Silver ions as EM marker of congo red ligation sites in amyloids and amyloid-like aggregates

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Congo red (CR) is a known selective amyloid ligand. The focus of our work is identification (by EM imaging) of dye binding sites and their distribution in amyloids and amyloid-like aggregates formed in vitro. In order to produce the required contrast, CR has been indirectly combined with metal via including Titan yellow (TY) by intercalation which exhibits a relatively strong affinity for silver ions. The resulting combined ligand retains its ability to bind to proteins (which it owes to CR) and can easily be detected in EM studies thanks to TY. We have found, however, that in protein aggregates where unfolding is stabilized by aggregation and therefore is irreversible, TY alone may serve as both, the ligand and the metal carrier. The formation of ordered structures in amyloids was studied using IgG light chains with amyloidogenic properties, converted into amyloids by shaking. The resulting EM images were subjected to interpretation on the basis of the authors’ earlier research on the CR/light chain complexation process. Our results indicate that dimeric light chains, which are the subject of our study, produce amyloids or amyloid-like complexes with chain-like properties and strong helicalization tendencies. Cursory analysis suggests that the edge polypeptide loops belonging to unstable light chains form intermolecular bridges which promote creation of loose gel deposits, or are otherwise engaged in the swapping processes leading to higher structural ordering.

Key words: Congo red, Titan yellow, amyloids, supramolecular dyes, metal markers, light chain, amyloid-like aggregates, edge loop

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INTRODUCTION

Amyloids are protein aggregates which resist biological degradation mechanisms due to their structural ordering. The formation of such structures (often referred to as amyloidogenesis) has been the subject of numerous studies (Dealwis, 2004; Barrow et al., 1992; Kelly, 1997; Bross et al., 1999; Teng et al., 2012; Pellarin et al., 2010). Amyloid deposits are formed from unstable, partly unfolded proteins, but aggregation usually results in tight packing and the resulting structures become impervious to natural degradation. As can be expected, amyloid aggregates are chainlike structures consisting of interlinked proteins, which – by way of successive transformations – produce higher-order units. In vivo, amyloids form slowly and, much like crystals, exhibit a high degree of structural ordering, which is revealed by birefringence in polarized light. In contrast, amyloids (or amyloid-like aggregates) rapidly synthesized under laboratory conditions exhibit variable levels of ordering. Despite these differences, experimental work provides much insight into the intermediate stages of protein aggregation. Amyloidogenesis remains a complex issue and there is no consensus regarding its course and the overall structure of its products. Some of the observed insoluble aggregates do not qualify as amyloids under the generally accepted criteria, since they do not attain sufficient structural ordering. It seems that this issue merits further attention (Fink, 1998; Khurana et al., 2001; Helms et al., 1996; Maji et al., 2009).

As amyloids are not classic crystals and do not lend themselves to crystallographic analysis, EM remains the method of choice for studying their properties. However, applying EM in molecular research requires a suitable contrast and staining which can reveal the location of specific target structures. The study presented here relies on silver ions, introduced into an amyloid-specific ligand (CR) in order to reveal the distribution of sites binding the dye in the in vitro produced amyloid-like aggregates. CR is doped with silver in an indirect manner, i.e. by including TY which in turn binds Ag⁺ ions. The structure and properties of TY resemble those of CR; hence TY can either be used on its own or introduced into micellar CR through intercalation, yielding complex ligands. The work presented here is a continuation of our earlier research on labeling amyloid-specific ligands with metal ions (Chłopaś et al., 2015).

Amyloid-like aggregates are obtained through shaking – a process which exploits the amyloidogenic properties of IgG light chains isolated from urine of patients suffering from multiple myeloma. Immunoglobulin light chain L. Lambda dimers were used in this work as a material with well-known structural properties and amyloidogenic tendencies.

Conclusions from the analysis of ion-stained amyloid-like aggregates under electron microscopy are presented in the Results and the Discussion sections.

MATERIALS AND METHODS

Reagents. CR with dye content of 97% was purchased from Aldrich Chemical Company Inc. It was used without further purification. TY was obtained from

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BDH Chemicals Ltd – England. The true content of the silver binding dye in the commercial material was measured by titration with AgNO₃ at 407 nm based on TY: Ag⁺:1:1 molar ratio (Chłopaś et al., 2015). The presence of some inactive impurities was deemed to have no impact on the staining procedure.

Other reagents used were of analytical grade.

