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Painting protein misfolding in the cell in real time with an atomic-scale brush

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The direct observation of specific biochemical events in living cells is now possible as a result of combined advances in molecular biology and fluorescence microscopy. By genetically encoding the source of a unique spectroscopic signal, target proteins can be selectively detected within the complex cellular environment, with limited interference from background signals. A recent study takes advantage of arsenical reagent-based methodologies to monitor *in vivo* protein misfolding and inclusion body formation in real time. This approach promises to yield important information on the kinetics of aggregate formation in living cells and its relation to the time-course of protein expression and post-translational processing. The ability to follow protein self-association in real time accurately from its early stages is unique to this method, and has far-reaching implications for both biotechnology and misfolding-based disease.

Introduction

Cell biology has recently witnessed spectacular advances as a result of novel technologies that enable the fate of individual proteins to be followed with high specificity in living cells. Macromolecular interactions are often monitored by the *in vivo* yeast two-hybrid system screening assay and related variants [1]. This methodology indirectly reports on target protein interactions through the restored function of reconstituted reporter enzymes. A more direct and quantitative strategy to map both macromolecular localization and protein–protein interactions in the cell involves the combination of fluorescence microscopy imaging (or spectroscopy) and the genetic incorporation of fluorogenic amino acid sequences into a target protein. Fusion constructs combining a highly fluorogenic protein reporter with the sequence of the biomolecule of interest are commonly used. The reporter moiety describes the intracellular properties and spatial distribution of the protein of interest with high selectivity [2,3]. The most popular amino acid sequence used as a source of intense fluorescence emission is the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its numerous variants [4,5]. Related proteins from other organisms with complementary spectroscopic properties have also been employed [6]. Cellular localization of individual proteins is monitored by simple fluorescence emission, whereas colocalization of multiple proteins and protein–protein interactions is typically monitored by fluorescence resonance energy transfer (FRET) variants of this approach.

In general, the reporter protein-based approach is robust and widely used. However, it has the disadvantage that the large size of the reporter protein (e.g. GFP has 238 amino acids) might perturb the intracellular properties of the protein of interest. Although this has not generally been a problem, investigations involving intermolecular interactions with large surface contacts, such as extensive protein aggregation, must take this issue into account. For example, it has been reported that protein aggregation alters the fluorescence of a covalently bound GFP reporter by preventing its proper folding [7].

The *in vivo* post-translational maturation of GFP and its analogs (involving an intramolecular oxidative cyclization) is very slow and the apparent half-life for the generation of GFP fluorescence ranges between 30 and 90 minutes [8]. Faster maturing proteins from jellyfish and other organisms are under development [9], including enhanced versions of yellow fluorescent protein (YFP) and GFP, denoted as Venus and T-sapphire, which acquire fluorescence with a remarkable apparent half-life of only 1.5 and 5 minutes, respectively [10,11]. Therefore, kinetic studies of intracellular processes occurring on the minute timescale or faster are not possible unless a Venus-fusion protein approach is used. However, the bulky size of the added fluorophore is not ideal in kinetic and mechanistic studies involving extensive intermolecular contacts. These investigations are better served by an alternative fast-maturing system using a smaller fluorophore.

FlAsHing light into specific biochemical processes

Tsien and coworkers have developed a clever arsenic–fluorescein-based approach employing an engineered receptor–ligand pair that becomes fluorescent upon complexation [12]. This method enables the specific fluorescent labeling of any desired protein expressed inside living cells with only moderate background signal. Figure 1 schematically illustrates the method [13]. Briefly, a short tetracysteine motif (Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa denotes any generic amino acid, preferably Pro-Gly [14]) is engineered into the target protein. Two properly positioned arsenic atoms in a custom-made biarsenical reagent selectively coordinate the tetracysteine motif. The fluorophore-derivatized biarsenical ligand is known as FlAsH-EDT$_2$ (fluorescein arsenical helix binder, bis-ethanedithiol adduct). This compound is

