

A Short Historical Survey of Developments in Amyloid Research

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Abstract: One of the hallmarks of modern science is technically controlled experimentation. In this paper, we underline how technical developments over the last 150 years have repeatedly created new horizons in amyloid research. The main focus is on chemical and biophysical analyses of amyloid fibrils *in vivo* and *in vitro*. Investigations into mechanistic aspects of fibril formation and possible links with pathogenesis are also discussed.

Keywords. Amyloid, history, milestones, experimental method, experimental technique.

1. INTRODUCTION

Investigations by using dyes and light microscopes of a macroscopic abnormality in brain tissue retrospectively mark the birth of amyloid research in mid-19th century. One hundred and fifty years later, the field of amyloid research is very active (Fig. 1) and our knowledge about the physico-chemical properties of the deposits that cause such tissue abnormalities and the way they are formed is growing at an increasingly fast pace. In this article, we highlight some of the key findings along the path to our present understanding and illustrate how critically the discoveries depended on the availability at the time of appropriate experimental approaches. Due to the importance of this latter aspect, we first summarize some historical facts about the origin of technique-driven approaches, that are typical of modern science and in particular of biomedical research. In the last section, we briefly outline our current knowledge of protein aggregation and amyloid fibril formation and how they are thought to be linked with human disease.

2. ON THE ROLE OF EXPERIMENTAL RESEARCH AND TECHNICAL INNOVATIONS IN SCIENCE

Before the Scientific Revolution of the 16th and 17th centuries, reason was considered the only reliable source of human knowledge and had priority over experience. According to scholarly natural philosophical tradition, laws about nature had therefore to be proposed from generally accepted, changeless and self-evident principles by deductive reasoning. The enduring persistence of such scientific practice through the Middle Ages is likely to be one of the main reasons why, for example, Ptolemy's (geocentric) view of the solar system dominated European astronomy for over 1000 years. It was based on Aristotle's philosophical world picture claiming that the universe is spherical and finite, because: 1) the sphere is the most perfect shape and 2) a body with a center, which was believed to coincide with the center of the

earth, cannot be infinite. Using such principles as major premises, in combination with the observational fact that the outer planets display retrograde motion once in a while, Ptolemy concluded that planets move along small circles (epicycles), whose centre moves along another, larger circle around the Earth (deferent). The compound motion of the two circles would then account for the looping of the planets.

By contrast to this *a priori* (and possibly arbitrary) choice of such first principles, modern science emphasizes the primacy of observations and experiments as the source of ideas and knowledge; in this case the first principles are therefore drawn by true induction from the results of observations and experiments. One of the best-known early examples that demonstrates the increasing power of experimental facts in philosophical and scientific discourses about nature is provided by the work of Galileo (1546-1642). Using his new telescope, he observed Venus changing its appearance and showing a full set of phases like the moon. This observation provided the first conclusive evidence that was in clear disagreement with the Ptolemaic (geocentric) worldview and that was instead in favour of a heliocentric model, as previously proposed by Copernicus in 1540. Francis Bacon became later the advocate of technically controlled experimentation and the new inductive natural philosophy, strongly insisting that experimental facts must be accepted, however much they might seem to be against common-sense reason. Experimental results and knowledge gained using artificial devices, however, are not self-evidently true to everyone and, indeed, scepticists may find it therefore even harder to accept counter-intuitive results on faith, without having the possibility of repeating the observation or experiment themselves. Consequently, credible witness testimony became an increasingly important issue to natural philosophers whilst their attention shifted from universal statements about nature to statements about particular natural and experimental events.

Experimental knowledge was only gradually accepted as scientific practice within academia. Industry and military helped to promote the social and cultural changes that were needed to establish scientific laboratories in traditional academia, mainly by stressing the utilitarian side of the new experimental science, that is, the material interests of the

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state in terms of the common welfare. Indeed, in contrast to “ancient” science, “modern” science has allowed the construction of locomotives, aeroplanes and the production of drugs, etc. [1]. By 1900, the art of experiments had eventually been developed to the most powerful art of knowing within physical science, in which theory was now understood as “condensed experience” [2].

