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Heterotypic interactions in amyloid function and disease

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Abbreviations

AA, Amyloid A amyloidosis; AApoAI, ApoAI amyloidosis; AApoAII, ApoAII amyloidosis; Acys, Acys Amyloidosis; AD, Alzheimer's disease; LC, immunoglobulin light chain; AL, Light chain amyloidosis; ALS, Amyotrophic lateral sclerosis; ApoAI, Apolipoprotein AI; ApoAII, Apolipoprotein AII; ApoE, Apolipoprotein E; α -synuclein, alpha-synuclein; APRs, Aggregation-prone regions; ATXN, ataxin; A β , beta amyloid peptide; BRCA1, Breast cancer type 1 susceptibility protein; CA, Cerebral atherosclerosis; CJD, Creutzfeldt–Jakob disease; CMV, Cytomegalovirus; COPI, Coat protein I; cryo-EM, Cryogenic electron microscopy; CsgA, Major curli subunit; CsgB, Minor curli subunit; CsgF, Curli production assembly/transport component CsgF; CsgG, Curli production assembly/transport component CsgG; cysC, cystatin C; DLB, Dementia with Lewy Bodies; DRA, Dialysis related amyloidosis; EAAT, Excitatory amino acid transporter; EC, entorhinal cortex; FAC, Familial amyloid cardiomyopathy; FAD, Familial Alzheimer's disease; FAP, Familial amyloid polyneuropathy; FTD, Frontotemporal dementia; FTDP17, Frontotemporal dementia and parkinsonism linked to chromosome 17; FUS, RNA-binding protein FUS; GCIs, Glial Cytoplasmic Inclusions; GFAP, Glial fibrillary acidic protein; GWAS, Genome-Wide Association Studies; HD, Huntington's disease; HIV, Human immunodeficiency virus; HSP70, Heat shock protein 70; HSP90, Heat shock protein 90; htt, Huntingtin; IAPP, Islet amyloid polypeptide; iPSCs, Induced pluripotent stem cells; LB, Lewy Bodies; MLKL, Mixed lineage kinase domain-like protein; MSA, multiple system atrophy; NLR, Nod-like receptor; PAP, prostatic acid phosphatase; PD, Parkinson's disease; PGRP-LC, Peptidoglycan-recognition protein LC; PGRP-LE, Peptidoglycan-recognition protein LE; PrP, Prion protein; PS1, Presenilin-1; PSP, Progressive Supranuclear Palsy; RBPs, RNA binding proteins; RHIMs, Rip homotypic interaction motifs; RIPK1, Receptor-interacting serine/threonine-protein kinase 1; RIPK3, Receptor-interacting serine/threonine-protein kinase 3; rpAD, rapidly progressive Alzheimer's disease; SAA, Serum amyloid A protein; sAD, sporadic Alzheimer's disease; SAP, Serum amyloid P; SCA1, Spinocerebellar ataxia type 1; SCA2, Spinocerebellar ataxia type 2; SCA3, Spinocerebellar ataxia type 3; SCA8, Spinocerebellar ataxia type 8; SCA10, Spinocerebellar ataxia type 10; SOD1, Superoxide dismutase 1; SSA, Senile systemic amyloidosis; Sup35, Eukaryotic peptide chain release factor GTP-binding subunit; T2D, type 2 diabetes; TDP43, TAR DNA-binding protein 43; Tia1, Nucleolysin TIA-1 isoform p40; TTR, Transthyretin; ZBP1, Z-DNA binding protein 1; ZP, Zona pellucida; β 2M, Beta-2 microglobulin

Keywords: heterotypic aggregation; co-aggregation; cross-seeding; amyloid co-deposition; functional amyloids; amyloidosis; prion transmissibility; aggregation-prone regions; selective

vulnerability; supersaturated proteins; amyloid polymorphism; amyloid strains; phase transition; phase separation; sequence specificity;

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Abstract

Amyloid aggregation results from the self-assembly of identical aggregation-prone sequences into cross-beta-sheet structures. The process is best known for its association with a wide range of human pathologies but also as a functional mechanism in all kingdoms of life. Less well elucidated is the role of heterotypic interactions between amyloids and other proteins and macromolecules and how this contributes to disease. We here review current data with a focus on neurodegenerative amyloid-associated diseases. Evidence indicates that heterotypic interactions occur in a wide range of amyloid processes and that these interactions modify fundamental aspects of amyloid aggregation including seeding, aggregation rates and toxicity. More work is required to understand the mechanistic origin of these interactions, but current understanding suggests that both supersaturation and sequence-specific binding can contribute to heterotypic amyloid interactions. Further unravelling these mechanisms may help to answer outstanding questions in the field including the selective vulnerability of cells types and tissues and the stereotypical spreading patterns of amyloids in disease.

Introduction

Protein aggregation has been connected to more than 30 proteinopathies, ranging from neurodegeneration to Type 2 Diabetes (T2D) and systemic amyloidosis [1]. Despite the diversity in clinical presentation between these diseases and the heterogeneity of the affected tissues, common pathological hallmarks and principles unite them [2, 3]:

First, the tissues/organs that are most affected characterize each disease and dominate its symptoms. For example: Degeneration of motor neurons is at the heart of Amyotrophic Lateral Sclerosis (ALS)[4], resulting in the gradual weakening of the muscles, and in particular, the involuntary muscle groups. Degeneration of specific volumes of the brain stem causes Progressive Supranuclear Palsy (PSP)[5], leading to loss of balance, slowing of movement, difficulty moving the eyes, and dementia. Alzheimer's Disease (AD) is associated with degeneration in the hippocampus and cortex and hence leads to symptoms related to memory and behavior[6].