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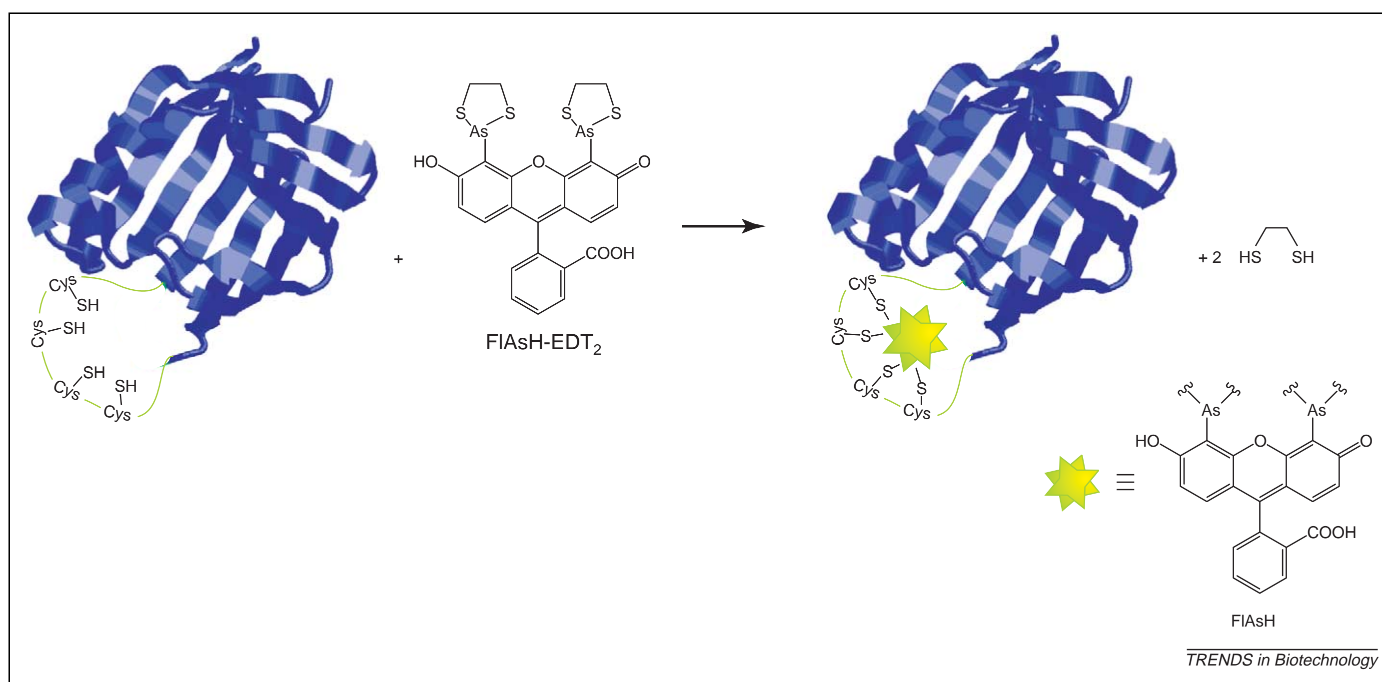

Figure 1. Site-specific protein labeling by the FlAsH fluorescent arsenical reagent. The primary sequence of the protein of interest is genetically engineered by inserting the CCXXCC sequence (C=cysteine and X=any other amino acid) at the amino- (or carboxy-) terminus or an exposed surface loop. Upon addition of the fluorescein arsenical hairpin binder (FlAsH™-EDT$_2$, green star) labeling reagent, the engineered cysteine residues coordinate the arsenic, displacing ethane dithiol (EDT) and converting the dye to its fluorescent state. The figure displays FlAsH labeling of the cellular retinoic acid-binding protein I (CRABP I) carried out in the work by Ignatova and Gierasch [36], discussed in this review. The protein image was generated with the software Protein Explorer from the Protein Data Bank (PDB) file 1CBI [44].

generally cell membrane-permeable and it can be conveniently preloaded into cells before expression of the target protein. Remarkably, the ligand is virtually non-fluorescent until bound to its tetracysteine receptor. Binding takes place rapidly (with a half-life of ~1.5 minutes). Upon addition of the FlAsH reagent, some undesired background fluorescence is always generated *in vivo*. This is primarily due to the presence of endogenous cellular proteins displaying a weak affinity for FlAsH. These generally bear one or more cysteines at variable positions. The background signal is minimized by treating the cells with moderate amounts of ethane dithiol (EDT), thus ensuring that excess FlAsH is more likely to undergo exchange with EDT, rather than binding to endogenous cysteine-containing proteins*.

