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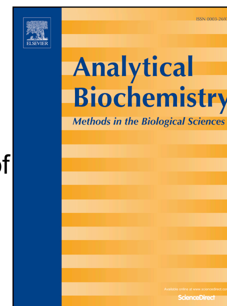

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Preparation of pure populations of covalently stabilized amyloid β-protein oligomers of specific sizes

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ABSTRACT

Evidence suggests that amyloid β-protein (Aβ) oligomers may be seminal pathogenic agents in Alzheimer's disease (AD). If so, developing oligomer-targeted therapeutics requires an understanding of oligomer structure. This has been difficult due to the instability of these non-covalently associated Aβ assemblies. We previously used rapid, zero-length, *in situ* chemical cross-linking to stabilize oligomers of Aβ40. These enabled us to isolate pure, stable populations of dimers, trimers, and tetramers and to determine their structure-activity relationships. However, equivalent methods applied to Aβ42 did not produce stable oligomers. We report here that the use of an Aβ42 homologue, [F10, Y42]Aβ42, coupled with sequential denaturation/dissociation and gel electrophoresis procedures, provides the means to produce highly pure, stable populations of oligomers of sizes ranging from dimer through dodecamer that are suitable for structure-activity relationship determination.

INTRODUCTION

Alzheimer's disease is a fatal neurodegenerative disorder in which abnormal assembly of the amyloid β-protein (Aβ) appears to be a seminal pathogenic process [1]. Assembly produces a number of structures, including fibrils [2], protofibrils [3-5], annuli [6], and oligomers [7] (for a review, see [8]). A substantial body of evidence suggests that oligomers, in particular, may be important pathogenic agents [9-16]. The findings that oligomers of α-synuclein, huntingtin, superoxide dismutase 1, prion protein (PrP), islet amyloid polypeptide, transthyretin, and β_2-microglobulin have been implicated in causation of Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, prionoses, type 2 diabetes mellitus, familial amyloid polyneuropathy, and dialysis-related amyloidosis, respectively, supports the hypothesis that

pathologic oligomerization may be widespread among neurodegenerative diseases [13]. These facts support the significance of establishing structure-activity relationships (SAR) as a first step toward the proper targeting of therapeutic agents. SAR studies in the Aβ system have been challenging for a variety of reasons [17], including: (1) Aβ is intrinsically disordered, and therefore a native monomer fold does not exist; (2) Aβ self-assembly produces a population of polydisperse and metastable oligomers; (3) oligomers exist in a rapid equilibrium among monomers and higher-order species, including protofibrils and fibrils.[1]

To overcome these problems, Bitan *et al.* [19] applied the method of Photo-Induced Cross-linking of Unmodified Proteins (PICUP) to the Aβ system [20]. PICUP is a zero-length, photochemical cross-linking chemistry that can be performed in biological buffers in the absence of exogenous cross-linking agents (for a review, see [21]). The method enabled the discovery that the oligomer distributions of Aβ40 and Aβ42 were distinct [9]. PICUP, followed by oligomer isolation from bands on SDS gels, also enabled SAR studies of Aβ40 monomer through tetramer, revealing a disproportionate increase in neurotoxicity with Aβ40 oligomer size [22]. Attempts to apply this approach to SAR studies of Aβ42 oligomers were not successful because, unexpectedly, not all Aβ42 oligomers were found to be covalently cross-linked (see [22], footnote on page 14745).

The PICUP chemistry involves the photooxidation of a Ru(II)-tris(bypyridyl) complex to Ru(III), producing a strong one-electron abstraction agent that can mediate production of free radicals on the phenolic side-chains of Tyr residues. These free radicals then can attack adjacent reactive groups, including other Tyr side-chains, to produce C-C (zero-length) bonds. This

[1] It has been suggested that "Aβ neurotoxicity is mediated by an ongoing nucleated polymerization process rather than by discrete Aβ species" [18]. However, first principles of semantic logic dictate that a process *per se* cannot be toxic. An entity or entities involved in the process must be the neurotoxins. These are the entities for which SAR must be determined.