Second, amyloid deposits in the disease-defining tissue(s) are a unifying hallmark of the proteinopathies [7]. These amyloids are ordered proteins aggregates, characterized by the self-assembly of the proteins into cross-beta structured deposits. Together with the organ/tissue, the identity of the main proteins in the amyloid deposits defines the disease. Examples include TDP43 aggregates in ALS, tau aggregates in PSP, α -synuclein aggregates in Parkinson's Disease (PD) and both A β and tau aggregates in AD.

Third, unique stereo-specific spreading patterns are another characteristic of several of these diseases [2]. The aggregates appear first in a defining area and spread from there to functionally connected areas, notably in the brain, likely through synaptic connections. For example, α -synuclein aggregates first appear in PD patients in the olfactory bulb and dorsal motor nucleus and spread from there to the *substantia nigra* and eventually to the neocortex[8]. This spread correlates to a progressive worsening of the clinical symptoms, of which the earliest often go unnoticed, such as a loss of the sense of smell in early PD.

These patterns of deposition and spreading have led to the proposal that the aggregates are the disease-causing agents and that their inherently cytotoxic properties disrupt cell- and tissue homeostasis [9]. Since the mechanisms through which aggregates achieve this remain poorly understood [10], the alternative view can however not be fully

dismissed that aggregation in some cases may be a mere consequence of some other, not yet identified cause [11].

Protein aggregation is thus defined as a process of self-recognition and thus homotypic interactions, which is justified since the aggregates are typically strongly enriched in a single protein. However, protein deposits have long been known to contain a complexity of other components, both proteinaceous and other, and recently heterotypic interactions are increasingly being considered as potential modulators of both the aggregation itself and of its functional consequences. Heterotypic amyloid interactions might provide the mechanistic underpinnings of still insufficiently understood phenomena such as protein co-deposition in amyloid plaques [12], polymorphism [13], transmissibility and prion-like behaviour [14]. Furthermore, heterotypic aggregate interactions could have beneficial functions in a cellular environment, such as sequestering toxic aggregates or mediating new functions. This apparent duality of heterotypic interactions makes them of great interest to further understand protein aggregation, and subsequently may also provide tools to manipulate the aggregation process to our advantage.

Evidence of cross talk between pathological amyloidogenic proteins

Although amyloid diseases are usually associated with one or two amyloid proteins such as mentioned above, there is growing evidence that amyloid pathology can co-occur in the same tissue and this is often associated with more severe disease progression [15]. This co-occurrence may be an outcome of direct interactions between the aggregating proteins, such as co-aggregation or cross-seeding, or could be an indirect effect resulting from an overall decline in protein homeostasis in the affected tissues. Since the interplay of amyloid cross-interactions have extensively been reviewed recently [16-18], we limit this section to few notable examples (Fig. 1).

Alzheimer disease as such is a case in point as this neurodegenerative disease has been defined from the outset by the co-existence of amyloidogenic proteins in the same tissue. The main pathological hallmarks of AD are the presence of both extracellular A β aggregates and intracellular tau tangles [19]. Although the aggregation of A β precedes the aggregation of tau by some time in the course of the disease [20], it is unclear if and how the

aggregation is related to either, but there is evidence to suggest that the aggregation of one may directly affect the other, even if one is primarily intracellular (tau) and the other is primarily extracellular (A β) [21]. It has been shown *in vitro* and in cells that pre-aggregated A β can induce fibril-formation of tau [22]. Moreover, familial Alzheimer's disease (FAD) mutations in the amyloid precursor protein or presenilin-1 (PS1) can promote high levels of phosphorylated tau aggregates in 3D neuronal cell cultures [23]. Interestingly, upon inhibition of A β production, both A β and tau pathology was reduced in these models [23]. A similar observation was made in neurons derived from induced pluripotent stem cells (iPSCs) of AD patients [24]. Furthermore, evidence suggests that A β aggregation enhances the aggregation of tau in triple-transgenic mouse models [25]. Studies in human brains of patients with AD have also shown that the presence of A β pathology influences tau propagation [25].

It appears A β aggregation may have other molecular links. Type 2 diabetes (T2D) is linked to the aggregation of the Islet amyloid polypeptide (IAPP), which causes loss of β -cells in the pancreatic islets of Langerhans. T2D has been associated with an increased risk of dementia, including AD and vascular dementia [26]. Although the specific mechanism behind this connection is not yet fully understood, several lines of evidence suggest that cross-talk between the protein aggregation that exists in both diseases may play a role. Indeed, cross-seeding of A β and IAPP has been shown *in vitro*, with IAPP increasing A β aggregation [27]. Moreover, injection of A β fibrils in IAPP transgenic mice successfully induced the aggregation of IAPP in the islet of Langerhans when compared to control groups [28]. Interestingly, IAPP has been found to co-deposit in amyloid plaques of AD patients, even in absence of a diabetes diagnosis [28, 29]. A potential explanation of the connection between A β and IAPP may be found in their primary sequence, since both proteins share 50% sequence similarity, and have an even higher degree of similarity in the regions known to be important for self-aggregation [30, 31], suggesting this may play a role in cross-seeding and co-deposition in disease.