Medical faculties have to some extent always promoted an experiential approach to understand nature, even before the Scientific Revolution. In Italy and France, students studied practical aspects of medicine by local practitioners, and the university provided the means for theoretical studies and access to new and expensive instruments. Thus, experimental methods were an intrinsically important part of medicine and the revolution of the life sciences took place almost entirely within a university context [3]. The birth of modern medicine is usually said to start in the second half of the 19th century. At that time, not much more was known of the actual causes of the great plagues, the fevers and the pestilences, than what was inherited from the Greeks. This changed fundamentally when Louis Pasteur and Robert Koch established the germ theory of disease. By 1880, bacteria were identified as agents of many diseases, and the role for technology and scientific laboratories in the diagnosis of disease grew with these scientific advances.

In the light of these developments it is perhaps not very surprising that the history of amyloid research also started around mid-19th century, and that it is to a large extent a story of the inventions of new techniques and new methods. It happened at a time when people started to watch out for infectious agents of diseases by experimental methods, and it has left its marks on amyloid research ever since.

3. MILESTONES IN THE RESEARCH ON AMYLOID FIBRILS

Our survey starts around mid-19th century with the introduction of the term “amyloid” in medicine by Rudolph Virchow [4] (Table 1). In those early days of research on the origin and nature of amyloid, the available techniques merely enabled medical doctors to detect macroscopic abnormalities on autopsy of tissues from patients and to classify the clinical symptoms associated with these macroscopic observations. The term “amyloid” was originally used to describe normal amylaceous constituents of plants that produced a red or violet colour upon staining with iodine. Because particles found in diseased nervous system exhibited the same characteristic reaction with iodine, Virchow considered them identical to starch and coined the term amyloid to refer to them. Funny enough, the term “amyloid” survives up to the present day despite the fact that Friedreich and Kekulé found in 1859, that amyloid deposits contained no material that was chemically similar to starch or cellulose but that the high nitrogen content rather pointed towards proteins [5]. Various more selective metachromatic stains were subsequently found and used to identify amyloid deposits. It was the aniline dye Congo red, however, that ultimately enabled the most specific distinction between amyloid and non-amyloid plaques [6]. Moreover, Divry and Florin found that Congo red-stained amyloid plaque from the brains of deceased patients of Alzheimer’s disease (AD) exhibited a characteristic apple-green birefringence with respect to the long axis of the

deposits when viewed under polarized light [7]. This observation suggested that *in situ* amyloid is not structurally amorphous but rather possesses fibrillar features. This result was consistent with the first electron micrographs of various amyloid tissues recorded in 1959 by Cohen and Calkins [8]. Similar fibrillar submicroscopic structures were indeed observed in sections of different abnormal tissues supporting the idea of the existence of a generic architecture of amyloid deposits.

With the advent of the techniques of biochemistry and molecular biology it became possible in the mid-sixties to further purify fibrils from tissue and to clarify their molecular composition. The morphology of amyloid fibrils isolated from tissues was found to be virtually identical to their appearance in the tissue prior to purification as probed by Congo red staining and electron microscopy (EM). Amyloid deposits in tissues, however, consist not only of fibrils but contain also non-fibrillar components including serum amyloid P component (SAP), heparan sulfate proteoglycans, and sometimes apolipoprotein E. In fact, in 1988 Pepys and co-workers introduced radiolabelled SAP as quantitative and generic *in vivo* tracer for amyloid deposits enabling a new way of clinical diagnosis and monitoring of the progression of amyloid disorders in patients [9]. In 1968, X-ray diffraction studies revealed that all amyloid fibrils isolated from tissue were structures rich in β -sheet sharing the so-called cross- β motif as a common core [10]. The diffraction patterns of all amyloid fibrils show two major reflections: The strong and sharp meridional reflection at 4.7 Å is interpreted as hydrogen-bonding distance between individual β -strands that constitute a β -sheet along the axis of the fibril. Consistent with this interpretation is the fact that it is virtually identical in all amyloid fibrils. The equatorial reflection at about 10 – 12 Å, on the other hand, is variable and is thought to arise from the stacking of different β -sheets perpendicular to the fibril axis; the precise value of this reflection depends at least in part on the precise amino acid composition of the constituent protein [11]. Amino acid composition and sequence analysis of the protein components recovered from isolated and denatured amyloid fibrils from the 1970s onwards revealed that each amyloid disorder was linked with a unique protein or peptide [12]. Today more than 20 amyloid diseases are identified. Fig. 1 shows the percentage of papers on amyloid during each year between 1982 and 2004. The rapid development of the field observed in the late 1980s is probably somehow related to the discovery of the A β peptide in 1984 in association with AD [13] and of Prusiner’s prion hypothesis put forward in 1982 [14] along with the emergency of the bovine spongiform encephalopathy (BSE) crisis in Europe [15].

