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The use of Titan yellow dye as a metal ion binding marker for studies on the formation of specific complexes by supramolecular Congo red

Abstract: Congo red (CR) and other self-assembling compounds creating supramolecular structures of rod- or ribbon-like architecture form specific complexes with cellulose and also with many proteins, including antibodies bound to the antigen and amyloids in particular. The mechanism of complexation and structure of these complexes are still poorly recognized despite the importance of the problem for medicine. This work proposes the progress in electron microscopy studies of amyloid-dye complexes by labeling supramolecular ligand CR with silver ions as a marker. Silver ions are introduced to CR carried by the strongly binding silver dye Titan yellow, which in addition form comicellar structures with CR. Silver carried by self-assembled dye molecules forms in the resulting metal nanoparticles, making the specific amyloid ligand CR perceptible in EM studies.

Keywords: amyloids; Congo red; dye/metal nanoparticles; silver ion complexation; Titan yellow.

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Introduction

Due to its specific interactions with a variety of proteins, Congo red (CR) constitutes an interesting research subject. This dye can be viewed as representative of a whole group of compounds with attractive scientific and therapeutic properties [1–4]. CR has been known for many years as a specific cellulose and amyloid binding dye [5–9].

The major difference between CR and commonly used pharmaceuticals involves its supramolecular properties that determine its interaction capabilities. The amazing ease with which CR molecules bind to one another, producing a relatively stable supramolecular structure, means that it also interacts with proteins as a supramolecular ligand [10, 11]. In this form, CR does not bind to the protein's active site unlike the vast majority of monomolecular ligands (including most drugs). Rather, the dye seeks out areas of relative structural instability, preferentially anchoring itself to the β-structure [12–16]. The stability of CR/protein complexes is ensured due to the ribbon-like structure of this supramolecular dye (ensuring exposure of nonpolar components) as well as its great plasticity, which is a result of the noncovalent interaction between individual monomers. By accommodating distortions and adjusting its own structure to that of the target protein, CR can easily bind to partly unfolded polypeptide chains but also to a wide range of native proteins that are characterized by instabilities (usually related to their biological function) [17, 18]. In many proteins, local structural instabilities are transient in nature and associated with function – the requirement to bind a natural ligand such as a substrate (in the case of enzymes) or antigen (in the case of antibodies) [19, 20]. The complexation of a supramolecular ligand (triggered by structural changes) renders the corresponding biological process irreversible. As a result, transient phenomena, such as enzymatic action, are arrested, but processes that lead to the formation of persistent structures are up-regulated – this includes, for example, the formation of immune complexes [21, 22].

Clearly, supramolecular ligands may constitute a valuable tool in biological research and clinical studies.

The supramolecular character of CR means that its complexes are notoriously difficult to analyze because the nonuniform nature of the ligand prevents crystallization. Electron microscopy (EM) is often also incapable of resolving protein/CR complexes due to the relative similarity of the dye and its target protein (in terms of electron density). Thus, many theories regarding the interaction of CR with amyloids and other proteins remain within the realm of speculation [23–28]. This work focuses on the possibility of enriching CR with metal ions, providing a suitable contrast for EM studies. Although pure CR does not readily bind metal ions, such ions may be introduced by means of a carrier molecule that intercalates itself in the CR micelle. The best candidates for this purpose seem to be planar organic molecules composed of benzene rings and groups capable of binding metal ions. From among a large number of compounds that were taken into account, we have selected Titan yellow (TY) as the carrier. TY reactivity toward magnesium has been known for many years [29, 30]. However, its structure with azo-bridge connecting symmetrical halves of the molecule convincingly suggests the larger possible metal complexation activity, including silver in particular. Hence, the silver ions were chosen first as the metal marker and used for study.

This work presents the properties of the resulting complexes and their potential applications.

Materials and methods

Reagents

TY was purchased from BDH (Bristol, UK) and CR was purchased from Sigma-Aldrich Comp (Millwaukee, USA). Other reagents used were of analytical grade.

Electrophoretic analysis

For some experimental purposes, TY was purified by chromatography in a solvent composed of chloroform, acetic acid, and water (5:1:1), with methyl alcohol added to ensure clarity. The effect of the combination and properties of combined dye moieties were studied by agarose gel electrophoresis using Tris-HNO_3 buffer (pH 8.2) as well as spectrophotometrically. Standard molar dye relation used in the experiments was in the range of 1.33:2.24 (TY/CR) and 1:1–2:1 (silver ions/TY).