Since the original implementation, other FlAsH derivatives with complementary spectroscopic properties have been designed [14,15], including an analog with an environmentally sensitive fluorophore on the biarsenical ligand for *in vivo* imaging of protein-conformational changes [16]. Widespread applications of the FlAsH technology include selective labeling of cell-surface proteins, affinity purification [17] and direct in-gel detection of expressed proteins [14]. One of the modified biarsenical analogs, ReAsH, is both fluorescent and suitable for detection by electron microscopy [14], providing a powerful tool for correlating optical and electron microscopy images. FlAsH–tetracysteine labeling has been used to investigate several *in vivo* biochemical events. These include gap junction assembly and turnover [18], protein expression levels [19], protein function probed by selective *in situ* photoinactivation [20,21] and trafficking of an Ebola virus matrix protein in live cells [22]. In the specific case of *in vivo* protein misfolding kinetics, FlAsH-type labeling offers competitive advantages over GFP and other available fluorescence-based methods (see Table 1 for a comprehensive overview). The fluorogenic moiety is small and it is rapidly co- or post-translationally incorporated, provided that cells are preloaded with FlAsH-EDT$_2$ before target protein expression.

* The biarsenical FlAsH reagent is currently marketed by Invitrogen (http://www.invitrogen.com) under the Lumio™ trade name.

From inclusion bodies to Lewy bodies: protein misfolding in biotechnology and disease

Protein misfolding is a highly undesirable process in the context of living cells. In biotechnology and basic research, overexpression of target genes often generates macroscopic solid deposits known as inclusion bodies. These usually require laborious procedures for their conversion to soluble native-like active protein [23]. The generation of heavily misfolded proteinaceous aggregates is directly linked to the pathology of several human diseases. For example, nuclear inclusions, amyloid fibrils or Lewy bodies are generated intracellularly in the case of Huntington's, Alzheimer's and Parkinson's neurodegenerative disorders [24], respectively. *In vitro* studies monitoring the presence and formation of self-associated species have been instrumental in revealing fundamental aspects of this process. However, it is only through *in vivo* investigations that the actual cellular localization and biologically significant self-association mechanisms can be elucidated.

Several studies have been successful at mapping the distribution of macroscopic protein aggregates in the cell by imaging techniques, mostly based on fluorescence

Table 1. Available methods for tracking macromolecular behavior *in vivo* by genetically encoded site-specific fluorescence labeling

Reporter system	Nature of the modification to the target protein	Cell-permeable cofactor:		Low background fluorescence	Fast (≤ 5 min) apparent half-life for generation of fluorescent signal	No large fusion protein required	Refs
		Non-fluorescent until bound	Inherently fluorescent				
GFP (and analogs)	GFP tag (238 amino acids)	n.a	n.a		✓[a]		[8,10, 11,40]
DNA repair protein (hAGT)-O^6-benzylguanine-fluorophore	hAGT tag (207 amino acids)		✓	✓[b]	✓[c]		[41]
Oligohistidine-Ni^{2+}-nitrilotriacetate-fluorophore	10 amino acid tag		✓	✓[d]	✓	✓	[42]
FlAsH-tetracysteine	6 amino acid tag	✓		✓[e]	✓[f]	✓	[12,14]
Intein-generated N-terminal cysteine-thioester-fluorophore	Single cysteine tag		✓	✓[b]		✓	[43]

[a]Only for Venus and T-Sapphire variants.

[b]After cell washing.

[c]In CHO cells; fluorescent probes have low permeability in *E. coli* and yeast cells.

[d]Only a small excess of unbound fluorophore cofactor is present in the cell at equilibrium. Data are based on fluorescence emission and FRET experiments.

[e]With EDT treatment.

[f]Cofactor must be preloaded into cells before expression.

microscopy. For example, the senile plaques of Alzheimer's disease have been studied by multiphoton microscopy upon thioflavin S staining in live mice [25]. Details on intermolecular interactions among Parkinson's disease-related Lewy bodies have been obtained by *in vivo* detection of aggregates tagged by fluorophore-labeled secondary antibodies in brain tissues [26]. Rajan *et al.* have used GFP-based FRET combined with deconvolution microscopy to monitor the specificity of inclusion body formation in intact eukaryotic cells [27]. GFP-based studies monitoring fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP) and FRET have characterized the diffusive motions and interaction among polyglutamine–huntington protein (Htt) aggregates and other glutamine-rich proteins or the Hsp70 chaperone *in vivo* [28].