In 1971 Glenner and co-workers demonstrated that lysosomal extracts can convert some human Bence Jones proteins (light chains of immunoglobulin proteins) into amyloid fibrils *in vitro* by proteolytic digestion of the immunoglobulin light chain precursor protein into amyloidogenic fragments (known as amyloid AL proteins) [16]. This finding supported the hypothesis at the time that the protein component of fibrils formed *in vivo* was indeed sufficient to produce the characteristic features of amyloid fibrils. It is well-known today that many amyloidogenic peptides are in fact produced only upon proteolytic processing of a

Table 1. Milestones in Amyloid Research from 1850 - 2000¹

Year	Achievement	Technique/Approach	Reference
1854	Coining of the term 'amyloid' (Virchow) to describe macroscopic abnormalities on autopsy of organs/tissues associated with clinical symptoms	Staining with iodine and sulfuric acid	[4]
1859	Main component in 'amyloid' is protein rather than carbohydrate	Determination of nitrogen content	[5]
1895	Experimentally induced amyloid in animals		[26]
1907	Plaques and neurofibrillary tangles in brain of a demented patient		[27]
1922	Specific staining of amyloid fibrils	Congo red	[6]
1927	Ordered submicroscopic structure of amyloid deposits in a variety of tissues	Congo red staining and apple-green birefringence by polarized light microscopy	[7]
1959	Fibrillar ultrastructure and dimensions of amyloid fibrils	Electron microscopy	[8]
1964	Isolation of amyloid fibrils from tissues	Differential centrifugation	[28]
1968	Beta-pleated sheet conformation oriented perpendicular to the fibril axis of isolated amyloid fibrils	X-ray diffraction analysis	[10]
1971	Biochemical composition of amyloid fibrils and identification of proteins involved	Amino acid composition and protein sequence analysis	[12]
1971	Amyloid fibril formation <i>in vitro</i> upon proteolysis of precursor protein	Proteolytic digestion of human amyloid AL protein	[16]
1982	Single proteinaceous infectious particle in both CJD of humans and scrapie of sheep. The prion hypothesis.	Biochemical analysis and intracerebral inoculations of isolated components into animals	[14]
1984	Identification of the A peptide	Purification and biochemical characterisation of the A peptide from <i>ex-vivo</i> aggregates	[13]
1988	Scintigraphy with ¹²⁵ I-SAP as quantitative <i>in vivo</i> tracer for amyloid deposits	SAP becomes resistant to catabolism upon binding to amyloid deposits	[9]
1991	Trinucleotide CAG repeat expansion coding for polyglutamine associated with some inherited neurodegenerative diseases	Statistical analysis of gene mutations in patients exhibiting spinal and bulbar muscular atrophy	[29]
1992	Amyloid fibril formation <i>in vitro</i> from full-length amyloidogenic protein upon partial denaturation of quaternary and tertiary structure	pH denaturation of tetrameric transthyretin and biophysical and chemical characterization of the associated processes	[17]
1992	Nucleation-dependent polymerization model for the formation of amyloid fibrils <i>in vitro</i>	Turbidity measurements, kinetic analysis of amyloid formation by a protein sequence from <i>E. coli</i>	[20]
1992	Prion protein deficient mice are resistant to infection by disease-causing prion protein preparations	Prion protein knock-out mouse	[23]
1994	Prion analogue in <i>Saccharomyces cerevisiae</i>	Genetic manipulations with <i>S. cerevisiae</i>	[24]
1998	Amyloid fibril formation <i>in vitro</i> from full-length proteins unrelated to any known amyloid disease	PI3-SH3 domain and fibronectin type III module	[18, 19]
1999	Structural arrangement of the protofilaments in an amyloid fibril	Cryo-electron microscopy	[30]

¹For more detailed and comprehensive articles on the history of amyloidosis we refer to the reviews of Jean Sipe [31, 32].