Dye-protein complexation

Protein-dye complexation was performed under unfolding conditions (heating to 63 °C for 20 min). Human lyophilized immunoglobulin (Baxter Health Care Corp. USA) was used. The effect was tested by measuring the loss of the ability to bind to the bio-gel P10. The analysis was performed using small columns (Bio-Rad Bio-Spin Disposable Chromatography columns) filled with gel and centrifuged. The dye emerging from the columns was analyzed spectrophotometrically and by electrophoresis on agarose gel plates. The binding of silver ions to TY was studied spectrophotometrically at the 407 nm wavelength.

EM tests

The idea of possible silver ion introduction to CR binding objects was checked using cellulose powder (Whatman) in the form of its tiny fragments selected for staining and EM studies by sedimentation fractionation. For this aim, cellulose was incubated with the TY solution and, after washing with water or Tris-HNO_3 buffer, submitted to complexation with Ag^+ ions ($AgNO_3$). Depending on the conditions used, silver-marked dye nanoparticles may have in EM images different sizes collecting more or less self-assembled dye molecules. The careful washing of stained material was used to remove the excess of dye molecules from supramolecular ligands seen as nanoparticles. High-resolution scanning microscopy JEOL JSM-7500F with an energy-dispersive X-ray spectroscopy (EDS)-INCA PentaFETx3 accessory was used in the EM tests.

Complexation of lead ions (Pb^{2+})

The complexation of TY and CR with lead ions (Pb^{2+}) was studied by spectrophotometric analysis and specific staining after electrophoretic separation using H_2S atmosphere after short exposure to acetic acid vapor.

Results

Properties of the TY dye

Much like CR, TY is a flat, symmetrical molecule composed of outlying polar groups and nonpolar benzene rings in its central part favoring the formation of supramolecular structures similar to that of CR (Figure 1). Visible spectra

of both dyes are presented in Figure 2. The structural characteristics of these dyes permit self-assembling and the expected co-association (Figure 3). Yet, despite structural similarities, TY has different functional characteristics than CR. For example, TY exhibits a much greater ability to bind metal ions due to the presence of a thiazole ring but particularly due to the presence of a peculiar triazo-bridge that links both halves of the molecule. Self-association and the formation of supramolecular structures of TY is, however, to some measure less pronounced than in the case of CR. The evidence of this is provided by the somewhat poorer adhesion of TY to different surfaces, adsorption on biogels, and binding to cellulose (a property that is mediated by the formation of ribbon-like micellar structures with the exposed nonpolar moieties). Supramolecularity-derived properties may, however, be strengthened by complexation with CR and the formation of comicellar structures.

Complexation of silver ions by TY

The proof of strong complexation of silver ions is furnished by a change in the absorption spectrum of TY following the combination of both substances (Figure 4).

Such a vivid spectral change permits the titration of TY with a silver nitrate solution, revealing the dye-metal complex (Figure 5). The shape of the presented curve unequivocally indicates a rather strong complexation. One silver ion bound to one TY molecule was found in the result. Lower than the predicted value obtained by titration is the result of impurities (approximately 20%) difficult to be removed. The single silver ion binding to the TY molecule clearly indicates on azo-bridge the complexation locus in the molecule; it fits well to the standard silver ion complexation specificity.

CR is also able to bind silver ions, but the resulting complexes are relatively unstable and tend to dissociate under electrophoresis unlike the complexes formed by TY.

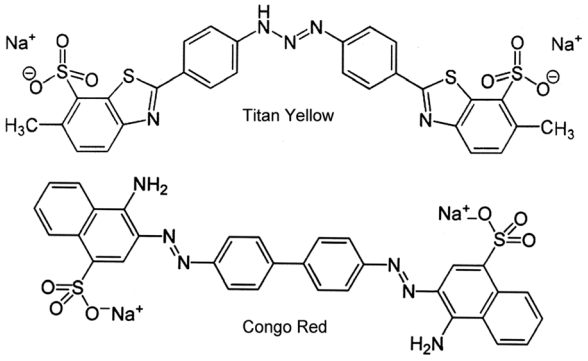

Figure 1: Structural formulas of TY and CR.

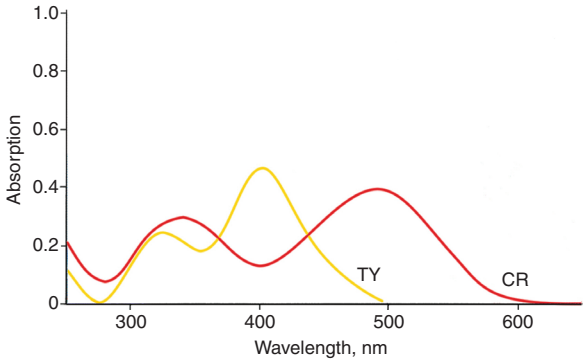

Figure 2: Spectra of CR and TY.