chemistry can be quite rapid (ms time regime) and efficient (>90%). However, chemical accessibility of reactants and reactive atoms is required. One explanation for the incomplete cross-linking of Aβ42 relative to Aβ40 [22] is that the reactive Tyr10 is less accessible in Aβ42 than in Aβ40. Extensive comparative experimental and computational studies of Aβ40 and Aβ42 have revealed that their folding landscapes [23] and oligomerization pathways are distinct [9, 24-26] suggesting that local conformational differences may indeed affect the ability of a covalent bond to form between two tyrosines. To explore this question, scanning Tyr substitution studies were done to determine whether covalent cross-linking could be achieved if the reactive Tyr residue were positioned at different sites within Aβ42 [27]. The results showed that replacing Ala42 with Tyr, while simultaneously replacing Tyr10 with Phe ([F10, Y42]Aβ42), resulted in the formation of oligomers of substantially greater stability without substantially affecting peptide secondary structure dynamics, oligomer frequency distributions, fibril formation kinetics, or fibril morphology as shown by circular dichroism, thioflavin T, and electron microscopy respectively [27, 28]. We report here a sequential denaturation/dissociation and gel electrophoresis procedure that when applied to an initial heterodisperse, quasi-stable population of cross-linked peptides produces pure populations of [F10, Y42]Aβ42 oligomers, ranging in size from dimer through dodecamer, that are suitable for use in structure-activity studies.

MATERIALS AND METHODS

Chemical and reagents

Water was obtained using a Milli-Q system (Millipore, Billerica, MA, USA). Reagents were obtained in the highest purity available from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise noted below.

Preparation of peptides

Aβ42 and [F10, Y42]Aβ42 were synthesized using solid phase peptide synthesis and Fmoc chemistry on an Applied Biosystems model 433A peptide synthesizer (Foster City, CA, USA), as described previously [3]. Peptide lyophilizates (200 µg of approximately 80% peptide by weight) were dissolved in 25 µL of 60 mM sodium hydroxide (Fisher, Waltham, MA, USA) in water to increase solubility and decrease *de novo* peptide aggregation [29]. Immediately thereafter, 112.5 µL of water and 112.5 µL of 22.2 mM sodium phosphate, pH 7.4, were added, and the solution was sonicated in an ultrasonic water bath (model 1510, Branson Ultrasonics Corp., Danbury, CT, USA) for 1 min at 22°C. The pH of the solution at this stage of peptide preparation is ≈11, which facilitates peptide manipulation by increasing solubility and preventing spontaneous self-association [29]. Protein concentration was determined at 22°C by UV absorbance (ε_{274}=1280 $cm^{-1}M^{-1}$) using a 1 cm quartz cuvette (Hellma, Plainview, NY, USA) and a Beckman DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). Peptide concentration was adjusted to 80 µM using 10 mM sodium phosphate, pH 7.4. This yielded a final pH of 7.4.

Photochemical cross-linking of Aβ

Peptides were cross-linked using the method of Photochemical Cross-linking of Unmodified Proteins (PICUP) [20, 21], essentially as described [19]. Briefly, 3 µL of 2 mM Tris(2,2′-bipyridyl)-dichlororuthenium (II) hexahydrate [Ru(II)] and 3 µL of 40 mM ammonium persulfate, both dissolved in water, were added to 54 µL of 80 µM Aβ. This mixture was vortexed briefly and irradiated for 1 s with visible light from a 150 W source (model 170-D, Dolan-Jenner, Lawrence, MA, USA), after which the free radical reaction was quenched by

addition of 1 µL of 1 M dithiothreitol (DTT) (Fisher) in water, followed by brief vortexing. This reaction was performed six times, yielding a final volume of 366 µL.

Fractionation of mixtures of cross-linked oligomers

Following cross-linking, 183 µL of 2X Tricine sample buffer (LifeTechnologies, Carlsbad, CA, USA) and 183 µL of dimethyl sulfoxide (DMSO) were added to the 366 µL of cross-linked peptide. (Note that DMSO percentages specified in the text and figure legends always are v/v). The resulting solution was placed in a heating block ("Reacti-Therm" heating/stirring apparatus, Pierce, Rockford, IL, USA) for 10 min at 100°C, after which half of the solution was electrophoresed on each of two precast 1 mm thick 10-20% T Tricine SDS gels (LifeTechnologies). We modified the precast 12-well gels to have one wide lane (width 6.4 cm) into which the cross-linked peptide was placed and a second lane (width 3.5 mm) into which 2 µL of Mark 12 molecular weight markers (LifeTechnologies) were placed.