The above work has significantly contributed to our understanding of the distribution of aggregates *in vivo*. However, the fundamental reasons behind intracellular self-association are far from being understood, and more studies are urgently needed to explore the mechanistic aspects of this issue in a biologically relevant context. A first important step in this direction relies on improved methods to monitor *in vivo* aggregate formation in real time, including the early events that take place before macroscopic fibrils or other large complexes are generated. Previous work was based primarily on studies employing GFP as an aggregation reporter. Specifically, the time-course of aggregate appearance was monitored for various versions of the Htt-bearing C-terminal 'enhanced' green fluorescence protein (EGFP) tags [29]. The kinetics of polyglutamine aggregate formation has also been monitored in COS-7 cells upon expression of GFP-containing constructs reporting on the self-association of polyglutamine-containing atrophin-1 protein [30], whose misfolding is linked to dentatorubral-pallidoluysian atrophy (DRPLA), a severe neurodegenerative disorder. The above GFP-based reporters bear the disadvantage of acquiring fluorescence very slowly after biosynthesis, thereby masking the aggregation stages taking place during the initial one or two hours. Although early aggregation events might be captured with Venus- or T-Sapphire-like GFP-derivatives, the large size of the tag might interfere with the self-association process, and no studies of this type have been published to date. An alternative FlAsH-based method is described in the next section.

Watching protein misfolding in real time

In order to properly understand the mechanisms of protein misfolding in the cell and its implications for biotechnology and disease, it is necessary to follow the process from its initial steps. Early intermediates and small transient aggregates are believed to play important roles in the metabolism of the cell and to affect the ultimate outcome of self-assembly processes [31,32]. Therefore, observation of the protein aggregation time-course within a cell is best achieved by probes capable of tracking the full evolutionary history of a protein. This starts from the generation of ribosome-bound nascent polypeptides up to full-length chains and inclusion bodies, as illustrated in Figure 2.

The ability to follow aggregation from its early stages poses the most stringent technical challenges. As seen above, approaches based on GFP, its improved mutants and other GFP analogs from different organisms suffer from undesirable drawbacks. Alternative approaches based on radioactive labeling [33,34] or antibody tagging [35]

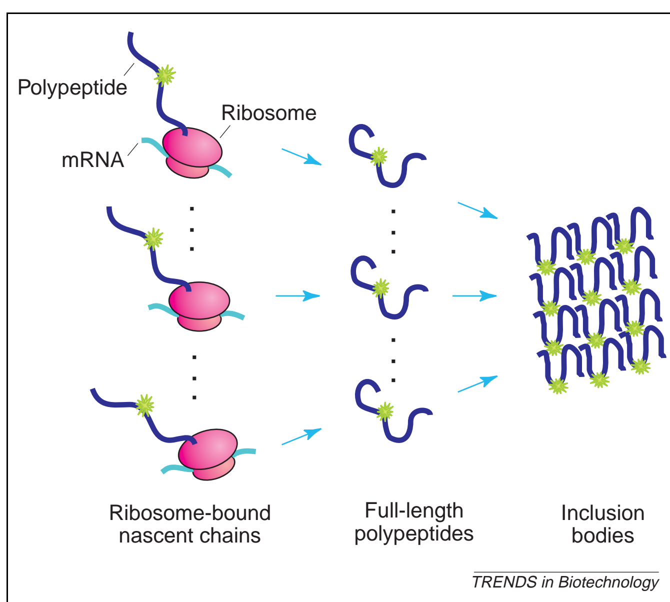

Figure 2. Schematic representation of *in vivo* inclusion body formation. Ribosomal protein biosynthesis leads to accumulation of full-length polypeptide chains. In some cases, the newly synthesized polypeptides do not properly fold to their native state, and form macroscopic aggregates known as inclusion bodies. Co- or post-translational incorporation of the FlAsH fluorescence reagent (green star) enables monitoring of the time course of *in vivo* protein synthesis and the subsequent inclusion body formation.

might perturb the cell's equilibrium because they involve the physical separation of cellular components after self-association has taken place.