The ability of TY and CR to form complexes with each other enables us in the effect to embed silver ions in CR micelles. This phenomenon beyond the spectral effects is clearly evidenced by the change in CR migration properties under electrophoresis (specifically accelerated migration, as seen on Figure 6). On the contrary, binding silver ions slows down the corresponding migration of the CR/TY complex, which again proves the existence and stability of the dye-metal association. The direct evidence of strong silver ion binding to TY was obtained using sodium dithionite to reduce silver ions and CR separated by electrophoresis dye/silver complexes performed to reveal the presence of metal atoms. The reduction turns silver ions to their metal form seen as the development of the black

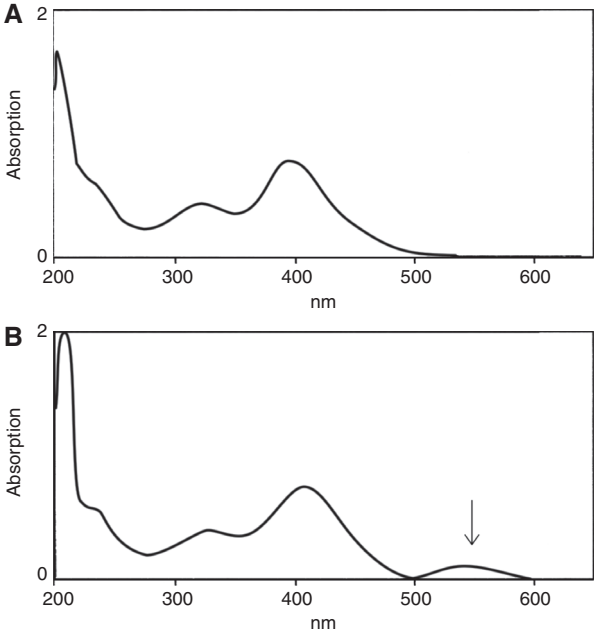

Figure 3: Spectrum of TY (A) and differential spectrum of CR and TY complex versus CR exposing a new band specific for the complex of dyes (arrow) (B).

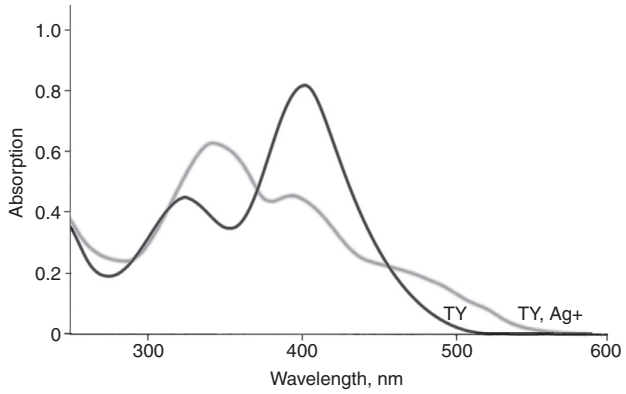

Figure 4: Spectral effect of silver ion complexation by TY.

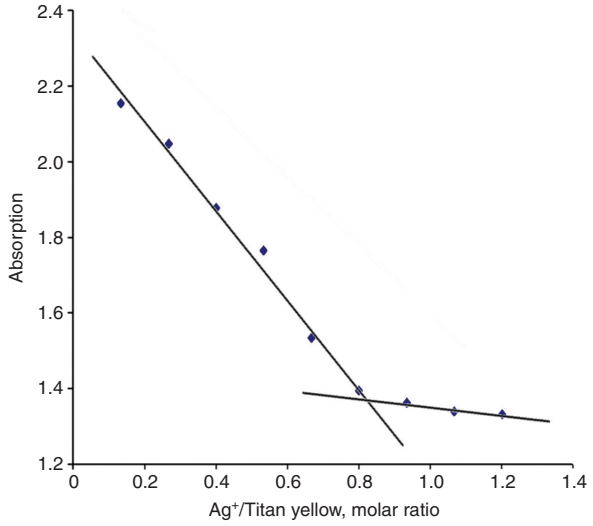

Figure 5: Titration curve – the evidence of strong TY and silver ion complexation measured at 407 nm wavelength.

color while it simultaneously causes CR to disappear as the stain. The reduction effect, as revealed in Figure 7A and B, is the resulting black color developed in the electrophoretic spots of dyes migrating to the anode and carrying complexed silver.

The reduction that also makes CR sports independently disappear confirmed that TY binds silver ions with strength sufficient to survive the electrophoretic separation. In contrast, no silver migrating with CR in the absence of TY was observed in the electrophoresis (Figure 7B).