Protein bands were visualized using reversible Zn/imidazole stain staining. To do so, 200 mM imidazole and 200 mM zinc sulfate (Acros Organics, New Jersey, USA), both in water, were prepared and filtered through a 0.22 µm pore size vacuum filter system with a polyethersulfone membrane (Corning, Corning, NY, USA). The gel was incubated at room temperature for 20 min in 200 mM imidazole on a "Tabotron" orbital agitator plate (Infors AG, Bottmingen, SUI) at 70 rpm, washed briefly with water, and then agitated for ≈3 min in 200 mM zinc sulfate. The lowest twelve horizontal areas (bands) were then excised using a #10 feather surgical scalpel blade (Fisher) and placed into separate 1.5 mL conical siliconized microcentrifuge tubes (Fisher). Two-hundred µL of 2X Tris-glycine sample buffer

(LifeTechnologies) were added to each tube and then the tubes were boiled for 10 min in a heating block set to 100°C.

The boiled peptide-containing gel pieces then were subjected to a second electrophoretic fractionation in urea-containing SDS gels, similar to the procedure of Cleveland et al. [30]. To do so, a 1.5 mm thick PROTEAN II xi Cell gel (Bio-Rad, Irvine, CA, USA) was cast with an 18% T separating gel and a 4% T stacking gel, both containing 6 M urea (Fisher). Gel pieces were loaded into 1 cm wide lanes, after which 35 μL of 2X Tris-glycine sample buffer were added. Eight μL of Mark 12 unstained standard (Thermo Fisher Scientific) were placed in a separate lane. The gel was electrophoresed at 45 V for 1.5 h at room temperature and then the voltage was increased to 75 V for 17-19 h. To avoid overheating, a cooling system using a "Variable Speed Pump" (Bio-Rad) was connected with Tygon tubing to a cooling coil (Bio-Rad), which was placed in a bucket of ice, continuously pumping cooled water through the gel apparatus. Upon completion, the gel was zinc/imidazole stained, as described above. The oligomer bands were then excised and cut into pieces of dimensions ≈1.5 mm × ≈2 mm × ≈2 mm.

Electro-elution and analysis of the stability of cross-linked oligomers

Protein was electro-eluted from each set of gel pieces using Bio-Rad Model 422 electro-eluters. Electro-elution was done in 25 mM Tris-glycine, pH 8.4, containing 192 mM glycine and 0.1% (w/v) SDS in water. To avoid overheating, electro-elution was done in a cold room at 4°C, using a constant current of 5 mA per electro-eluter tube for 18 h (a total of 30mA for the six-sample apparatus used). Each tube yielded ≈400 μL of oligomer solution, which then was used in experiments. We note that, at this stage, the oligomer solutions also can be dialyzed against any buffer/solvent of choice, depending on the experiment to be performed.

Quantification of Purity

A 5 µL aliquot of each electro-eluted oligomer was added to 5 µL of 2X Tricine sample buffer and boiled for 10 min, as described above. Samples then were electrophoresed in a precast 15-lane, 1 mm thick, 10-20% T Tricine gel (LifeTechnologies). The gel was silver stained (Silver-Xpress; LifeTechnologies) according to the manufacturer's instructions and then desiccated overnight using a DryEase® Mini-Gel Drying System. Desiccated gels were scanned at 400 dpi in color mode (Canon "CanoScan 9950F"). The resulting image was converted to grayscale. Band intensities were quantified by densitometry, using ImageJ 1.43r (http://imagej.nih.gov/ij/), after boxes were placed manually around each band. Percent purity $P = (I_i \Big/ \sum_{1}^{n} I_i) \times 100$, where $\boldsymbol{I_i}$ is intensity of band of order ***i*** and ***n*** is the total number of oligomer bands.