Aggregation of α -synuclein into Lewy bodies (LB) has been connected to Parkinson's disease (PD). Although LB are the pathological hallmark of PD, 50% of AD patients also exhibit α -synuclein aggregates, which is associated to a more aggressive progress of the disease [32-34]. NMR studies have shown that A β peptides can interact with α -synuclein, while kinetic aggregation studies suggest that the presence of A β can accelerate the aggregation of α -synuclein *in vitro* [35, 36]. A similar observation was made in double transgenic mice expressing human A β and α -synuclein mimicking Lewy Body Alzheimer disease, with those

mice having more α -synuclein inclusions than the ones expressing only α -synuclein [37]. In addition, crossing 3xTg-AD mice, that are expressing human APPsw, human P301L tau and PS1^{M146V} knock-in, which can develop plaques and tangles, with transgenic mice bearing the α -synuclein A53T mutation, simulated DLB-AD successfully and resulted in a drastic increase in cognitive dysfunction and amyloid pathology [38]. A-synuclein also enhanced aggregation of tau, and both proteins affect each other's aggregation [39]. Moreover, tau positive aggregates have been identified in transgenic mice bearing A53T α -synuclein mutation. Furthermore, α -synuclein inclusions were found in double transgenic mice bearing wild type α -synuclein and P301L tau [39]. Co-expression of α -synuclein and tau in different cell models also showed that those proteins colocalize and that tau affects the number and pattern of α -synuclein aggregation [40].

The presence of more than one amyloidogenic proteins is also found in other proteinopathies, such as Huntington's disease (HD). HD is a neurodegenerative disease caused by a mutation in the huntingtin (htt) gene, resulting in a CAG nucleotide expansion [41]. Many studies have shown that HD coexists with ALS and AD [42, 43]. Indeed, increased aggregation of α -synuclein, tau and TDP-43 has been connected to HD brains [44]. Moreover, α -synuclein aggregates have been colocalized in htt aggregates both in human and mouse brains [45]. Tau pathology has also been connected to HD. In detail, tau aggregates have been found to partially colocalize with htt aggregates and also exist in early-onset HD patients, indicating that it is not caused by normal aging [46]. In addition, studies in cells and mouse models showed that mutant htt affects phosphorylation of Tau, a process connected to tau aggregation [47].

Finally, SCA2 is a neurological disease caused by mutation in the ATXN2 gene resulting in a CAG expansion [48]. Evidences from a SCA2 patient's brain showed the presence of TDP43 aggregates, similar to those seen in ALS. Although, SCA2 and TDP43 aggregates were not found to co-deposit, the connection of TDP43 to many polyQ diseases suggest that there is a possible mechanism behind this co-existence [49]. Similarly, the RNA-binding protein FUS have been localized to intracellular inclusions in human brains of SCA1,2 and 3 [50].

Heterogeneity of amyloid deposits

In addition to the co-occurrence of main pathology-driving proteins such as described above, there is also considerable additional heterogeneity in the deposits themselves, both in terms of the proteins found as well as in the other types of biological macromolecules (Fig. 2). Although the mechanism behind this co-deposition is not well understood, recent advances in mass spectrometry allows ever better elucidation of this complexity, in particular in terms of the protein components of those aggregates. For example, amyloid plaques, of which the main component is the A β peptide, have been found to be composed of a significant number of other proteins. Indeed, mass-spectrometry studies on amyloid plaques from AD patient brains have revealed the number of plaque-resident proteins to range from 400 to 1000 [51, 52]. Although the number of proteins identified in each study differed, likely due to the specific procedures followed or the tissues analyzed, there appears to be similarities in the proteins identified: Glial related proteins like GFAP, EAAT and vimentin have been found repeatedly to reside in amyloid plaques and potentially provide a link to gliosis, a well characterized feature of AD [52, 53]. Extracellular matrix proteins like glypican, testican, and cystatin have also been found in amyloid plaques [51, 52]. Moreover, proteins of the complement system, for example C1q, C1r, C1s and C5-C9 have been associated with A β aggregates, possibly as result of the activation of the complement system during the course of the disease [51]. Furthermore, proteins connected to a variety of cellular processes like actin organization, chaperones, membrane trafficking and degradation have also been connected to A β deposits [51-53]. Synaptic proteins and synaptic vesicle proteins have also been found in amyloid plaques, comprising 15% of the proteins found in them [53]. Interestingly, comparison of amyloid plaques from patients with rapidly progressive AD (rpAD) and sporadic AD (sAD) showed that rpAD plaques contain more neuronal and synaptic proteins and less astrocyte-related ones [54]. These lines of evidence suggest that a significant number of proteins can co-deposit with A β aggregates and that plaque composition is in some way related to disease progression.