The excess, free silver ions migrating to the cathode are seen below the starting line in Figure 7B as black spots of different intensity, confirming again the complexation of dyes.

To practically validate our approach, we have introduced the presented complex (consisting of CR, TY, and silver ions) into a partly unfolded form by heating a protein (human IgG). The complexation was further assisted by ultrasound. The resulting protein complex was introduced to the column filled with the bio-gel P10, which eagerly binds uncomplexed CR adsorbing it without affecting proteins. In control samples, pure dyes (TY and CR) were also treated using the same method, separately as well as in complex with each other. The full complex (IgG-CR-TY-Ag^+) passed through the column unhindered (Figure 8A), whereas pure dye silver complex deprived of protein was entirely adsorbed (Figure 8B). TY itself, with its somewhat poorer adhesion versus CR properties, was able to slowly penetrate the column and could be detected in the analyte. However, when TY was bound to CR, it too remained within the column, further confirming the strong mutual affinity of both dyes and validating our choice of CR/TY complexes as a uniform ligand.

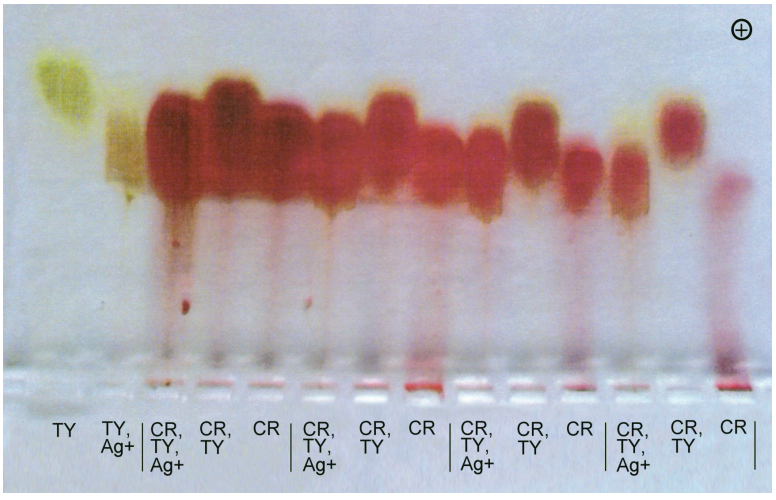

Figure 6: CR migration rate accelerated upon complexation of TY and CR/TY complex migration rate reduced by adjoining silver ions. Evidence of CR and TY mutual complexation as well as silver binding.

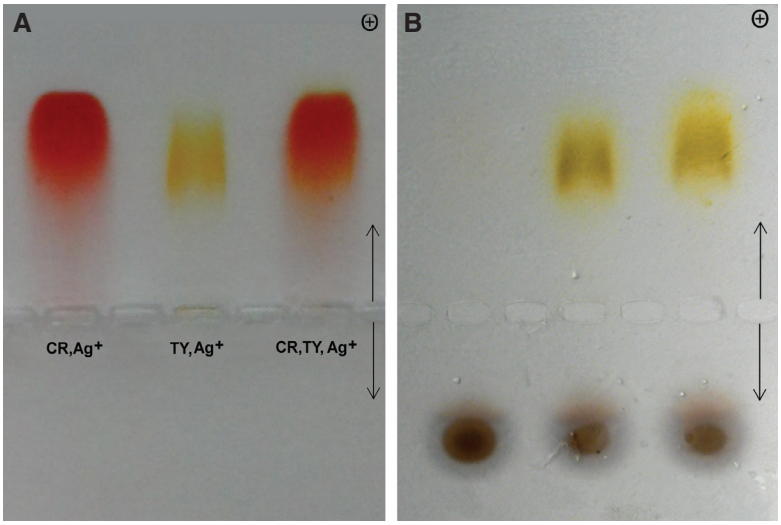

Figure 7: Evidence of possible CR labeling (for EM studies) using silver carried by TY introduced as a guest dye to supramolecular CR and revealed by reduction.
(A) Before reduction. (B) After reduction. Black color in the electrophoretic spots of dyes migrating to anode developed by reduction using sodium dithionite is an evidence of silver present in the complex. Excess of free silver ions migrating to the cathode revealed as black spots below the starting line and the disappearance of CR spots are also both the result of strong reduction.

The idea of using silver as a specific CR contrast for EM studies was initially verified by staining cellulose with TY/silver complex as well as with TY/CR complex. Cellulose rather than amyloid was chosen for initial tests, as it is a well-known CR as well as a TY binding material of more predictable structure than amyloids. In nonswollen and crumbled cellulose, only selected fragments appear susceptible for dye adherence. Binding indicates that supramolecular ligand attaches as nanoparticles to exposed cellulose threads and not to well-packed fragments. The spots seen in the EM pictures of TY/silver-stained cellulose represent, as may be expected, nanoparticles formed by the binding to cellulose thread micellar pieces of the dye composed of several or more associated dye molecules each one carrying a silver ion (Figure 9).

