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Review

Camelid single-domain antibody fragments: Uses and prospects to investigate protein misfolding and aggregation, and to treat diseases associated with these phenomena

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ABSTRACT

The deposition of misfolded peptides and proteins in the form of amyloid fibrils is the hallmark of nearly fifty medical disorders, including Alzheimer's disease, Parkinson's disease, prion diseases and type II diabetes. These disorders, referred to as amyloidoses, generally become apparent late in life. Their psycho-sociological and economic incidence in western societies will be therefore considerable in the coming decades due to the ageing of the population. Neither preventing nor curative treatments are available yet. These disorders constitute therefore a medical challenge of great importance. Thus, an extensive research is being carried out to understand, at the molecular level, (i) how amyloidogenic proteins misfold and convert from their soluble form into amyloid fibrils, and (ii) how these aggregates or some of their oligomeric precursor species are toxic. The formation of amyloid fibrils proceeds through a complex nucleation/polymerisation mechanism with the formation of various species, including small oligomers. . In this review, we focus on how V_HHs or *nanobodies*, the antigen-binding domains derived from camelid heavy-chain antibodies, are being increasingly used to characterise each of the species formed on the pathway of fibril formation in terms of structure, stability, kinetics of formation and toxicity. We first introduce the characteristic features of *nanobodies* compared to those of conventional antibody fragments. Thereafter, we discuss how *nanobodies*, due to their unique properties, are used as probes to dissect the molecular mechanisms of misfolding and aggregation of six proteins associated with diseases, i.e. human lysozyme, β2-microglobulin, α-synuclein, prion, polyadenylate binding protein nuclear 1 and amyloid β-peptide. A brief general presentation of each disease and the associated peptide/protein is also provided. In addition, we discuss how *nanobodies* could be used as early diagnostic tools and as novel strategies to treat diseases associated with protein misfolding and aggregation.

Abbreviations: Ab, antibody; Aβ, amyloid β-peptide; AD, Alzheimer's disease; ANS, anilino naphthalene-sulfonic acid; AP, alkaline phosphatase; APP, amyloid precursor protein (APP); αSyn, α-synuclein; β2m, β2-microglobulin; BBB, blood–brain barrier; CDR, complementarity determining region; C_m, concentration of mid-denaturation; CR, Congo Red; CSF, cerebrospinal fluid; ΔN6β2m, a truncated form of β2m lacking the six N-terminal amino acids; DLS, dynamic light scattering; DRA, dialysis-related amyloidosis; FR, framework; FTIR, Fourier transform infrared spectroscopy; GFP, green fluorescent protein; HCAb, heavy-chain antibody; H/D, hydrogen/deuterium; HSQC, heteronuclear single quantum coherence; Htt, huntingtin; HuL, human lysozyme; IAPP, islet amyloid polypeptide; IMAC, immobilised metal ion affinity chromatography; ITC, isothermal titration calorimetry; LB, Lewy bodies; MCH1, light chain of the type I major histocompatibility complex; MM, molecular mass; NAC, non-Aβ component; NLS, nuclear localisation signal; NMR, nuclear magnetic resonance; OPMD, oculopharyngeal muscular dystrophy; PABPN1, polyadenylate binding protein nuclear 1; PD, Parkinson's disease; PrP, prion protein; PrP^C, normal cellular prion protein; PrP^{Sc}, misfolded form of the prion protein associated with disease; QCM, quartz crystal microbalance; sdAb, single-domain antibody; SPR, surface plasmon resonance; ThT, thioflavin-T; TEM, transmission electron microscopy; T_m, temperature of mid-denaturation; TSEs, transmissible spongiform encephalopathies; V_H, variable domain of the heavy chain of conventional antibodies; V_HH, variable domain of heavy-chain antibodies; V_L, variable domain of the light chain of conventional antibodies.

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1. Introduction

The amino acid sequence of each protein newly synthetised by ribosomes contains all the information required for the protein to acquire its native tridimensional structure and, therefore, its functional biological state. Nature has in fact developed sophisticated mechanisms to ensure the correct folding of proteins, including the evolution of specific polypeptidic sequences or the involvement of factors such as folding catalysts and molecular chaperones [1]. The latter help the polypeptide chains to correctly and efficiently fold but they do not however determine the final native conformation of the protein. Despite these mechanisms, some proteins, however, fail to fold correctly or to maintain their native state, because of different factors such as mutations or increased concentration. These proteins are normally rapidly recognised by the quality control system and degraded by the proteasome or other proteolytic machineries [2]. In spite of these quality control and degradation machineries, the probability that some proteins fail to adopt or to maintain their functional conformation is however not negligible. Given the crucial roles of proteins in virtually any cellular processes, this failure generally leads to serious diseases which are known as protein conformational diseases or protein misfolding diseases [3,4]. In some cases, not only misfolded proteins are not anymore able to exert their normal biological activity (and this could be the direct cause of some diseases such as in cystic fibrosis [1]), but they can also interact with each other to form pathological insoluble, highly organised and stable aggregates called amyloid fibrils. The formation of such aggregates is associated with nearly fifty diseases referred to as amyloidoses [3].