Toxicity of Aβ assemblies

Rat pheochromocytoma (PC12) cells were cultured in 75 cm^2 Canted Neck Flasks (Corning Inc., Corning, NY) in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) HS, 2.5% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, 0.1 mg/mL of streptomycin, and 25 µg/mL amphotericin B at 37°C in 5% (v/v) CO_2 in air. The cells were passaged at least four times before use. To prepare the PC12 cells for toxicity analysis, the medium was removed and the cells were washed once gently with DMEM medium containing 0.5% (v/v) FBS, 2% penicillin/ streptomycin. A cell suspension was then made with this media supplemented with 150 µg/mL of nerve growth factor (NGF). The cell concentration was determined by Trypan blue staining, after which cells were plated at a concentration of 20,000 cells/well (100 µL of total volume per well) in Costar 96-well white clear-bottom plates (Corning, Corning, NY). The NGF-

induced differentiation of the cells was allowed to proceed for 48 h. To perform the toxicity assays, 25 μM of Aβ solution was prepared in DMEM medium containing 0.5% (v/v) FBS and 2% penicillin/ streptomycin. Forty μL aliquots of the Aβ solution were added to the wells to yield a final Aβ concentration of 10 μM. The cells then were incubated for 48 h. To determine the effects of Aβ on LDH activity, we used the Promega CytoTox-ONE Homogeneous Membrane Integrity assay (Madison, WI). The positive toxicity control was 1 μM staurosporine. One hundred microliters of LDH reagent was added to each well and the plate was incubated in the dark for 10 min, after which 50 μL of stop solution was added and the fluorescence was measured using the Synergy HT plate reader with an excitation wavelength of 560 nm and emission wavelength of 590 nm. Six replicates were used for each treatment group and the data were reported as mean ± SE. Percent toxicity $T = ((F_{A\beta} - F_{medium})/(F_{full\ kill} - F_{medium})) \times 100$, where $F_{A\beta}$, F_{medium}, and $F_{full\ kill}$ were fluorescence values from Aβ containing samples, negative control (buffer volume equivalent with medium), and 1 μM staurosporine alone, respectively.

RESULTS AND DISCUSSION

[F10, Y42]Aβ42 forms oligomers of increased stability

We sought to develop a method to separate covalently cross-linked Aβ42 oligomer populations from non-covalently cross-linked populations. To facilitate cross-linking, we used a novel Aβ42 analogue, [F10, Y42]Aβ42, that is more efficiently cross-linked than is wild type peptide [27]. Figs. 1a and 1b show the results of experiments in which Aβ42 and [F10, Y42]Aβ42 were cross-linked using PICUP, oligomers were isolated by standard SDS-PAGE, and then each oligomer band was excised and re-electrophoresed in a second SDS gel. Substantial dissociation of oligomers occurred in the Aβ42 sample, in addition to self-association to form higher-order

oligomers (Fig. 1a). However, the extent of each process occurring with the [F10, Y42]Aβ42 peptide was reduced (Fig. 1b). We then electro-eluted the oligomers present in each of the bands from the second gel and evaluated their purity in a third gel (Fig. 1c and Table 1). Monomers and dimers were relatively pure (≳90%). Purities of the remaining oligomers ranged from 24-64%.

In neurotoxicity studies of Aβ40, we observed that oligomers of a specific size, e.g., dimers, trimers, or tetramers, each possessed an intrinsic neurotoxic activity that did not change regardless of whether the oligomers were tested in pure form or existed within mixtures of different sized oligomers [22]. This observation is particularly important with respect to proper definition of SAR in biological systems, such as the Aβ system, in which absolute purity of analytes is difficult to achieve. For example, if two different Aβ oligomers were present in equimolar amounts in a sample to be tested for neurotoxicity, and each possessed substantial intrinsic neurotoxic activity, assignment of the observed biological effects to one or the other of the oligomers would be problematic. However, if the most abundant oligomer were present in amounts significantly larger than the amount of the second most abundant oligomer, then the SAR problem becomes tractable because, *ceteris paribus,* any effects observed must be due primarily to the most abundant oligomer. This argument is made more compelling by the fact that the toxic potential intrinsic to a particular Aβ40 oligomer subtype has been found to increase disproportionately with order [22]. If such a phenomenon were found to occur with Aβ42 oligomers, then lower order oligomers contaminating an otherwise pure oligomer population would contribute much less to an observed biological effect than would be predicted simply by their relative abundance. Whether these arguments are true or not will need to be determined in subsequent experiments. Nevertheless, it is clear that the use of [F10, Y42]Aβ42 produces purer populations of each of the isolated cross-linked oligomers than does use of Aβ42.