Finally, dye-silver complex appears promising as a specific marker for use in amyloid and immune-complex EM studies. Hence, CR and TY supplement each other in constituting the joint silver-labeled supramolecular specific ligand of proteins but immune complexes and amyloids in particular.

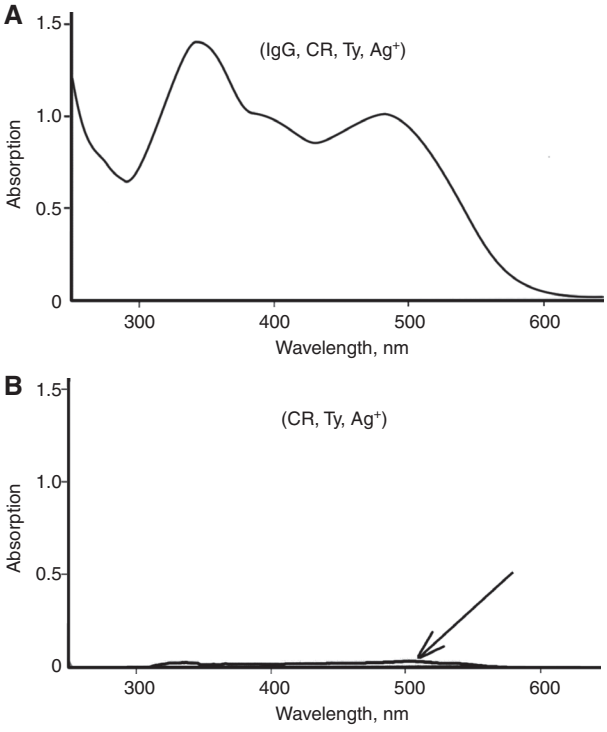

Figure 8: Evidence of binding combined dyes and silver (CR-TY-Ag^+) complex by protein (partly unfolded IgG) revealed using bio-gel P-10 as the material strongly adsorbing free dyes while allowing them to pass and not hindered through the column in the complex with protein.
(A) Spectrum carried by protein dyes and silver complex emerging from the bio-gel column. (B) Absence of dyes in the effluent introduced to the column without protein.

Lead complexation

The lead ion, being twice as massive as its silver counterpart, constitutes a more suitable contrast in EM studies. Hence, lead was also proposed in our work as the EM

marker of CR. No spectral changes similar to that of silver were, however, observed upon complexation studies, indicating that the complexation of TY and Pb^{2+} ions is poor or its mechanism is at least different. The electrophoretic test also appeared negative. Surprisingly, the complexation property of CR itself was found sufficient to bind lead ions after all. Binding efficiency seems to increase with the concentration of CR, indicating that the supramolecular structure, and perhaps its ribbon-like architecture, affects complexation (Figure 10). Hence, the mechanism of complexation looks interesting. Future experiments will bring more details.

Discussion

Amyloids have long been the subject of scientific research and are well known for their ability to specifically bind CR. This has clear implications for diagnostics as well