**Electron Microscopy.** Transmission electron micrographs were collected using the JEOL 2100 HT microscope. Typical nominal magnifications ranged from 30–75,000. Stained samples were deposited on Formvar 300-Mesh copper grids for analysis. Aggregates were analyzed without using negative staining. The introduced dye contrast was solely of silver origin.

**Light microscopy.** A Carl Zeiss Jena light microscope with polarization and fluorescence equipment was used for initial analyses preceding EM analysis.

**Dynamic light scattering.** LS – Dyna-Pro800 produced by Protein Solutions INC, with a measuring range of 0.5–50 nm was used to verify the supramolecular nature of CR and TY dyes.

**Other laboratory equipment.** Standard laboratory equipment was used to complete experimental studies.

**Proteins.** Immunoglobulin light chains were obtained from the urine of myeloma patients. They were salted out and, following intensive dialysis, further purified by molecular gel filtration.

Amyloid and amyloid-like aggregates were prepared by shaking at 40°C deg in 0.05 M acetate buffer, pH 5.2, or 0.05 M glycine/HNO₃ buffer, pH 2.15, using protein concentrations of 8–10 mg/ml. Aggregation was performed with or without dithiotreitol added at a concentration of 0.6 mg/ml.

The precipitate was washed with 0.3% KNO₃ or by 0.05 M Tris/HNO₃ buffer, pH=8.2. In selected cases, a 0.05 M acetate buffer, pH=5.2, was used instead.

**Light chains.** The Lambda light chains used in this work adopt a dimeric form. They originate from different patients and thus differ in terms of stability. This factor was evaluated on the basis of CR binding susceptibility, since CR is known to form complexes by penetrating into the destabilized protein domains. Complexation occurs at room temperature or may require suitable heating. The stability of V domains is greater in dimers due to interface interaction of the V domains. The purity of light chains was evaluated by analysis using agarose and polyacrilamide gel electrophoresis (without SDS) performed in a 0.06 M veronal buffer (pH=8.6) or 0.05 M Tris buffer (pH=8.2).

**Fibril formation by Beta-amylloid peptides.** 0.5 mg of amyloid Beta-peptides was solubilized in 100 µl H₂O. Subsequently, 30 µl of this solution was mixed with 30 µl of 0.5 M Tris/HNO₃ buffer (pH=7.35) and incubated over four days at 37°C.

**Staining of L chain (immunoglobulin) aggregates.** The silver-labeled stain for EM studies was prepared by forming the combined micellar structures composed of TY, Ag⁺ and CR, mixed at molar ratios of 1:1:1 or 1:1:0.5 respectively, with initial concentrations of TY equal to 4 mg/ml.

To obtain a proper dye combination, the mixture of TY and CR was heated for 20 min at 60°C and then cooled slowly to room temperature. Silver ions (AgNO₃) were added with stirring to form the complex. The concentration of dyes used for staining of protein precipitates was generally at the level of 2 mg of each dye per ml. The silver-containing stains (TY/Ag⁺/CR or TY/Ag⁺) were added to the aggregates and incubated for 30 min. The stained aggregates were then washed with 0.025 M Tris/HNO₃ buffer (pH=8.2) or, in some experiments, with 0.025 M acetate buffer (pH=5.2). This was done to verify the stability of the complex at different pH levels. The stained precipitate was suspended in water directly prior to the EM analysis. Silver-containing stains were kept in the dark before use.

To verify that silver added to dyes forms part of the complex, the stain and free silver ions were separated by agarose electrophoresis using 0.05 M Tris/HNO₃ buffer (pH=8.2) in order to verify that the silver ions migrate to the anode (together with dyes) and not to the cathode (as free ions). To localize the silver in the plate, agarose plates are placed in a solution of sodium dithionite to discolor CR spots and simultaneously reduce silver ions (Stopa et al., 2003). Reduction continues until CR spots disappear. TY is then transferred to the filter paper by blotting. Insoluble silver (if present) remains in the agarose as black remnants of dye spots. The control sample (AgNO₃) and the excess of silver ions in stained samples manifest as spots migrating toward the cathode. The reduction-mediated blackening of stained aggregates may also be observed under a light microscope.

**Models of aggregates.** Hypothetical models of aggregates were constructed on the basis of former studies regarding CR location in the V domains of immunoglobulin light chains (Stopa et al., 2003), as well as current experimental results.