Effect of DMSO and urea on oligomer distributions

We reasoned that sequential SDS-PAGE might not yield bands comprising only cross-linked oligomers because avid non-covalent association of monomers might partially protect these assemblies from SDS-induced dissociation. In addition, for Aβ42 but not Aβ40, we have found previously that SDS can *induce* oligomerization into dimers and trimers [9]. For these reasons, we explored the possibility of treating populations of cross-linked Aβ with DMSO or urea alone, or in combination, to determine if such treatment would facilitate dissociation of non-covalently cross-linked species.

We first added DMSO, at concentrations ranging from 12.5-50%, to cross-linked [F10, Y42]Aβ42 oligomers prior to SDS-PAGE. DMSO altered the oligomer distribution in a concentration-dependent manner (Fig. 2a). The intensities of oligomers of orders 2-8 increased in the presence of 12.5% DMSO. With 25% DMSO, we observed an increase in oligomers of orders 6-11 and smearing at higher molecular weights. DMSO also caused an increase in monomer, dimer, and trimer, suggesting a dissociation of non-covalently crossed-linked oligomers into monomers, dimers and trimers. With 50% DMSO, monomer, dimer, trimer, and tetramer displayed increased intensity, but significant smearing above 55.4 kDa also was apparent.

We next determined how the presence of 6M urea during SDS-PAGE (added during the casting of the gel) affected the oligomer distributions. Urea caused a shift in the apparent molecular mass (M_r) of the Aβ42 monomer, from ≈5 to ≈9 kDa (cf. Figs. 2b and c), as previously reported [31]. Non-cross-linked Aβ42 electrophoresed on a 6 M urea gel no longer displayed three distinct bands (of ≈5, ≈17, and ≈20 kDa), but rather a more bulbous monomer band at ≈9 kDa and one

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band at ≈18 kDa (*cf.* Aβ42 nXL in Figs. 2b and 2c). The distribution of cross-linked Aβ42 oligomers displayed a node at pentamer/hexamer as well as decamer/ dodecamer bands at ~55 kDa (Fig. 2b, Aβ42 XL), consistent with previous studies [9]. However, on a 6 M urea gel, cross-linked Aβ42 displayed distinct bands only at 7, 9, 26, 28, and ~55 and ~60 kDa, as well as smearing (Fig. 2c, Aβ42 XL). This supports the conclusion that the bands visualized on the gel contain both covalently and non-covalently linked oligomers. Non-cross-linked [F10, Y42]Aβ42 produced a distribution qualitatively similar to that of Aβ42, but with lesser amounts of monomer and greater amounts of putative trimer and tetramer (Fig. 2b [F10, Y42]Aβ42 nXL). When examined on a gel with 6 M urea, the largest band was observed at ≈20 M_r, which was more intense than either of the lower molecular weight bands (Fig. 2c [F10, Y42]Aβ42 nXL). Interestingly, when cross-linked [F10, Y42]Aβ42 was electrophoresed with or without 6 M urea the distribution of oligomers was similar, with a slight increase of the bands above 31 kDa, though the M_r values of the lowest molecular weight bands were higher (Fig. 2b and Fig. 2c, [F10, Y42]Aβ42 XL). The highest intensity bands of cross-linked [F10, Y42]Aβ42 were between 21.5 and 31 M_r, corresponding to tetramer through heptamer, similar to cross-linked Aβ42.

We then examined how the combination of DMSO pre-treatment and SDS-PAGE with 6M urea affected the oligomer distributions. To do so, 25% DMSO was added to non-cross-linked and cross-linked [F10, Y42]Aβ42, which then was electrophoresed with or without the addition of 6 M urea. We chose a 25% DMSO concentration because it appeared to facilitate oligomer dissociation, improve resolution of oligomers of orders 6-11, and produce less smearing than did 50% DMSO. The addition of DMSO to cross-linked Aβ42 caused a decrease in the oligomers 4-9 and an increase in the monomer and dimer bands, while simultaneously increasing the staining intensity at bottom of the stacking gel (Figs. 2c and e, Aβ42 XL). When we examined non-cross-