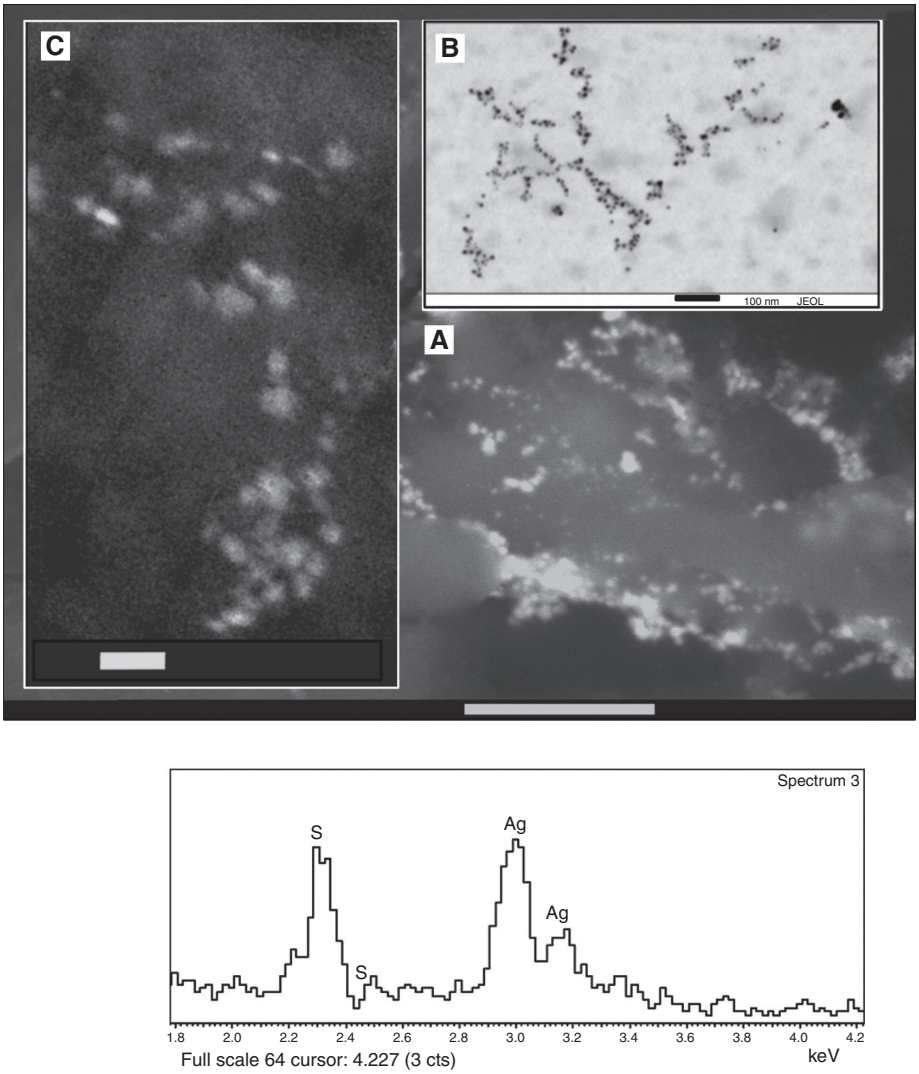

Figure 9: EM images made for the initial verification of the proposed technique designed to introduce silver ions to CR binding materials (amyloid and cellulose) as the contrast for EM studies and using the supramolecular dye TY itself or in complex with CR as the specific carriage.
(A) Silver ions introduced to cellulose (Whatman powder) by TY. EM/backscattered electron technique. (B) Silver ions introduced by TY to crumbled and swollen cellulose by heating tiny threads of cellulose obtained by sedimentation fractionation. EM transmission technique. (C) Silver ions introduced to cellulose by TY/CR complex. Large particle size seen in the image may likely result from the high self-assembling tendency of carriage dyes and/or insufficient washing intensity. Bottom, evidence of silver present in the studied material (EM-EDS detector) [31, 32].

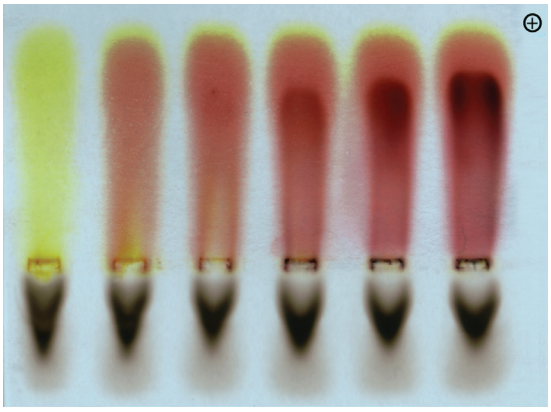

Figure 10: Evidence of lead complexation by dyes (CR in particular) used for agarose electrophoresis.
A new electrophoretic fraction in lead-containing sample revealed first by hampered blotting of dye excess to filter paper B (arrow) and then emerged as the black-stained fraction B′ (arrow) after exposure to the dried electrophoretic plate to H_2S/acetic acid atmosphere.
A and A′ control samples (dyes without lead). Below starting line: excess of lead (Pb^{2+}) migrating to cathode.

as therapy of amyloidogenic diseases. Unfortunately, however, neither amyloids themselves nor their complexes with CR undergo crystallization due to their lack of structural uniformity. EM can potentially address this issue as long as a method is devised to enhance the detection of CR (the electron density of which approximates that of the target proteins). This can be affected by embedding metal ions in CR micelles. CR itself exhibits, however, rather poor affinity for metal ion complexation, including silver ion that seems attractive for the designed purpose in particular.

An alternative solution is to attach the ions to other high-affinity organic compounds that can then form stable complexes with CR producing a uniform comicellar structure.