**RESULTS**

**Silver-carrying EM stain for amyloids.**

The formation of ordered structures in the amyloidogenesis process protects peptide bonds from natural degradation. Such degradation is further inhibited by the tight packing of proteins in amyloid plaques. As a rule, amyloids consist of partly unfolded proteins. This means that parts of the protein molecule are not involved in the unfolding process. The aggregation mechanism is hence more specifically targeted and the final product is expected to be ordered rather than amorphous (Jiang et al., 2001; Mizobata et al., 1994; Quintas et al., 2001; Colon et al., 1992).

As can be expected, amyloid aggregates are chain-like structures which consist of protein monomers wound into higher-order structures. In this sense they resemble peptide chains; however instead of amino acids their building blocks are entire protein molecules.

Understanding of this problem enables us to study the onset and further progress of amyloid aggregation (Quintas et al., 2001; Colon et al., 1992). Our work is based on studying this process using electron microscopy (EM) as the principal analytical tool.

We trace the presence of amyloid aggregates using Congo red (CR) – a well-known selective amyloid ligand which helps to reveal the presence of its binding sites and therefore facilitate molecular studies of amyloids (Quintas et al., 2001; Colon et al., 1992; Inouye et al., 2000).

In order to achieve a suitable contrast for EM, the ligands have been doped with silver ions; however the metal is not directly bound to CR (which has poor complexation properties) – instead CR is intercalated as a complex with another, similar substance which can bind silver ions and readily associates with CR – Titan Yellow (TY) (King et al., 1967) (Fig. 1, Fig. 2). The supramolecular nature of both dyes and their ability to form
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TY’s ability to bind silver ions is due to its centrally located tri-azene group, attracting one ion per dye molecule (Chłopaś et al., 2015). The supramolecular ligand binds to proteins as a package consisting of several self-assembled molecules, resulting in local concentration of silver nanoparticles, recognizable under EM.

The TY/Ag\(^{+}\) binding strength is verified by testing the stability of the complex in the presence of anions which, when mixed with silver ions, form poorly soluble salts with a known solubility product constant. It was found that the TY/Ag\(^{+}\) complex forfeits its silver ion to iodates and thiols, but in the presence of Tris as an additional silver complexation component it survives chlorides even at a concentration of 0.15 M. Electrophoretic migration of the TY/Ag\(^{+}\) complex in 0.05 M Tris/HNO\(_3\) buffer (pH = 8.2) in the direction of the anode proves its stability under these conditions. It was found that the use of an acetate buffer (0.05 M; pH = 5.2) is also possible although the resulting complex is less stable.

The tri-azene bridge, centrally located in the TY molecule, seems essential for complexation of silver ions. Direct Yellow 28 (DY28), a similar dye, lacks such a bond and does not form stable complexes with Ag\(^{+}\).

The presence of complexed silver ions in the experimental sample can be easily determined by reduction with sodium dithionite, with the resultant darkening of samples serving as a direct evidence of the presence of silver.

TY is a supramolecular system with a ribbon-like structure, commonly assumed by symmetrical, planar molecules which exhibit polarity in their distal fragments and contain a nonpolar central fragment composed of...
aromatic rings (Fig. 1). Unlike CR, TY does not readily penetrate into the protein complexes. The formation of a complex requires irreversible aggregation-stabilized unfolding of the protein molecule – here, TY is outcompeted for access to the binding site by the trans-located polypeptide loop present in the reversible dynamic structure of the protein (Fig. 5). The polypeptide loop effectively occupies the binding locus in preference to TY (but not to CR) and causes removal of the dye from the complex.

Fortunately, the penetration abilities of TY are substantially improved when it forms a co-micellar structure with CR (Fig. 6) – although some aggregates which depend upon intrinsically stabilized unfolding of proteins may be tagged by TY alone in the EM studies.

**EM visualization of dye-silver-stained amyloid-like aggregates**

Loose strips of gel formed in vitro by IgG light chains, observed at the edges of more densely packed structures (where the gel is less dense, allowing penetration), easily bind the TY/Ag⁺ complex, revealing the molecular structure of aggregates. The silver-doped ligand can be detected as clusters of granularities, reflecting the distribution of dye particles in the gel (Fig. 7).

According to the common definition, an amyloid is a protein aggregate with a fibrillar structure which binds CR and exhibits birefringence in polarized light (Howie et al., 2009; Picken 2001). Amyloid-like gels formed by shaking at 45–50°C deg manifest themselves under light microscope imaging as veils, often lacking birefringence (although they retain their ability to bind CR). When the temperature drops below 35°C (and then further below 30°C) birefringent structures become more prevalent and many fibrillar structures begin to emerge (Fig. 8).