Neurofibrillary tangles, consisting mainly of tau aggregates have also been shown to contain many cellular factors. Colocalization studies have shown that filamin-A and myosin VI localize with tau tangles in AD and FTDP-17 [55]. Moreover, BRCA1, a DNA nuclear protein, has been found to mis-localize and deposit with tau in different tauopathies, possibly causing loss of function and reducing DNA repair [56]. Furthermore, incubating different aggregated species of tau with rat brain lysate revealed a distinct interaction pattern compared to

monomeric tau [57], suggesting aggregate-specific interactions occur. Interestingly, interactions changed during the course of aggregation. Early interactions contained proteins connected to Coat Protein I components, which in late stage aggregates were decreased in abundance [57]. In contrast, during these later stages of aggregation, proteins connected to RNA, phosphorylation and microtubules were able to interact highly with tau fibrils [57]. Co-immunoprecipitation of tau from microsomes isolated from AD and control brains showed that 42.9% of the proteins identified were only found in AD brains, with proteins involved in RNA translation having higher abundances [58]. In addition, proteomics analysis of neurofibrillary tangles isolated from AD brains identified around 542 proteins [59]. These proteins include chaperones (HSP70, HSP90), proteins of peroxiredoxin family, proteins with RNA recognition motifs, 14-3-3 proteins, etc [59]. Interestingly, a significant number of the identified interactors of phosphorylated tau (a hallmark of the pathological form) were also present in tau tangles [59]. In addition, some of the interactors found in tau tangles were in fact only identified to be present in some of the cases, indicating that variation in the interactome between patients may hold important information for disease progression [59].

Similar observations have been made for other protein deposits, for example in Lewy bodies (LB), which mainly consist of α -synuclein, but also been shown to contain a number of different proteins. These proteins have been connected to extracellular matrix, signaling and apoptosis, cytoskeleton, inflammation, proteins synthesis or degradation [60]. Moreover, synaptic vesicle proteins have been associated with the formation of LB in DLB and Glial Cytoplasmic Inclusions (GCI) in multiple system atrophy (MSA) [61]. Htt inclusion bodies have been found to be enriched in ubiquitin in living cells [62]. Moreover, cell culture experiments showed that insoluble htt aggregates are enriched in protein quality control proteins while soluble htt oligomers have the ability to interact with more than 800 different proteins connected to different cellular pathways [63].

Deciphering the role of brain heterogeneity in aggregation diseases

Despite being widely expressed in the brain, amyloid-forming proteins seem to follow specific spatiotemporal patterns of self-propagation that do not always appear to match disease progression [64]. For example: In AD patients, amyloid plaques spread along different areas of the brain, while the regional pattern of neurofibrillary tangles better resembles the progression of clinical symptoms [65, 66]. Recent work using iPSCs from patients harbouring

an amyloid precursor protein mutation revealed a differentiated response to A β and tau by individual neuronal cell types, suggesting that cell-autonomous factors partially mediate spatiotemporal patterns of selective regional vulnerability [67]. This is supported by a plethora of information focused on charting cellular susceptibility, as regions located in the outer layer of the entorhinal cortex, basal forebrain and hippocampus are exceptionally vulnerable in AD, while glial cells, cortical neurons and inner layers of EC are more resistant, respectively [68-73]. Analysis of neuron-specific GWAS expression profiles linked cellular vulnerability with genes involved in processes of axon plasticity, tau splicing and aging [74].

The prominent role of the proteome heterogeneity per cellular type cannot be ignored, although the basis of this apparent selective vulnerability is still largely unknown [64]. Reelin and SMI32-immunoreactive EC neurons show increased vulnerability in both transgenic mice and human AD patients [75]. The former is an extracellular protein involved in synaptic transmission pathways that are pivotal for synaptic plasticity, neuronal migration and dendritogenesis [76]. Reelin has been reported to directly influence A β aggregation, by prolonging oligomer lifetime and delaying A β fibrillation, while overexpression in transgenic mice has been shown to rescue memory recognition by delaying the formation of amyloid plaques [77]. Despite this and the fact that A β oligomerisation has been linked to increased expression of Reelin, co-aggregation entrapment was found to diminish the levels of Reelin and is directly associated to advanced Braak stages in AD patients [78], while additional studies indicate that Reelin impairment also compromises functional down-regulation of tau phosphorylation [79]. In another direct example, tau and lysozyme share a common mechanism of induced ribosomal sequestration through co-aggregation which causes dysregulation of protein translation pathways [80, 81], while cross-interaction with RNA molecules has been shown to regulate prion aggregation [82, 83]. In this line, selective vulnerability to α -synuclein aggregation has been linked with mitochondrial dysfunction and chronic inflammation in PD [84-86]. Co-aggregation of multiple defective proteins is a hallmark behind selective motor neuron dysfunction in ALS, as reviewed by Gomes et al. and others [87-89].

Taken together, the above have led to the notion that the temporal instability of the cellular proteome composition can mediate selective vulnerability through direct heterotypic interactions between the (pre-)amyloid aggregates and specific proteins, potentially

exacerbated by a general decline in protein homeostasis due to aging or external stress [90]. Direct interactions between differentially controlled proteins are also compatible with amyloid cross-talk [91], as well as with the amyloid strain hypothesis [92]. However, it still remains possible that other more generalised mechanisms, such as metal binding or widespread collapse of cellular proteostasis may also explain why specific cell types are more susceptible to toxic aggregates [93-96].