Ignatova and Gierasch have taken advantage of the fast incorporation of the biarsenical FlAsH reagent into an engineered tetracysteine motif (Figure 1) to monitor the time-course of protein misfolding in *Escherichia coli* cells [36]. Two time points are shown in Figure 3. FlAsH-EDT_2 is only fluorescent upon binding to the tetracysteine motif, and unincorporated FlAsH makes a negligible contribution to background fluorescence. The FlAsH–tetracysteine acceptor sequence can, in principle, be appended to either the amino- or carboxy-terminus or inserted anywhere in the interior of the sequence of a protein, provided that no significant structural perturbations are introduced.

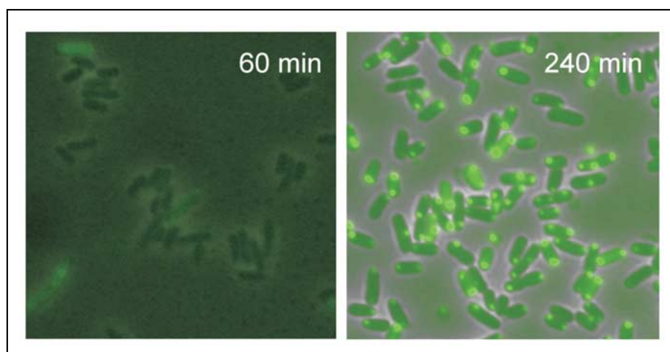

Figure 3. *In vivo* visualization of inclusion body formation in real time by fluorescence microscopy. Images of agarose-immobilized cell suspensions obtained 60 (left) and 240 (right) minutes after isopropyl-beta-D-thiogalactopyranoside (IPTG)-promoted P39A tetra-Cys CRABP I expression. The inclusion body fluorescence is due to FlAsH-EDT_2 probe incorporation. After 60 minutes, very small fluorescent aggregates are visible for only a few cells. By 240 minutes, the majority of the cells have accumulated a large number of highly fluorescent aggregates. Reproduced, with permission, from Ref. [36]. Copyright (2003) National Academy of Sciences, USA.

Insertion into α-helical secondary structure motifs has proven to be quite effective [12]. Ignatova and Gierasch showed that flexible internal loops are also good insertion sites for the FlAsH binding motif [36].

Given that the FlAsH-EDT_2 dye is only moderately cell permeable, intact cells had to be pretreated with moderate amounts of lysozyme and then washed, before isopropyl β-D-thiogalactoside (IPTG) induction. This procedure partially disrupts the peptidoglycan layer and it facilitates the intracellular incorporation of the biarsenical FlAsH-EDT_2 dye. The *E. coli* outer cell membrane recovers its original nature within one generation, once lysozyme has been removed. Cells pretreated by this method appear morphologically intact by fluorescence microscopy (Figure 3) and are as viable as untreated controls. The cellular retinoic acid binding protein I (CRABP I) is mostly composed of β-sheets and it usually expresses in soluble form. However, its P39A mutant forms inclusion bodies. This protein pair is therefore a good system for monitoring variations in the FlAsH fluorescence reporter as a function of protein aggregation. Self-association and inclusion body formation is monitored by both fluorescence microscopy imaging and fluorescence emission spectroscopy in bulk cell suspensions. Assuming that the biarsenical dye has been internalized at the time of protein synthesis initiation, and considering the fast time scale of FlAsH binding, the aggregation time-course is followed from its very early steps. The P39A mutant aggregates fast and to a much larger extent than wild-type CRABP I. Interestingly, this *in vivo* overexpressed mutant can be refolded to a native-like monomeric conformation under diluted *in vitro* conditions. However, this process occurs significantly more slowly than for the wild-type protein [37], suggesting that the extra time intrinsically required for P39A folding might enable kinetically competing misfolding processes to take over and dominate the conformational search *in vivo*.

A key issue in these studies is that the quantum yield of FlAsH-labeled CRABP I fluorescence depends on its conformation and chemical environment. For example, urea-unfolded FlAsH-labeled CRABP I has a significantly higher fluorescence than its native state. In whole cells, the fluorescence signal increases upon inclusion body formation. This suggests the possible presence of relatively unfolded pre-inclusion bodies or mature aggregates. This aspect of the work will undoubtedly be enriched by additional future investigations taking the quantum yields of individual species into account. The approach by Ignatova and Gierasch is simple and powerful, and holds promise to become routinely employed to follow protein self-association *in vivo*, starting from its early stages.