EM imaging reveals loosely packed amyloid-like structures as short chains of aggregates randomly strewn across the field of vision. Some of the longer chains are positioned in parallel with respect to one another, or form spirals (Fig. 9 – arrows). This suggests a degree of ordering which, in turn, may provide a starting point for the onset of birefringence. A typical higher-order structure is a circular shape comprising tightly wound spiral chains of aggregates (Fig. 10, 10A). The observed threadlike structures are formed from loosely bound grains of contrast. These can be assumed to correspond to light-chain molecules interlinked by exposed polypeptide loops. On the other hand, EM imaging also reveals other types of ordered
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Structural interpretation of dye silver stain binding by the L chain V domain

In an attempt to explain the observed aggregation mechanism at the molecular level, we refer to the V domain structure of the IgG light chain. This domain comprises two β-sheets linked by a disulfide bond, usually referred to as the upper core and the lower core, respectively (Fan et al., 1992; Ewert et al., 2004) (Fig. 11). Loops which form the lower core are additionally stabilized in a dimeric form via interactions with the V domain of the complementary chain. Amyloid aggregation is therefore mostly due to polypeptide chains forming the upper core. The relative instability of the upper core is also due to the presence of the N-terminal fragment which, being a terminal fragment, is unstable by nature and easily displaced from its packing locus. As has been revealed in our study, displacement of the N-terminal fragment creates a cavity which the supramolecular CR ligand eagerly occupies, preventing the domain from reverting to its initial, native form (Piekarska et al., 2001).

Figure 11 shows a schematic diagram of the V domain with β twists forming the upper core represented by black arrows. Displacement of the N-terminal fragment from its native locus (e.g. under heightened temperature) destabilizes the upper core, strongly affecting the edge loop (D–E) which may hence protrude from the domain (Yang et al., 2003; Sinha et al., 2001). As expected, mutual association of the exposed D-E loops and formation of β-bridges produces a chain-like aggregate (Fig. 11D). The resultant link comprises two D–E loops, i.e. four polypeptide chains, with preserved β conformation.

The involvement of the N-terminal fragment in the β-bridge is unclear as its participation would seemingly promote the formation of a six-chain bridge.

Displacement of the polypeptide loops and their involvement in aggregation creates gaps, which are further occupied by supramolecular ligands (Fig. 11) (Piekarska et al., 2001; Jagusiak et al., 2014; Król et al., 2005). The dyes penetrate and bind in between β-structural polypeptide chains (Roterman et al., 1998). Our previous studies confirm that CR attaches to the V domain of the light chain. The four-molecule dye ligand engages the domain once the N-terminal fragment has been displaced. The resulting complex is found to be slow-moving in electrophoretic studies and will be referred to as such. Under favorable conditions (higher temperature; higher CR concentration) four additional CR molecules may attach to the complex, resulting in a fast-moving fraction (Fig. 5; Fig. 6) (Piekarska et al., 2001; Jagusiak et al., 2014). This complexation progresses in a stepwise fashion and with limited efficiency as it requires displacement of the C–D and E–F loops, which is more troublesome than in the case of the N-terminal fragment. The overall process produces a streak-like pattern on...
the electrophoretic plate. It seems that the complex may bind ligands consisting of five, six, seven or eight dye molecules (Pickarska et al., 2001; Jagusiak et al., 2014; Król et al., 2005).

Swapping as the hypothetical mechanism of the ordered L chain aggregation

Aggregates formed by swapping may prove unsuitable for binding supramolecular ligands in their basic form, since their domains are often fully packed. This conclusion is supported by detection of nonbinding or weakly binding CR amyloid-like structures in the aggregation products (Fig. 12) (Wood et al., 1996; Heegaard et al., 2000; Liu et al., 1998). The coupling of V domain chains by swapping is illustrated in Fig. 13.

The relative structural freedom of amyloid-like aggregates and their variable binding mechanisms (formation of bridges and/or swapping) indicate that the phenomenon is quite complex and requires an in-depth analysis. Additionally, the means by which aggregates bind the CR molecules is unclear and has been the focus of varying interpretations (Frid et al., 2007; Turnell et al., 1992; Jin et al., 2003; Elhaddaoui et al., 1995; Klunk et al., 1999; Kim et al., 2003). The proposed use of metal ions as a contrast carried by protein ligands may shed more light on this important matter.