Protein supersaturation profiles

Perhaps what best ties together co-aggregation and selective vulnerability is the proposed role of the supersaturation, i.e. accumulation to levels beyond their intrinsic solubility of specific sub-proteomes, in driving additional protein components to collapse into aggregate deposits (Fig. 3) [97-101]. This hypothesis was first formulated in a bid to explain selective susceptibility of neuronal cells in neurodegenerative diseases [97]. An intricate balance between protein supersaturation, external stress and the inherent metastability of subcellular proteome contents against proteostatic quality control machinery of cells was suggested to modulate neuronal susceptibility [97]. Apart of obvious supersaturation levels for major amyloidogenic proteins [98], it was shown that many other proteins exceed their solubility thresholds and indeed are found in many deposits associated with the major aggregation-related diseases, such as AD, PD and HD [99]. Follow-up analysis of single-cell transcriptomics and subcellular proteomics data revealed an enrichment of supersaturated proteins related to synaptic function and mitochondrial metabolism, that showed a remarkable overlap to regional vulnerability patterns observed in AD, PD, FTD and ALS [100]. Differential analysis of transcriptional regulation in AD, compared to aging, revealed that certain supersaturated protein populations have an increased propensity for aggregation and strongly correspond to patterns of down-regulated gene expression, suggesting that this might be a controlling mechanism against neurodegeneration [101]. A breakdown of the protein content of ALS-related inclusion bodies indicated that co-aggregating proteins are either interaction partners of the major protein constituents (TDP43, SOD1, FUS), or display supersaturated expression levels and increased metastability towards aggregation, suggesting that progressive impairment of the proteostatic mechanisms can trigger their co-deposition in motor neurons [102]. Recently, it was shown that protein supersaturation levels are not only linked to neurodegenerative disorders, as a similar metastable subproteomic

population was shown to dictate the formation of inclusions in muscular proteinopathies [103].

Heterologous biomolecular condensation

The formation of membraneless cellular inclusions through phase separation has been interchangeably linked to promoting, as well as to disfavoured the development of proteinopathies. Major amyloidogenic proteins, including α -synuclein, tau and ALS-related proteins form heterogeneous condensates through phase separation that can contain up to hundreds of proteins [104-106]. Two basic modes of action have been proposed for stress granule-induced toxicity which is associated to neurodegenerative disorders [107, 108]. In the first mode, gradual conversion of dysregulated condensates towards fibrillar aggregates can drive the entrapment and accumulation of their additional components, which causes cellular depletion and subsequent loss of function. Given that important autophagy-related proteins are recruited for their disassembly and clearance and that the majority of condensate hetero-components refers to RNA-binding molecules [107, 108], sequestration and loss of their function can critically impair vital cellular activities, such as translation and protein quality control [109]. On the other hand, stress granule formation has also been depicted as an intermediate metastable state towards the formation of pathological amyloid aggregates [104, 110, 111]. In an interesting change of perspective, however, Mathieu et al. recently pointed out that stress granules can actually utilise heterotypic interactions as a molecular crowding scheme to prevent homotypic association of amyloidogenic components, which is the driving force of liquid-to-solid phase transition [112]. This buffering heterotypic interplay, as the authors mention, is an intricate balance that can be tipped by a change in the relative concentrations of the granule constituents, pathological mutations or additional competitive heterotypic interactions [112].

Is sequence specificity a determining factor in amyloid heterotypic interactions?

Early experiments indicated that cross-aggregation is somehow related to the sequence similarity between seeding and co-aggregating proteins [113]. In a landmark study, sequence conservation between neighbouring Ig-like domains of large multi-modular proteins was shown to determine their co-aggregation potential, as detectable interactions were observed only between immunoglobulin domains with more than 70% sequence

identity [114]. As a result, neighbouring domains retain exposed aggregation-blocking conserved residues as an evolutionary strategy to prevent lateral aggregation in multidomain, β -folded or β -solenoid proteins [115-120]. ESI-IMS-MS experiments revealed that variants of IAPP can co-aggregate and form hetero-oligomers in pairwise mixtures, yet remain incapable of cross-seeding each other, suggesting that these are two separated processes that do not necessarily co-occur [121]. Cross-seeding mechanisms have been highlighted for disease-related variants of several other amyloid-forming proteins. Differential kinetic analysis of heterologous seeding between five conserved immunoglobulin light chains revealed that acceleration or deceleration of the nucleation process of one protein by another relied heavily on the conformational properties of the nucleator [122]. Cross-seeding studies between α -synuclein and single-position variants, namely A30P and A53T, indicated that the wild-type protein aggregated faster and formed morphologically distinct fibrils in the presence of either A30P or A53T preformed seeds, whereas no cross-seeding was detected between the two variants [123]. This morphological alteration can probably be tracked-down to the diverse structural layout of fibrils formed by single mutants such as in the case of E46K [124], A53T [125] and H50Q [126], compared to WT α -synuclein [127]. Different structural polymorphs have also been identified for other amyloid disease variants, such as the Alzheimer β -peptide Iowa (D23N) [128] and Osaka [129] mutants, as well as the IAPP S20G mutant [130], when compared to wild type A β [131, 132] and IAPP [130, 133] fibril structures, respectively. In contrast, species complementarity was disclosed by cryo-EM structures of pathological amyloids involved in systemic AA amyloidosis, despite an evident difference in the handedness of their fibril twist [134]. Sequence selectivity has also been related to the structural compatibility that affects transmissibility of prions, although the limits of this dependence have been hard to determine in past studies [135, 136].

