, 2016; Prusiner, 2013; Wickner et al., 2010). Depending on their morphology amyloid aggregates of lower eukaryotes, especially prions, can change cellular metabolism without cell death (Baxa et al., 2006; Liebman and Chernoff, 2012; Wickner et al., 2010). Due to that a huge number of works are devoted to studies of structure and properties of amyloids (see, for example, Adamcik and Mezzenga (2012), Breydo and Uversky (2015), Knowles et al. (2014), Liebman and Chernoff (2012)).

The Sup35 protein (Sup35p) is one of the first amyloids found in *Saccharomyces cerevisiae* yeast. Its aggregation leads to the emergence of prionogenic factor [PSI^+] (Glover et al., 1997; King et al., 1997; Patino et al., 1996; Paushkin et al., 1996). This determines greatly the interest to studying the Sup35p (Bondarev et al., 2015). Conventionally, this protein is divided into three domains: the amino-terminal (N), the carboxy-terminal (C) and the middle (M) (Kushnirov et al., 1988). The C-proximal domain is necessary and sufficient for the translation termination and the cell viability. The N-proximal domain is not essential for the viability or the termination, but its deletion abolishes the prion propagation and aggregation (Liebman and Chernoff, 2012). This domain forms a core in Sup35p aggregates while the carboxy-terminal domain is offset of this spine (Baxa et al., 2011). The M domain is considered as a linker between the N and the C domains (Baxa et al., 2011) or as a part of the fibril (Shewmaker et al., 2006). The Sup35NMp (fragment of Sup35p comprising N and M domains) or shorter fragments are used in many experiments because only these regions are actually implicated in amyloid formation (Baxa et al., 2011; Glover et al., 1997; King et al., 1997).

Despite the widespread usage of the transmission electron (TEM) and the atomic force (AFM) microscopy to study amyloid structures, including Sup35p fibrils, many methodological issues still need to be studied. Firstly, the distinctive features of Sup35p fibrils fixation on different surfaces have not been yet characterized in detail. Secondly, the influence of the substrate material on the fibrils morphology is not considered accurately enough. Thirdly, the comparison of the results of

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Table 1
Results of different measurements of the Sup35p fibrils diameters.

Method	Substrate	Protein	Diameter, nm	References
TEM	Carbon	Sup35N (2–114)p	3	King et al. (1997)
		Sup35NMp	8	Kishimoto et al. (2004)
		Sup35p	9 ± 1	Baxa et al. (2011)
		Sup35NMp	10	Shewmaker et al. (2008)
		Sup35NMp	11 ± 3	Liu et al. (2011)
		Sup35NMp	11.5 ± 1.5	Glover et al. (1997)
		Sup35NMp	13.5 ± 0.5	Inoue et al. (2004)
	Quantifoil™	His5-Sup35(1 −6 1)-GFP-Strep(II)p	17.5 ± 0.5	Diaz-Avalos et al. (2005)
	–	Sup35NMp	9 ± 1	Shewmaker et al. (2009)
		Sup35p	10	Perutz et al. (2002)
AFM	Mica	Sup35p	4.5	Collins et al. (2004)
		Sup35NMp	31 ± 2	Xu et al. (2001)

measurements obtained with the aid of various types of microscopy on different surfaces was not yet carried out. Fourthly, there does not exist a unified method for measuring linear characteristics of the fibrils (Sokolov et al., 2017). Fifthly, a statistical analysis that would show the minimal number of measurements necessary to obtain the values in a given confidence interval still was not carried out. Along with the presence of amyloid variants with different geometric dimensions (Krishnan and Lindquist, 2005; Lyubchenko et al., 2006; Rubin et al., 2013), it could cause significant discrepancies in the definition of Sup35p fibrils diameters. As it can be seen in Table 1, this parameter varies greatly in different studies.

In this paper, we analyze in detail the adsorption of monomeric Sup35p and its fibrils on the surface of the mica, the silicon, and the gold substrates, and the formvar film. It was shown that the monomeric protein forms layers on the mica surface that affects the adsorption of fibrils and their visualization. A comparison of the results obtained by AFM, TEM and the scanning electron microscopy (SEM) with the usage of different substrates was carried out. For each type of microscopy and of the substrate material the minimal number of measurements required to produce statistically significant results were found.