Figure 14 presents the use of dye-silver staining (CR, TY, Ag⁺) to reveal the standard dye-amyloid complex in the case of amyloid β-peptide derived fibrils (Wu et al., 2012; Lu et al., 2013; Serpel, 2000; Tycko, 2004). Periodic distribution of contrast density along fibrils suggests a twisted ribbon structure (Pedersen et al., 2010; Petkova et al., 2006; Bose et al., 2010; Lendel et al., 2010; Lührs et al., 2005). The formation of amyloids from peptides differs from the previously discussed light-chain aggregation process as there is no protein ballast; however the underlying self-organization mechanism and the resulting structure appear to be similar.

The periodicity observed in fibrils suggests a twisted ribbon-like nature of amyloid threads (Petkova et al., 2006; Bose et al., 2010; Lendel et al., 2010; Lührs et al., 2005; Skowronek et al., 1998). Coating of fibrils by silver may be interpreted as adhesion of the supramolecular dye (carrying silver ions) to the amyloid fibril ribbon. Contrast is provided by precipitated silver following reduction of silver ions under EM, revealing the structural characteristics discussed above.

DISCUSSION

In our research, we have attempted to analyze the formation of ordered structures in the process of amyloidogenesis based on the distribution of specific amyloid ligands which have been imbued with contrast properties by intercalation of Ag⁺ ions. Our ligands include supramolecular dyes — CR and/or TY (Skowronek et al., 1998; Iconomidou et al., 2003; Stopa et al., 1998). Both dyes, when mixed together, are capable of selective binding to amyloid-like aggregates and the distribution of such combined ligands can be studied under EM thanks to the silver’s contrast properties.

Metal ion complexes have many applications in diagnostics and therapy. Complexation of metal ions increases their solubility while also reduces their toxicity. In this work, however, silver ions are used for an entirely different purpose: to reveal the distribution of Congo red in amyloid aggregates under electron microscopy, even though the dye itself does not readily form complexes with this metal. In order to overcome this difficulty, we have created a supramolecular ligand consisting of two distinct organic compounds: Congo red and Titan yellow, the latter of which exhibits strong affinity for silver ions. Both dyes must combine to form a ligand, but the presence of Congo red is arguably more important since it is a well-known amyloid ligand and its distribution is the principal focus of EM analysis. The silver-binding component (TY) should not appreciably alter the supramolecular structure of Congo red and should not interfere with its ability to bind to proteins.

TY appears to fulfill the above conditions. The TY molecule is structurally similar to Congo red and it forms similar supramolecular structures (although its complexes with proteins appear to be less stable in the absence of Congo red). Both dyes readily form combined micellar structures. It appears that combination of both dyes does not alter the specific complexation profile of Congo red and the aggregate supramolecular dye represents a useful means of revealing the structure of amyloids under electron microscopy.

Even though CR is widely known for its selective affinity for amyloids, its binding mechanism has not been unequivocally elucidated (Wu et al., 2007; Demeule et al., 2007; Khurana et al., 2001; Pollack et al., 1995). Controversy surrounds the means of complexation of individual
CR molecules which bind to β-pleated sheets, as well as the penetration of self-assembled molecules as protein ligands into unstable β areas (Wu et al., 2007; Demeule et al., 2007; Khurana et al., 2001; Pollack et al., 1995). Adhesion appears to play an important role in this process, since – in the presence of water – CR forms long ribbon-like chains with large nonpolar surfaces, shaped by associated aromatic rings of the central part of the dye molecule (Woodcock et al., 1995). Ultimately, however, the specific mechanisms of amyloid formation and Congo red binding remain unclear and continue to attract the researchers’ attention (Barrow et al., 1992; Kelly 1997; Jiang et al., 2001; Qin et al., 2007).

In the study presented here, amyloid and amyloid-like structures were obtained from IgG light chains via shaking. Light chains were selected as the study subject due to their well-known structural properties, ability to bind CR and overall clinical importance (Spólnik et al., 2004). Standard amyloid fibrils were obtained using commercial 1–40 amyloid β-peptides.

Displacement of polypeptide chain fragments in the unstable V domain creates suitable conditions for supramolecular ligands to penetrate in between polypeptide chains of β-pleated sheets where they can anchor themselves with their nonpolar fragments, while the distal polar groups protrude outside the protein (Roterman et al., 1998).