The above criteria are met by the dye (TY). Our work presents the basic properties of this substance and characterizes its complexation property. The quantitative analysis of the complexation properties of TY is hampered, however, by the presence of some derivatives that are difficult to separate from the target compound due to structural similarities and the supramolecular nature of the target itself [29, 30]. Derivatives are produced by the structural modification of the dye molecule or its cleavage. Fortunately, however, derivatives are deprived of silver complexation capability and do not exhibit an absorption band of 407 nm, which is a characteristic property of TY. As the result, this spectral band may be used to identify unmodified dye molecules; hence, the presence of derivatives does not preclude its application in EM studies.

A preliminary verification of the elaborated method designed to use silver in the complex with supramolecular dyes as the EM contrast was done by staining cellulose playing a role of the specific target for TY or TY+CR. However, TY without CR was basically used in the initial tests to simplify the interpretation.

The grainy character of the contrast seen in EM images (Figure 9) indicates that forming grain nanoparticles composed of self-assembled dye molecules carrying the silver bind to recurrent acceptor sites of cellulose; hence, they are attached to cellulose threads showing linear arrangement. Self-assembling dyes that form supramolecular structures of ribbon-like architecture similar to CR easily adhere to fibrillar materials including cellulose. The significant improvement of EM technique allows now for better nanoparticle imaging, particularly in the study of metal or metal-containing materials [33, 34]. Here, silver was used to form metal nanoparticles specific for biological targets due to the combination with structures determining affinity for supramolecular ligands. A different size of particles seen in images likely depends on the number of particles carrying the silver self-assembled dye molecules participating directly or indirectly in the complex with target sites; it may be reduced by controlled washing to remove the excess of indirectly bound dye molecules. Future studies will involve the optimization of the procedure.

No clear complexation of TY to lead ions that we attempted to use as CR contrast in a way similar to silver ions was, however, observed, again confirming by the way that the azo-bridge in the TY molecule is the specific acceptor of silver ions. The binding of lead ions by CR itself appeared more promising. The supramolecular form of CR seems necessary for the efficient binding of this ion; it somewhat complicates its use. Hence, despite the apparent attractiveness of lead ions for the expected experimental effects, their application needs more studies and they cannot replace at the moment silver ions in this role.