Amyloidoses are neurodegenerative or non-neuropathic, depending on whether the amyloid fibrils deposit in the nervous system or in other tissues or organs; in the latter case, the disease can be localised (i.e. affecting only one organ) or systemic (i.e. affecting various organs) [3]. The most common neurodegenerative amyloidoses are Alzheimer's disease (AD) and Parkinson's disease (PD). Amyloidoses generally appear late in life; due to the rising ageing of the population, they inflict enormous psycho-sociological and economic burdens on western societies and constitute therefore a great medical challenge. These age-associated disorders indeed affect several million people per year. For example, it is estimated that, in 2005, between 4.1 and 4.6 million individuals were living with Parkinson's disease in the 15 more populated nations worldwide, and this number will double by 2030 [5]. According to a report published in 2013, Alzheimer's disease is the sixth leading cause of all deaths in the United States. An estimated 5.2 million Americans have AD and, by 2050, the incidence of AD is expected to approach nearly a million new cases per year, with a total estimated prevalence of 13.8 million people [6]. So far, there is no preventive or curative therapy for these devastating pathologies, and the current treatments only alleviate the symptoms without modifying the course of the diseases. For example, in the case of AD, acetylcholinesterase inhibitors are used to reduce the loss of acetylcholine caused by the death of cholinergic neurons [7]. For the patients suffering from PD, most treatments aim at correcting the deficiency of the brain neurotransmitter dopamine [8]. There is therefore an intensive worldwide research effort to shed light on the mechanisms, at the molecular level, of protein misfolding and aggregation and their link to pathology. The identification of all the various species formed during the complex process of fibril formation as well as their characterisation in terms of structure, stability, kinetics of formation and toxicity are essential to develop some early diagnostic tools and to identify targets for therapeutic treatments.

Each amyloidosis is associated with the aggregation of a particular peptide or protein into amyloid fibrils (for example Aβ-peptide (Aβ) and Tau protein in Alzheimer's disease, α-synuclein (αSyn) in Parkinson's diseases and islet amyloid polypeptide (IAPP) in type II diabetes). The disease-associated proteins differ widely in their sequence, size and native structure; in their aggregated form, however they all share a similar highly organised structure, rich in β-sheets and known as cross-β structure [9]. Fibril formation is a complex phenomenon, generally described as a nucleation/polymerisation process, characterised by a sigmoidal growth profile (Fig. 1). This process involves a variety of conformational rearrangements and multiple steps of assembly [3,4]. First, a slow nucleation step is characterised by a lag phase; it involves the misfolding of the proteins into aggregation prone species (also referred to as amyloidogenic intermediate) and the association of these species into various oligomeric species, leading to the formation of nuclei or seeds of aggregation. These oligomers, also called prefibrillar species, are structurally diverse and some of them have been found to be more cytotoxic than mature fibrils [10]. During the polymerisation step (or elongation phase), the nuclei rapidly expand by addition of monomers or oligomers to form larger polymers, protofibrils and finally fibrils. In the final steady state or stationary phase, fibrils and monomers are in equilibrium and the structure of the fibrils can still evolve for example by rearrangement of the region of the protein that is not part of the core of the fibrils, leading to mature fibrils [11]. Besides, the amyloid fibrils can also associate laterally [12]. The lag phase can be shortened or even suppressed by the addition of seeds (i.e. preformed oligomers or fibrils), a phenomenon known as seeding [13]. Moreover, increasing evidence suggests that once a critical concentration of fibrils has formed, the primary nucleation/polymerisation pathway described above can be dominated by secondary pathway(s) such as fibril fragmentation, lateral growth and/or fibril-catalysed secondary nucleation [14–16]. Finally, recent findings suggest a prion-like spreading of amyloidogenic proteins: aggregates of tau, αSyn, Aβ and huntingtin (Htt) have indeed been found to be capable of moving between cells and trigger the misfolding of their normal conformers [17].

Because of their insoluble character, their heterogeneity in size and quantity, and/or their transient feature, the characterisation of most of the species formed upon fibril formation is extremely challenging. Indeed, these species cannot be easily studied by most of the conventional techniques available for soluble proteins because these require concentrated and homogeneous samples which are difficult to obtain for each individual intermediate species. Since a given protein generally adopts a different conformation in its native, oligomeric and amyloid forms, the development of specific and sensitive structural (or conformational) molecular probes for the characterisation of these distinct forms is therefore a very valuable approach. Monoclonal conformational antibodies can be extremely promising for this purpose since they could in principle be specifically raised against any conformational state of peptides or proteins [18–21]. Moreover, as illustrated in this review, the extent of their specificity can be adjusted through the judicious choice of antigens and of the screening procedures.

Antibodies can be used in several ways to investigate the mechanism of fibril formation. For example, the binding properties of a series of antibodies specifically raised against various epitopes at the surface of the native state of a protein can be compared before and after the conformational changes leading successively to amyloidogenic intermediates, oligomeric species and mature fibrils [21,22]. In this way, the regions of the protein that are structurally reorganised can be identified at each step. Alternatively, partial unfolding events expose to the solvent some regions of the protein, normally buried within the native fold; antibodies raised against

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