precursor protein. Examples include the islet amyloid polypeptide fragment, calcitonin and the A β -peptide. However, there are other amyloidogenic proteins such as α_2 -microglobulin, lysozyme, insulin and transthyretin which assemble into amyloid fibrils *in vivo* as full-length polypeptides. Kelly and co-workers showed in 1992 that purified transthyretin converts into amyloid fibrils *in vitro* after destabilization of the natively folded conformational state

merely by mimicking the low pH conditions of a lysosome [17]. This observation demonstrated that conformational changes were sufficient to trigger aggregation into amyloid fibrils and opened a new avenue of research. It indeed became possible to look in detail *in vitro* at the process of fibril formation from a kinetic and structural point of view using various techniques including those used to study protein folding. It was shown later that under appropriate conditions,

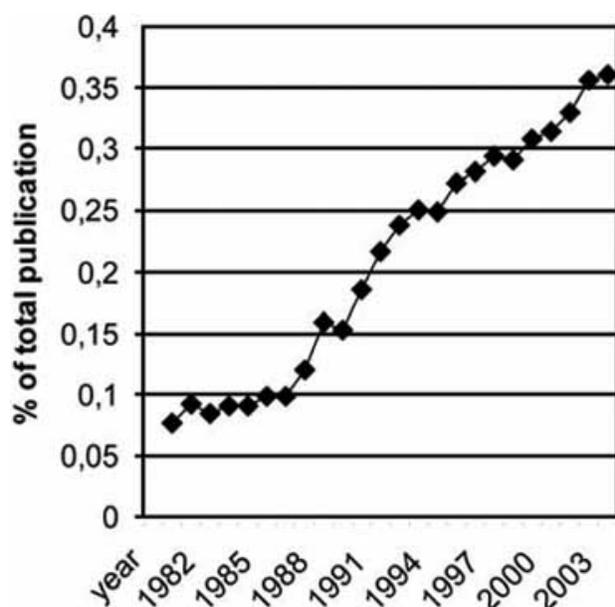


Figure 1. Percentage of papers published in PubMed data base each year between 1982 and 2004 with the term “amyloid” matched against the Medical Subject Headings (MeSH) and searched as a Text Word (including all words in the title, abstract, other abstract, MeSH terms, MeSH Subheadings, chemical substance names, personal name as subject, MEDLINE Secondary Source).

peptides and full-length proteins unrelated to any known diseases can form amyloid fibrils *in vitro* [18-20]. These observations were important from a practical point of view as they stimulated new investigations of many more proteins than those involved in diseases in order to eventually find generic structural and mechanistic features underlying protein misfolding and aggregation. For example, in many cases the behaviour observed *in vitro* can be described in terms of a nucleation and growth mechanism, as first proposed by Jarrett and Lansbury in 1992 [20, 21]. Moreover, from one of these proteins, an SH3 domain, the first three-dimensional reconstruction at 25 Å resolution of an amyloid fibril formed *in vitro* was determined in 1999 using cryo-electron microscopy and image processing [30]. The model, showing a double helix of four protofilaments consisting of pairs of closely interacting β -sheets, was proposed as a basis for understanding the structure of amyloid fibrils in general. From these observations, Dobson suggested that the amyloid fibril can be considered a generic protein structure as the formation of its core involves the main chain (backbone) that is common to all polypeptide chains, and indeed, there is an increasing body of experiments showing that the ability to form amyloid structures under suitable experimental conditions is a general feature of most – and perhaps all – proteins, although with different intrinsic propensities.

Among all aggregation disorders, the prion diseases (including scrapie, BSE, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia) are unique in that they have been shown to be transmissible in experiments using animals. A natural route of transmission includes ingestion, but it may also be possi-