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References

1. Rudyk H, Vasiljevic S, Hennion RM, Birkett CR, Hope J, Gilbert IH. Screening Congo red and its analogues for their ability to prevent the formation of PrP-res in scrapie-infected cells. J Gen Virol 2000;81:1155–64.
2. Lai L-L, Su F-Y, Lin Y-J, Ho C-H, Wang E. Synthesis and study of azo-dye compounds: various molecular stackings from different polarities of the molecules. Helv Chim Acta 2002;85:1517–22.
3. Rudyk H, Knaggs MH, Vasiljevic S, Hope J, Birkett C, Gilbert IH. Synthesis and evaluation of analogues of Congo red as potential compounds against transmissible spongiform encephalopathies. Eur J Med Chem 2003;38:567–79.
4. Spólnik P, Konieczny L, Piekarska B, Rybarska J, Stopa B, Zemanek G, et al. The use of the Congo red-related dye DBACR to recognize the heavy chain-derived abnormality of myeloma immunoglobulins. Arch Immunol Ther Exp (Warsz) 2006;54: 217–21.
5. Lorenzo A, Yankner BA. Beta-amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. Proc Natl Acad Sci USA 1994;91:12243–7.
6. Woodcock S, Henrissat B, Sugiyama J. Docking of Congo red to the surface of crystalline cellulose using molecular mechanics. Biopolymers 1995;36:201–10.
7. Benzinger TL, Gregory DM, Burkoth TS, Miller-Auer H, Lynn DG, Botto RE, et al. Propagating structure of Alzheimer's beta-amyloid(10–35) is parallel beta-sheet with residues in exact register. Proc Natl Acad Sci USA 1998;95:13407–12.
8. Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, et al. Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 1999;274:25945–52.
9. Lim A, Makhov AM, Bond J, Inouye H, Connors LH, Griffith JD, et al. Betabellins 15D and 16D, de novo designed beta-sandwich proteins that have amyloidogenic properties. J Struct Biol 2000;130:363–70.
10. Skowronek M, Stopa B, Konieczny L, Rybarska J, Piekarska B, Szneler E, et al. Self-assembly of Congo red – a theoretical and experimental approach to identify its supramolecular organization in water and salt solutions. Biopolymers 1998;46:267–81.
11. Stopa B, Piekarska B, Konieczny L, Rybarska J, Spólnik P, Zemanek G, et al. The structure and protein binding of amyloid-specific dye reagents. Acta Biochim Pol 2003;50:1213–27.
12. Król M, Roterman I, Piekarska B, Konieczny L, Rybarska J, Stopa B, et al. An approach to understand the complexation of supramolecular dye Congo red with immunoglobulin L chain lambda. Biopolymers 2005;77:155–62.
13. Piekarska B, Konieczny L, Rybarska J, Stopa B, Zemanek G, Szneler E, et al. Heat-induced formation of a specific binding site for self-assembled Congo red in the V domain of immunoglobulin L chain lambda. Biopolymers 2001;59:446–56.
14. Spólnik P, Konieczny L, Piekarska B, Rybarska J, Stopa B, Zemanek G, et al. Instability of monoclonal myeloma protein may be identified as susceptibility to penetration and binding by newly synthesized Congo red derivatives. Biochimie 2004;86:397–401.
15. Piekarska B, Skowronek M, Rybarska J, Stopa B, Roterman I, Konieczny L. Congo red-stabilized intermediates in the lambda light chain transition from native to molten state. Biochimie 1996;78:183–9.
16. Glenner GG, Yanes ED, Page DI. The relation of the properties of Congo red-stained amyloid fibrils to the β-conformation. J Histochem Cytochem 1972;20:821–6.
17. Roterman I, Rybarska J, Konieczny L, Skowronek M, Stopa B, Piekarska B, et al. Congo red bound to α-1-proteinase inhibitor as a model of supramolecular ligand and protein complex. Comput Chem 1998;22:61–70.
18. Krol M, Roterman I, Drozd A, Konieczny L, Piekarska B, Rybarska J, et al. The increased flexibility of CDR loops generated in antibodies by Congo red complexation favors antigen binding. J Biomol Struct Dyn 2006;23:407–16.
19. Jagusiak A, Konieczny L, Król M, Marszałek P, Piekarska B, Piwowar P, et al. Intramolecular immunological signal hypothesis revived – structural background of signalling revealed by using Congo red as a specific tool. Mini Rev Med Chem 2014 [Epub available in PubMed].
20. Stopa B, Jagusiak A, Konieczny L, Piekarska B, Rybarska J, Zemanek G, et al. The use of supramolecular structures as protein ligands. J Mol Model 2013;19:4731–40.
21. Rybarska J, Konieczny L, Roterman I, Piekarska B. The effect of azo dyes on the formation of immune complexes. Arch Immunol Ther Exp (Warsz) 1991;39:317–27.
22. Kaszuba J, Konieczny L, Piekarska B, Roterman I, Rybarska J. Bis-azo dyes interference with effector activation of antibodies. J Physiol Pharmacol 1993;44:233–42.
23. Rybarska J, Piekarska B, Stopa B, Zemanek G, Konieczny L, Nowak M, et al. Evidence that supramolecular Congo red is the sole ligation form of this dye for L chain lambda derived amyloid proteins. Folia Histochem Cytobiol 2001;39:307–14.
24. Carter DB, Chou KC. A model for structure-dependent binding of Congo red to Alzheimer beta-amyloid fibrils. Neurobiol Aging 1998;19:37–40.
25. Caughey B, Brown K, Raymond GJ, Katzenstein GE, Thresher W. Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and Congo red [corrected]. J Virol 1994;68:2135–41.
26. Klunk WE, Jacob RF, Mason RP. Quantifying amyloid by Congo red spectral shift assay. Methods Enzymol 1999;309:285–305.
27. Chrambach A, Chrambach A, Brining SK. Gel electrophoretic distinction between Congo red nonreactive beta-amyloid (1–42) and beta-amyloid (1–40). Electrophoresis 2000;21: 760–1.
28. Findeis MA. Approaches to discovery and characterization of inhibitors of amyloid beta-peptide polymerization. Biochim Biophys Acta 2000;1502:76–84.
29. King HG, Pruden G. The component of commercial Titan yellow most reactive towards magnesium: its isolatin and use in determining magnesium in silicate minerals. Analyst 1967;92:83–90.
30. Garner RJ. Colorimetric determination of magnesium in plasma or serum by means of Titan yellow. Biochem J 1946;40:828–31.

31. Perez S, Mazeau K. Conformations, structures, and morphologies of celluloses. In: Diversity and functional versatility. Dumitriu S, editor. New York: Marcel Dekker, Inc., 2005:41–157.
32. Moon RJ, Martini A, Nairn J, Simonsen J, Youngblood J. Cellulose nanomaterials review: structure, properties and nanocomposites. Chem Soc Rev 2011;40:3941–4.
33. Liao H-G, Zherebetskyy D, Xin H, Czarnik C, Ercius P, Elmund H, et al. Facet development during platinum nanocube growth. Science 2014;345:916–9.
34. Azubel M, Koivisto J, Malola S, Bushnell D, Hura GL, Koh AL, et al. Electron microscopy of gold nanoparticles at atomic resolution. Science 2014;345:909–12.