Whereas protein folding is determined by the cooperative formation of tertiary interactions, protein aggregation is determined by local secondary structural propensity of short sequence segments, 5-15 residues in length [137-141]. These aggregation-prone regions (APRs) act as the kinetic drivers of protein aggregation due to their tendency to self-associate into ordered intermolecular β -sheet arrangements [2, 142]. In this conformation, APRs are stapled together in β -sheets through a network of backbone hydrogen bonds and pack laterally via tightly interdigitated sidechains forming stable structures, known as steric

zippers [143-145]. While sequences with a strong β -propensity are compatible with the generalized backbone stacking of β -strands, the intricate inter-fitting of side chains directly correlates to sequence specificity. Atomic structures of APR segment homologs from major prion protein (PrP) highlighted that sequence specificity critically confers interspecies transmissibility barriers [146]. Rationally designed single-position APR variants can alter both the aggregation propensity and cytotoxicity of hIAPP [147, 148], with additional work supporting that sequence variants can disrupt self-association and suppress aggregation of other proteins [149, 150]. Co-expression studies of unrelated aggregating peptides, sharing no sequence similarity, resulted to the formation of separate inclusion bodies in mammalian, as well as *E. coli* cells [151]. Heterotypic steric zipper interfaces between distant APRs within amyloid sequences were initially predicted to shape the core of full-length protein amyloid fibrils [2, 152-155]. This has been validated by the recent burst of cryo-EM fibril structures [125, 127, 132-134, 156-158]. Indeed, cryo-EM structures reveal that intramolecular heterotypic interactions mediate the formation of different amyloid polymorphs by shaping internal zipper interfaces of the fibril core or by controlling the lateral interactions between protofibrils [159, 160]. Multiple interfaces have been reported even in the structural packing of single APRs [161, 162]. The role of amino acid composition has also been indirectly expanded to regions flanking APRs in protein sequences. Our recent work has shown that single residues flanking APRs in protein sequences can act as gatekeepers that attenuate aggregation propensity [138, 163-166], whereas sequence stretches of high disorder can affect the transmissibility of functional prion APRs by modulating aggregation morphology [167].

The first structure of a sequence-dependent heterotypic amyloid assembly has recently been solved by solid state NMR, and concerns the RIPK1-RIPK3 necrosome signalling complex, i.e. a functional amyloid that was evolved to form a stable hetero-amyloid structure [168]. Such heterotypic amyloid interactions are compatible with the strain hypothesis, according to which, a protein can form morphologically distinct strains that follow the structural characteristics of the seeding molecule, a concept that holds true in cases of hetero-assembly [13, 169, 170]. Exactly this was recently shown for two fragments of the A β peptide: the two peptides form hetero-amyloids, but the amyloid conformation of the one peptide was modified by the presence of the second through unfolded state interactions, showing that strain selection can be steered by proteins that do not become part of the final

aggregates [171]. Another familiar example of sequence-related cross-interacting APRs involves A β and IAPP, despite the fact that they are spatially and functionally unrelated proteins [31]. The former has been shown to promote unilateral nucleation of IAPP, albeit with a lower efficiency compared to homologous assembly, whereby these proteins harbour stretches sharing sequence homology [172]. Several additional proteins have been associated to templated amyloid cross-talk via sharing of common structural architectures [161, 173-175], with the list expanding even to cases of functional amyloids [176-182]. Finally, cross-seeding and heterotypic co-aggregation has also been demonstrated in cancer-associated aggregation pathologies, where mutant p53 causes sequence-dependent co-deposition of its homologs p63 and p73 by virtue of their similar APRs [183-185].

Hetero-aggregation in functional amyloids

Several examples have been documented by previous studies, elucidating that amyloid cross-talk can be involved in the development of important biological functions, with cases spanning from bacteria to humans. Biogenesis of curli amyloid fibres in bacteria requires the co-operative action of six proteins encoded by shared operons [186]. Two subunits, known as major (CsgA) and minor (CsgB) curli subunit co-aggregate together to form the main axis of the curli fibres that remain attached to the surface through a heterotypic complex formed by CsgB, CsgF and a secreting pore formed by CsgG [186-188]. Both subunits have been shown to self-assemble, however CsgB pre-formed aggregates are also important for CsgA nucleation [189, 190]. This explains why *csgB*⁻ strains that stably express soluble CsgA show remarkable interbacterial complementarity when growing in proximity to *csgB*⁺/*csgA*⁻ strains [191]. Computational studies indicate that this complementarity largely stems from the fact that CsgA and CsgB most likely adopt similar β -solenoid-like folds [178, 192, 193]. A similar mode of action has been proposed for FapC biofilm formation by *P. fluorescens* [194, 195], while amyloid hetero-interactions have also been proposed to contribute in the formation of aerial hyphae by chaplins [196].

The role of amyloids and their interplay with respect to the reproduction system has also been reviewed [197]. Early work highlighted that the protective egg coat of silkworm and fish oocytes is in fact a natural protective amyloid structure [198, 199]. Silkworm chorion is composed by hetero-fibrils composed of a large number of proteins that are divided in two

major families that share common central aggregation prone domains [200, 201]. Amyloid fibrils of fish egg coats, on the other hand, consist of heteromeric building blocks formed by proteins that share an evolutionarily conserved amyloidogenic domain with proteins of zona pellucida (ZP), the extracellular matrix that controls fertilisation and protects mammalian oocytes [202]. Follow-up studies later revealed that mouse ZP also exhibits typical amyloid-like properties [203], while structural analysis has shown that a conserved amyloidogenic interface is responsible for the heterotypic interaction of ZP proteins from fish to humans [177, 179, 180]. Impressively, the sperm acrosome which allows spermatozoa to penetrate the oocyte by interacting with ZP during fertilisation contains a proteinaceous matrix that is also composed of several amyloidogenic proteins such as cystatin-C, lysozyme and cystatin-related epididymal spermatogenic protein [204]. Finally, semenogelin and prostatic acid phosphatase (PAP) form functional amyloid fibrils, found in seminal plasma, that immobilise and clear damaged sperm cells and bacteria by communicating with immune cells [205, 206], however also bind to HIV virions thus enhancing HIV transmission [207]. Similarly, A β amyloid fibrils participate in hetero-interactions with receptors of HIV-1 enhancing viral attachment and infectivity [208, 209], although HIV-1 and *AviBirnavirus* have also been shown to utilize cross-reactive amyloids to increase their virulence [210, 211].