linked [F10, Y42]Aβ42, we observed a decrease in the ratio of band intensities for putative trimer and monomer, suggesting that the DMSO/urea combination dissociated trimers into monomers (Fig. 2c, [F10, Y42]Aβ42 nXL -/+ DMSO). Cross-linked [F10, Y42]Aβ42 produced a ladder of bands corresponding to monomer through pentadecamer (Figs. 2c and e, [F10, Y42]Aβ42 XL + DMSO). Relative to [F10, Y42]Aβ42 XL electrophoresed in 6 M urea, but without DMSO pre-treatment, we noted a decrease in the intensity of the tetramer, pentamer, hexamer, heptamer and octamer bands and an increase in the intensity of the bands corresponding to monomer through trimer (7, 9, and 18 kDa) (Figs. 2 c and d). We also noted the presence of clearly resolved bands at higher M_r values than were seen in the absence of urea (Fig. 2e). The observation of a ladder (in some cases smearing) of high molecular weight bands in the presence of DMSO suggests that the dissociated monomers are able to interact to form larger aggregates.

In summary, we found that adding DMSO to the cross-linked protein *prior to* SDS-PAGE was able to dissociate some non-covalent oligomers that SDS did not dissociate. In the subsequent SDS-PAGE, the urea appears to dissociate oligomers that were not dissociated by DMSO. The combination of DMSO and urea was most effective compared to either method alone.

Preparation of oligomer populations of specific order

We combined DMSO pre-treatment, SDS-PAGE and band excision, and urea/SDS-PAGE and electro-elution to create a method for production of pure populations of oligomers of specific order following photochemical cross-linking (Fig. 3). Fig. 4a shows a representative Zn/imidazole-stained SDS gel of the initial mixture of cross-linked oligomers after DMSO treatment. The bands from this gel were excised and electrophoresed in a urea/SDS gel,

producing the Zn/imidazole-stained gel shown in Fig. 4b. Bands corresponding to each oligomer order were excised from this gel and subjected to electro-elution and analysis by SDS-PAGE. The results show that highly pure (up to 98%) oligomer populations (*cf.* with Fig. 1) can be produced in this manner. Absolute purities are shown in Table 1. This table reveals two things: (1) substantial improvements in absolute purity are seen for all oligomer orders (except dimers, which could be purified well using the prior methods); (2) the new method enabled isolation of dodecamers, an oligomer order that repeatedly has been implicated in disease pathogenesis [8][2].

Toxicity of Aβ assemblies

To determine if the toxicity of the assemblies formed by [F10, Y42]Aβ42 was affected by the cross-linking or the amino acid substitutions, we performed LDH release assays on NGF-differentiated PC12 cells (Fig. 5). We observed that non-cross-linked wild type Aβ42 and [F10, Y42]Aβ42 were equally toxic, within experimental error. Cross-linked assemblies of each peptide also were equally toxic. The levels of toxicity of the cross-linked assemblies were lower than that of the non-cross-linked peptides. We also compared the toxicities of fibrillar forms of each peptide. The average toxicity of fibrils formed by [F10, Y42]Aβ42 appeared higher than that of wild type Aβ42 fibrils, but the difference was not significant statistically. We note that the absolute levels of toxicity in these assays were modest. Nevertheless, these side-by-side comparisons of peptide toxicity levels do show that substitution or cross-linking do not alter the biological activities of the resulting assemblies significantly.

[2]We also applied the oligomer isolation method developed herein to wild type Aβ42. Only dimer and trimer were isolable, whereas the remainder of the oligomers formed dissociated during the procedure. Nevertheless, the pure dimer and trimer populations now may be used in parallel with [F10, Y42]Aβ42 dimers and trimers to establish how the biophysical and biological properties of oligomers formed from these two peptides compare.

METHOD SUMMARY

We have developed a method for producing and isolating chemically stabilized Aβ42 oligomers of specific order (2-12) in pure form. The method involves: (1) zero length, *in situ*, photochemical cross-linking of Aβ; (2) dissociation of non-covalent complexes by DMSO treatment; (3) oligomer size fractionation by SDS-PAGE; (4) band staining with reversible Zn/imidazole stain followed by band excision; (5) re-electrophoresis of each band in gels containing 6 M urea; and (6) band staining with reversible Zn/imidazole stain followed by band excision, oligomer electro-elution and dialysis. The pure oligomer populations provide starting material necessary for SAR determination, antibody production, and other purposes.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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TABLE 1