2. Materials and methods

For Sup35NMp purification, the pET20b-SUP35NM plasmid (Allen et al., 2005) was used. This vector contains a sequence encoding Sup35NMp fused to a His6-tag under control of an inducible T7 promoter. For protein purification we used *Escherichia coli* strain BL21. Overproduction of recombinant proteins was carried out in 2TYa media with 1 mM IPTG. Cultures were grown at 37 °C for 4 h. Proteins were purified in denaturing conditions (in presence of 8 M urea) according to previously published protocols (Serio et al., 1999). A two-step purification procedure with Ni-NTA agarose (Invitrogen) and Q-Sepharose (GE Healthcare) columns was performed. Proteins were concentrated using a Centricon (30 kDa, Millipore). In order to obtain aggregates of Sup35NMp, the protein was diluted at least 100-fold in fibril assembly buffer (5 mM potassium phosphate, pH 7.4, containing 150 mM NaCl) to a final protein concentration of 0.5 mg/mL. Samples were incubated in-tube at 26 °C with slow overhead rotation (Bio RS-24 rotator, Biosan) during 24 h. In these conditions, Sup35NMp spontaneously aggregates. The Sup35NMp fibrils solution was used as stock. The fibril assembly buffer was employed to obtain the desired concentrations.

The Zeiss Merlin scanning electron microscope, the Jeol JEM-2100 transmission electron microscope and the Bruker Nanoscope V atomic force microscope were employed. Gold thermally sprayed on glass, n-type crystalline silicon, freshly cleaved mica and formvar coated copper grids were used as substrates. The surface of a silicon was cleaned in an ultrasonic bath in the presence of acetone, followed by treatment with the plasma cleaner. The negative staining with a 1% aqueous solution of uranyl acetate was used for TEM measurements. Samples were prepared by the placement of 5 µl drop of the Sup35NMp fibrils solution on a substrate, followed by washing with distilled water and air blowing.

NanoScope Analysis 1.50 and Gwyddion 2.45 were used for AFM images processing. MagicPlotPro software was used for data approximation, charting and obtaining histograms. FibrilJ plugin developed by our group for ImageJ/FiJi software was used for average diameter, length and persistence length estimation (Sokolov et al., 2017). Three-dimensional AFM images were converted into two-dimensional images by forming a cross section at a certain height. Then AFM, SEM and TEM images series were processed using a common algorithm. At the first stage the image series were processed using FibrilJ plugin. Algorithms D_3 and PL_2 were selected to determine the average diameter $\langle D \rangle$ and persistence length $\langle PL \rangle$ of fibrils, respectively. At the second stage the distributions of fibrils over diameters were fitted with normal distributions and their peaks were determined for each image. Images with outlier peaks were filtered out from further analysis. At the third stage, all measurements of fibrils with the remaining images were combined and a normal distribution fitting was carried out again. Adjusted coefficient of determination got values 0.95–0.98, that confirms the feasibility of using this processing. Then, the peak value $\langle D \rangle$ and the corresponding standard deviation σ were calculated. The formula $\Delta = \sigma \cdot t_{0.95}/\sqrt{(N)}$ (1) was applied to determine the confidence intervals Δ with a confidence level of 95%, where $t_{0.95}$ is the Student's coefficient for the corresponding σ value with the number of measurements N (William Sealy Gosset, 1908). A formula $N_{min}^{fibrils} = \sigma^2 \cdot t_{0.95}^2$ (2) was used to determine the number of fibrils to be measured in order to obtain results in a confidence interval of ± 1 nm with the confidence level of 95%. The minimal corresponding number of point measurements of the diameter (N_{min}^{points}) was defined as $N_{min}^{fibrils}$ multiplied by the mean number of point measurements per fibril in the series of images. The combined measurements were used to determine averages of the fibril lengths and the persistence length as well.

3. Results and discussion

In this study we analyzed adsorption of monomeric or aggregated Sup35NMp on the surface of various substrates. We conducted a comparative assessment of fibril linear characteristics using AFM, SEM and TEM. The minimal number of measurements required to obtain statistically significant results was evaluated. The conditions for the visualization of the protein being not included in their spines composition were investigated.

3.1. Sup35NMp forms a film on the surface of mica

We observed that portion of Sup35NMp remains soluble after formation of aggregates *in vitro*. Monomeric protein settling on a substrate simultaneously with fibrils should be taken into account when characterizing the aggregates. In order to assess the parameters of monomeric protein adsorption the solutions drops containing the incremental yet not aggregated protein concentrations were deposited on the freshly

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