Hybrid amyloid constructs also play a pivotal role in necroptosis-associated signalling pathways. Formation of RIPK1-RIPK3 hetero-oligomeric signalling complexes, known as necrosomes, triggers phosphorylation of mixed lineage kinase domain-like protein (MLKL), which in turn mediates programmed necroptosis [212, 213]. Interestingly, MLKL also self-assembles to form amyloid fibrils, however, no amyloid-related cross-talk between MLKL fibrils and necrosomes has yet been shown [214]. RIPK1-RIPK3 amyloid complex formation is controlled by conserved short Rip homotypic interaction motifs (RHIMs) [168]. Recently, a novel viral infection strategy has emerged indicating that the RHIM-associated necroptosis pathway can be repurposed by viral MLKL counterparts. Several proteins with high sequence similarity to the pseudokinase domain of MLKL were identified in poxvirus genomes and shown to circumvent phosphorylation of MLKL by competitive binding to necrosome amyloid complexes [215]. In this line, Sunde et al. recently showed that the murine cytomegalovirus (CMV) protein M45 contains a RHIM motif that directs its self-assembly into homo-oligomers. M45 oligomers can cross-interact with the RHIMs of RIPK1, RIPK3 and the Z-DNA binding

protein 1 (ZBP1) to form heterotypic amyloid assemblies that block the necroptosis pathway in mice, thus explaining the ability of murine CMV to block necroptotic death [216]. The presence of RHIMs can be tracked down the evolutionary line, even to single cell organisms [217, 218]. A similar aggregation motif has been identified in a Nod-like receptor (NLR) in *P. anserine*, which promotes its own amyloid-like self-assembly and participates in a similar amyloid-driven signalling pathway by catalysing the conversion of Het-s prions [219]. RHIM-like motifs found in peptidoglycan recognition proteins PGRP-LC and PGRP-LE also control signal transduction in *D. melanogaster*, while participating in hetero-aggregation with adaptor protein Imd to activate NF- κ B signaling [220].

Prion transmissibility has been extensively linked to cross-seeding [14] and is considered a leading mechanism behind cross-species prion conversion [221, 222]. Formation of functional hetero-prions is often vital for important biological functions. Recent evidence revealed that Sup35 co-aggregates with prions deriving from the self-assembly of the known stress granule component protein Tia1, which self-propagates via a Q/N rich domain. This hetero-amyloid assembly is a primary component of binding complexes that contribute to localised synthesis of tubulin by harbouring components of translation machinery [223]. Rnq1 initiates self-assembly of Sup35 through cross-talk interactions that are sensitive to single-residue changes [224], as other critical residues moderate cross-species Sup35 transmission [225]. Previous work highlighted a similar mechanism to control strain-dependent seeding specificity and transmission barriers between mammalian prions [226]. This suggests that prion cross-infectivity is generally adjusted through a template-assisted mechanism that depends on strain-relevant structural conformation compatibility [169, 227]. Unilateral cross-aggregation templating by alternative spliceforms or naturally occurring glycosaminoglycans is an additional conformation-specific strategy used for the formation of functional amyloid in higher organisms [228, 229].

Conclusions

The after-effects of amyloid-like protein aggregation have long been directly associated to the adverse self-assembly of single aggregation-prone components. However, evidence is accumulating that identifying aggregation spreading patterns alone does not suffice in order to understand toxicity and selective vulnerability in proteinopathies. In this

review, we discussed recent evidence that is gradually shifting interest towards the heterotypic interplay of amyloids between each other and with other proteins, as a framework to elucidate pathophysiological mechanisms of protein deposition diseases. More and more the realisation grows that pathological amyloidogenic proteins are involved in a dense network of heterotypic interactions that can either promote disease progression and severity, but can also have a beneficial effect by either reducing the aggregation propensity or toxicity of major amyloid constituents. This interconnectivity is further entangled when considering complementary studies indicating that even non-aggregating or disease-unrelated proteins can modulate the morphology or aggregation rates of amyloidogenic proteins [230].