ble that some of these diseases can be transmitted through contact with infected tissue, body fluids, or contaminated medical instruments [22]. In 1982, Prusiner put forward his protein-only hypothesis claiming that the prion diseases are caused not by viruses, bacteria, fungi, or some other mundane agent, but by misfolded proteins [14]. He invented the acronym “prion” (proteinaceous infectious particles) to refer to self-replicating proteinaceous species that he suggested to be the infectious agent of this family of neurodegenerative disorders from a comprehensive body of experimental evidence and observations. With his proposal, for which he was awarded the Nobel prize in 1997, Prusiner overturned the prevailing paradigm at the time that an infectious agent contained either DNA or RNA. Consistent with his hypothesis is the fact that prion knock-out mice were later found to be completely resistant to infection when exposed to disease-causing prion protein preparations [23]. It is presently assumed that the infectious principle of the prion diseases is a conformational change from the normal form of the prion protein (PrP^C) into the pathogenic form (PrP^{Sc}), whereby the pathogenic form autocatalyses the conversion of PrP^C into PrP^{Sc}. The mechanism by which normal PrP is converted into the pathogenic form, however, is still unknown. Moreover, in order to validate directly the protein-only hypothesis it remains to be conclusively proven that disease-causing prion protein preparations can as well be generated *in vitro* from purified PrP^C. Last but not least, a promising approach to investigations on the principle of infection in prion diseases has emerged in 1994 with the discovery of yeast prions, which are not pathogenic, but nevertheless produce changes in phenotype with patterns of non-Mendelian, protein-based inheritance [24]. Indeed, it is suggested that at least four fungal proteins of *Saccharomyces cerevisiae* and *Podospora anserina* can epigenetically modulate a variety of fundamental biological processes, including translational termination and nitrogen regulation by switching to their prion state [25].

4. RESEARCH ON AMYLOID AT THE TURN OF THE 21ST CENTURY

Over the past 20 years our understanding of *in vitro* protein aggregation and amyloid fibril formation has advanced dramatically. It is now well accepted that the molecular basis of protein aggregation into amyloid structures involves the existence of “misfolded” forms of proteins, i.e., proteins that are not in their native state in which they normally function *in vivo*. Sometimes these proteins are partially degraded and the resulting fragments are intrinsically unable to fold. Protein misfolding *in vivo* can be associated with various other causes including specific mutations, misprocessing phenomena, aberrant interactions with metal ions, change in environmental conditions or chemical modifications. A large body of evidence has shown that the process of amyloid formation follows a hierarchical path of assembly involving multiple steps of association through a series of small aggregates to more organised structures and eventually mature fibrils, and a variety of conformational rearrangements seems to be associated with aggregate formation [33]. A great deal of work is now under way to define in greater detail the structural, kinetic and thermodynamic landscapes of the various states populated during the process in order to further understand

the molecular determinants of protein aggregation as reviewed in more detailed by Dobson in this special issue. Despite these dramatic advances, there is, however, still a long way to go until the thermodynamic and structural picture of protein misfolding and aggregation is complete. Major advances in the future are hoped to emerge from synergistic approaches of novel experimental and computational techniques that are adapted to study increasingly more complex and subtle aspects of protein aggregation.

In addition, a crucial question is the definitive molecular and biochemical basis of the pathological conditions of protein deposition diseases. The presence of amyloid fibrils or some of their precursors is generally believed to be the cause, not a consequence of the clinical symptoms - this hypothesis is known as the amyloid hypothesis. In the case of systemic non-neurological diseases the presence of aggregates in huge quantities may directly damage organs by physical pressure or they may act more indirectly by hindering a proper flow of nutrients to the cells thus impairing tissue functions. As with regard to neuro-degenerative diseases, the emerging scenario is that small aggregates - which are precursors of mature fibrils - initiate a cascade of events that are eventually toxic to the cells. There is, however, an ongoing debate not only about the identity of the cytotoxic species and about the mechanism(s) through which these species are toxic, but also on the validity of the amyloid hypothesis itself [34, 35]. Based on the picture that has emerged from *in vitro* studies, a number of therapeutic targets have been defined and rational therapeutic strategies have been proposed (for reviews see [36, 37]). For globular proteins one strategy - and probably the safest in the absence of a definite clue about the identity of the pathogenic mechanism - is to stabilize the native state of the protein thereby preventing the protein from misfolding and thus reducing the concentration of the aggregation prone species [38]. Other strategies include blocking the nucleation and growth of aggregates or boosting the natural 'housekeeping' mechanisms in the body [36]. Some of these therapeutic strategies are now under trial and the success or failure of such treatments will constitute the definitive proof or disproof of the amyloid hypothesis.

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ABBREVIATIONS

AD	=	Alzheimer's disease
BSE	=	Bovine spongiform encephalopathy
CJD	=	Creutzfeldt-Jakob disease
EM	=	Electron microscopy
PrP	=	Prion protein

PrP ^C	=	The normal form of the prion protein
PrP ^{Sc}	=	The infectious (scrapie) form of prion protein
SAP	=	Serum amyloid P component

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