Increased temperature destabilizes the IgG light chains which form two types of complexes with supramolecular CR ligands. Such complexes are characterized by variable migration speed under electrophoresis and comprise ligands with either 4 or 5–8 dye molecules. These fractions are referred to as “slow-moving” and “fast-moving” respectively (Fig. 5; Fig. 6) (Piekarska et al., 2001). The slow-moving complex forms easily through displacement of the N-terminal fragment from its packing locus, which is instead occupied by a 4-molecule CR ligand. Formation of the fast-moving complex occurs with greater difficulty (Piekarska et al., 2001) and only when CR is used as the ligand. TY alone seems to be unable to form such complexes; however, the slow-moving complex may involve TY without any participation of CR, but only under unfolding conditions which induce oligomerization (60 deg C; 10 min – see Fig. 5), ensuring permanent displacement of the domain loops. The complex formed by slightly destabilized IgG light chains and CR/TY ligands are soluble and do not – by themselves – form aggregates. Instead, aggregation requires further destabilization and sufficient exposure of polypeptide loops, which can be achieved by shaking and/or reduction of disulfide bonds. Unstable domains become susceptible to penetration by supramolecular ligands.

Reduction with sodium dithionite reveals the presence of silver (via darkening), both in the electrophoretic spots and in the TY-stained deposits. This serves as proof that silver ions are present in the sample.

Somewhat unexpectedly, microscopic imaging of amyloid-like aggregates reveals aggregates which do not bind CR (or bind it poorly) but retain birefringence under polarized light, suggesting a high degree of ordering. This phenomenon may be explained by assuming that complexation of light chain molecules occurs by way of inter-molecular swapping of edge loops (D–E). Such aggregation may produce densely packed polypeptide clusters which do not offer suitable cavities for CR ligands (Fig. 13) The structural freedom and elasticity of the resulting aggregates is limited by their high degree of structural ordering. Tight spiral winding of chains is therefore very difficult (or outright impossible). Nevertheless, under favorable conditions (destabilization), the aggregates remain capable of interaction with CR (Churukian, 2000; Lakdawala et al., 2002). This is due to the fact that not all fragments of the target polypeptide chain are equally stable (Tycko, 2004), providing attachment points for dyes even in aggregates formed by swapping – especially under unfolding conditions, as observed in staining studies. CR may also bind to proteins by means of adhesion due to its ribbon-like supramolecular structure presenting a large non-polar surface which may attach to fibrillar protein aggregates.

The instability of myeloma-derived light chains presents an important study subject due to their propensity to form amyloid deposits in kidneys (Schormann et al., 1995; del Pozo et al., 2008; Hurle et al., 1994; Wall et al., 1999). Assessing the degree of such instability and prediction of danger is possible on the basis of the so-called fuzzy oil drop (FOD) model (Banach et al., 2014), which determines the involvement of polypeptide chain fragment in the formation of an ordered, polar protein. The FOD model expresses the degree of participation of individual polypeptide chain fragments (especially secondary folds) in a common hydrophobic core structure. As a result, the FOD model can be used to compute the relative stability of each fragment.

Accurate prediction of amyloidogenesis would be of fundamental importance in clinical practice. Nevertheless, the problem remains a challenging one and further studies are required to formulate a complete theoretical model with therapeutic applications.

CONCLUSIONS

The specific ordered structure of amyloids renders them resistant to natural degradation mechanisms. A thorough study of the reasons and mechanisms behind the formation of such structures is a necessary step towards understanding the phenomenon of amyloidogenesis.

In order to analyze the formation of amyloids and amyloid-like aggregates using EM we have applied specific amyloid ligands, consisting of CR and TY molecules tagged with silver ions, as a contrast medium. CR and TY are capable of associating with each other and, together, form complexes with light chain aggregates (although, under favorable conditions, each dye remains capable of complexation on its own). Unlike CR, TY can bind silver ions strongly enough to survive complexation with proteins. CR serves as a carrier, enhancing the penetration capabilities of TY.

Preliminary studies using the above mentioned technique lead to the conclusion that aggregation of the L chains (IgG) is strongly mediated by the edge loop of the V domain (60–75 aa). This loop can form intermolecular β-bridges by association with its counterpart from the V domain of the adjacent L chain, producing thread-like aggregates, or participate in swapping, which leads to rigid higher-order structures. The resultant domains may, under favorable conditions, be fully packed and become impenetrable to CR, much like native domains.

According to our research, supramolecular dyes bind to aggregates either by occupying sites which have been previously vacated by dislocated peptide chain fragments (in unstable domains), or adhere as supramolecular ribbon-like micellar forms to ordered fibrillar amyloid structures.

The distribution of silver-derived stains in amyloid structures produced by light chains and/or amyloid-β
peptides indicates their general tendency to form twisting chainlike structures.

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