Following the rapid development of whole genome-expression and single-cell transcriptomics, a lot of effort has recently been put into identifying tissue-specific loci related to amyloid pathology. Disease-induced changes in expression profiles have been previously proposed to occur as a result of an indirect cellular response to protein aggregation. Co-aggregation has been extensively linked to promoting sequestration of cellular components with a role in translation, the depletion of which can provide context on the differences seen in transcriptional profiles between normal and disease brains. As prime example of this process, HD aggregates which are usually localized as nuclear inclusions promote sequestration of multiple transcription factors [231], while altered RNA metabolism is common in spinal motor neurons of ALS patients, as a result of the extensive co-deposition of RBPs [88]. Similarly, tau promotes co-aggregation of important RBPs involved in nuclear transport and chromatin remodeling [232], whereas single-cell proteomic profiling has shown that cerebral atherosclerosis is consistent with reduced synaptic signaling, RNA splicing and increased oligodendrocyte markers that associate to AD [233]. More evidence is required in order to determine if protein components of these pathways contribute to disease as innocent bystanders or as active modulators of co-aggregation. Nevertheless, it seems that the role of heterotypic interactions in this process can no longer remain unnoticed, as amyloids are constantly reported to trap vital proteins involved in diverse, yet pivotal pathways of cellular homeostasis, including inflammation, proteostatic control and autophagy among others [234].

In this overview we highlighted the emerging role of hetero-aggregation as an acquired structural strategy that complements amyloid polymorphism and thus facilitates

prion-like properties, determines species-specific transmissibility barriers and controls cross-nucleation induced by disease variants. A deeper look into the assembly mechanisms of major functional amyloids pinpointed that these proteins also contribute to the formation of heterocomplexes through surface-exposed APR interfaces of their native folds which share high structural complementarity. Nowadays, the wealth of structural information on amyloid fibrils and their polymorphic variants is constantly expanding, supporting the notion that sequence specificity is an important modulator of this structural compatibility. This hypothesis was also validated by experimental data on the first actual hetero-amyloid structure of RHIM-like motifs, building on years of work validating sequence specificity as a driving force behind prion inter-species infectibility and functional amyloid heterocomplex formation. However, as other mechanisms have also been recently reviewed in terms of supporting unspecific hetero-templating by amyloids through lateral or surface interactions [217], future work is required in order to determine the thresholds of sequence-specific mediated amyloid cross-talk, as well as to define its importance in disease.

Author Contributions

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Table 1. Definitions of terms used throughout the text.

Proteinopathies: Pathologies associated with protein aggregation and deposition which is promoted by self-assembly of amyloidogenic proteins.	Heterotypic buffering: Biomolecular crowding effect induced by heterotypic interactions that reduce pathological homotypic aggregation and condensation.
Heterotypic interactions: Any type of transient or permanent physical interaction between protein molecules with different primary structures.	Phase transition: The process of abruptly changing the physical properties of a state of matter by cooperative local overcrowding of biomolecules.
Cross-seeding: Also defined as heterologous seeding, this process describes the ability of pre-formed seeds to promote the nucleation of a different protein. This can refer to species-related homologous sequences, disease variants or completely different protein sequences.	Phase separation: Physical separation of locally co-existing phases produced by phase transition. This involves the separation of liquid phases with different densities (liquid-liquid phase separation – LLPS) or liquid-to solid-phase separation.
Co-aggregation: The heterogeneous condition of protein co-assembly once different proteins are dissolved in pairwise mixtures. Differs from cross-seeding in the sense that there is an absence of pre-formed seeds that can promote nucleation of either protein component.	Prion transmissibility: The ability of infectious protein aggregates to spread between different tissues/organs and further promote aggregation. Cross-species prion infectivity is determined through barriers defined by sequence specificity and structural compatibility.
Cellular selective vulnerability: The process in which only particular cell-types are pathologically affected by the presence of protein aggregates.	Aggregation prone regions: Short sequence stretches with a strong tendency to form amyloid-like aggregates through homotypic assembly.
Supersaturated proteins: Subcellular accumulation of specific subproteomic populations to levels beyond their intrinsic solubility. This process can potentially promote additional protein components to collapse into aggregate deposits through heterotypic interactions.	Amyloid polymorphism/strains. The ability of amyloidogenic proteins to form amyloid fibrils of variable distinct structural morphologies that can potentially contribute differentially to disease or transmissibility.
Biomolecular condensation: Formation of membrane-less cellular inclusions by concentrated biomolecules. Their formation occurs through reversible phase transition.	Functional amyloids: Amyloid fibril formation that supports important biological functions in organisms spanning from humans to bacteria.

Figure Legends

Figure 1. Dense cross-talk of amyloidogenic proteins and their relation to proteinopathies.

Primary amyloidogenic components (red rhomboid nodes) of several major amyloidosis diseases affecting a variety of tissues and organs have been shown to engage in heterotypic interplay (solid gray lines) and are occasionally found as secondary components in deposits (dashed grey lines) [16-18, 28, 29, 32-34, 42-46, 50, 235].

Figure 2. Co-localization of disease-associated protein aggregates with additional cellular components.

Co-localization of secernin-1 with A β in sAD human brains [54], filamin-1 with Tau in NFTs from FTDP-17 [55], parkin and Huntingtin in human HD brains [236], sumo2 and ataxin-7 in human SCA7 brains [237], LRRK2 and α -synuclein in PD brains [238], clusterin and TTR in FAP [239] and proximity ligation assay of Bri2 and IAPP in T2D [240]. Figures were adapted with permission from [54, 55, 236-240].

Figure 3. Heterotypic interactions contribute to the proteostatic equilibrium of protein aggregation-induced toxicity.

Hetero-aggregation can promote the entrapment of proteins through phase transition events, by depleting selectively vulnerable proteins, by targeting supersaturated populations or as a combination of the above. In contrast, cellular proteostatic mechanisms and heteromolecular crowding has been suggested to work in parallel as protective mechanisms providing resistance to toxic effects of aggregation.