Challenges and future directions

Although the FlAsH technology holds great promise as a tool for the study of protein self-association kinetics in the cell, several potential drawbacks might somewhat limit its general applicability. For example, FlAsH-mediated fluorescence lacks a well-defined characteristic spectroscopic signature for conformationally distinct populations (i.e. the unfolded, folded and aggregated states), in that no significant emission frequency shifts are typically

observed for the different states. Fluorescence quantum yields vary in a somewhat unpredictable fashion. Therefore, careful parallel *in vitro* and *in vivo* control experiments are required to characterize the fluorescence contributions due to each state and the correlation between fluorescence response and aggregation-driven misfolding. Deconvolution of the observed *in vivo* fluorescence signal is straightforward only in the presence of a limited number of species bearing distinctly different fluorescence quantum yields.

Efficient FlAsH labeling requires the cysteine thiols to be present in reduced form, limiting the applicability of this method to reducing intracellular environments [13,14]. Therefore, FlAsH labeling of proteins targeted to organelles such as the endoplasmic reticulum and secretory vesicles, which support oxidizing conditions, is likely to be inefficient. In the presence of competing cysteine oxidation, undesired inter- or intramolecular disulfide bridges are formed. However, artificial alteration of local redox properties can be successfully applied to overcome the above complication, as demonstrated for the labeling of a vesicle-associated membrane protein in HeLa cells [14].

Another potential limitation of the FlAsH labeling system is the presence of background signal. Three types of background fluorescence are possible: nonspecific labeling of endogenous cysteines (discussed previously), nonspecific FlAsH binding to hydrophobic sites and cellular autofluorescence. In the majority of FlAsH-based studies, an acceptable signal-to-noise ratio is obtained by including EDT in the FlAsH labeling solution. This minimizes fluorescence due to nonspecific cysteine thiol labeling. However, in several cell lines, residual high background persists (e.g. in HEK 293 and CHO cells [38]). Thiol-independent background signal (due to FlAsH binding to hydrophobic protein and organelle pockets, as in ECV 304, HEK 293, CHO, 3T3 and 3T6 cells [13]) can be attenuated by the co-delivery of uncharged membrane-permeant dyes such as Disperse Blue 3 [13]. Although cellular autofluorescence and light scattering have not been cited as significant problems for the detection of FlAsH fluorescence, a red-shifted FlAsH analog such as ReAsH (608 nm emission maximum wavelength) reduces the potential for scattering-related background signal.

As discussed above, FlAsH delivery into *E. coli* is facilitated by increasing the bacterial cell wall permeability by lysozyme pretreatment [36]. However, FlAsH is readily membrane-permeable in a variety of eukaryotic cells [13], including *Drosophila* larvae [21], HeLa cells [12,13,18,20], Jurkat lymphocytes [12], skeletal muscle (dysgenic myotubes) [38], 293T cells [13,22] and yeast cells [39]. Although FlAsH treatment is typically carried out for 30 minutes up to several hours to enable complete cell permeation, dye preloading into cells reduces the experimental dead time and it enables fast labeling, to follow the early kinetics of intracellular events [36]. A creative experiment employing pulse-chase labeling of gap junction proteins with FlAsH and ReAsH dyes in HeLa cells suggests that the preloading strategy is also readily applicable to eukaryotic cells because protein expression continues effectively (for up to 8 hours) after initial FlAsH labeling [18]. FlAsH labeling has been extended to live dissected *Drosophila* larvae [21]. However, it is of limited utility in whole live organisms because the need for noninvasive dye delivery and removal of excess label prior to imaging poses serious challenges.

FlAsH-based technology promises to provide much needed insights into the kinetics of *in vivo* protein self-association, starting from its early stages. This is significant for both biotechnology and medicine because early aggregation stages are likely to be important in directing the fate of the newly synthesized cellular proteins. Sorting out and controlling the pathways leading to irreversible aggregation, degradation or rescuing by the many helpers available inside the cell (including chaperones) is one of the most exciting outstanding challenges in cell biology.

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