Size	1	2	3	4	5	6	7	8	9	10	11	12
Purity (%) (Fig. 1c)	95.8	89.6	24.4	46.8	60.9	62.6	63.7	56.7	56.1	29.7	46.5	ND
Purity (%) (Fig. 3c)	96.2	87.9	53.4	81.7	71.3	79.2	84.2	93.0	97.5	98.0	95.9	95.3

Percent purities of [F10, Y42]Aβ42 oligomers isolated by electro-elution from each of the bands in a "second gel." Purities from Fig. 1c are from two sequential gels. Purities from Fig. 3c are from the full isolation method (DMSO and urea/SDS-PAGE). "ND" is "not determined," as dodecamers could not be obtained using prior methods.

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FIGURE LEGENDS

Figure 1. Stability of cross-linked Aβ42 oligomers. Cross-linked (a) Aβ42 and (b) [F10,Y42]Aβ42 oligomers were electrophoresed by SDS-PAGE and excised. These excised oligomer bands were examined by SDS-PAGE and silver stained. Open arrowheads in images (a) and (b) denote the positions where the nominal pure oligomer band should be in each lane. (Reprinted with permission from Yamin *et al.* Biochemistry [27]. Copyright 2015 American Chemical Society.) (c) Oligomer bands of [F10,Y42]Aβ42 (corresponding to oligomers shown in panel (b) were excised and their component oligomers were isolated using electro-elution. Subsequent SDS-PAGE and silver staining reveals oligomer purity. The vertical white line between lanes labeled 2 and 3 in panel (c) comes from the deletion of the image of a lane that was blank in the original gel.

Figure 2. Effects of DMSO and urea on Aβ oligomer distributions. (a) 12.5, 25, and 50% DMSO were added to cross-linked [F10,Y42]Aβ42 after which SDS-PAGE and silver staining were performed. Non-cross-linked (nXL) and cross-linked (XL) Aβ42 and [F10,Y42]Aβ42 were analyzed, with or without DMSO pre-treatment, by (b) SDS-PAGE or (c) SDS-PAGE with 6 M urea. Within each of the images (a-c), all lanes were from the same gel. However, to make comparisons among treatments easier, the lanes have been reordered. The oligomer order noted on the right of each gel corresponds to the cross-linked oligomer size. (d) Intensity profiles of cross-linked [F10, Y42]Aβ42 with and without 25% DMSO. (e) Intensity profiles of cross-linked [F10, Y42]Aβ42, with and without 25% DMSO, electrophoresed in the presence of 6M urea.

Figure 3. Method for the isolation of individual cross-linked oligomers. Low molecular weight Aβ is cross-linked using PICUP. 25% DMSO is added to the cross-linked protein, which then is fractionated by SDS-PAGE. Individual oligomer bands are excised and separated on a second SDS-PAGE gel containing 6 M urea. Oligomer bands of interest are excised and their component oligomers are obtained by electro-elution, with or without dialysis.

Figure 4. Purity of oligomers at each step of the purification procedure. (a) Cross-linked [F10,Y42]Aβ42 was treated with 25% DMSO, subjected to SDS-PAGE, and the gel was stained using the zinc/imidazole method. The zinc/imidazole is a negative stain, thus the protein bands remain translucent upon visualization. (b) Oligomer bands were excised and electrophoresed on a second SDS-PAGE, containing 6 M urea, after which the gel was stained using zinc/ imidazole. Numbers below the images correspond to oligomer order (i.e., 2 is dimer, 3 is trimer, etc.). (c) Oligomer bands were excised from the urea containing gel and subjected to electro-elution. The isolated oligomers then were characterized by SDS-PAGE and silver staining.

Figure 5. Toxicity of wild type Aβ42 and [F10, Y42]Aβ42. LDH assays were performed on NGF-differentiated PC12 cells incubated for 48 h with freshly prepared, non-cross-linked (nXL) peptides, cross-linked (XL) peptides, or fibrils. Percent toxicity $T = ((F_{A\beta} - F_{medium})/(F_{full\ kill} - F_{medium})) \times 100$, where $F_{A\beta}$, F_{medium}, and $F_{full\ kill}$ were LDH activities from Aβ containing samples, negative control (buffer volume equivalent with medium), and 1 μM staurosporine alone, respectively.

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Fig. 1

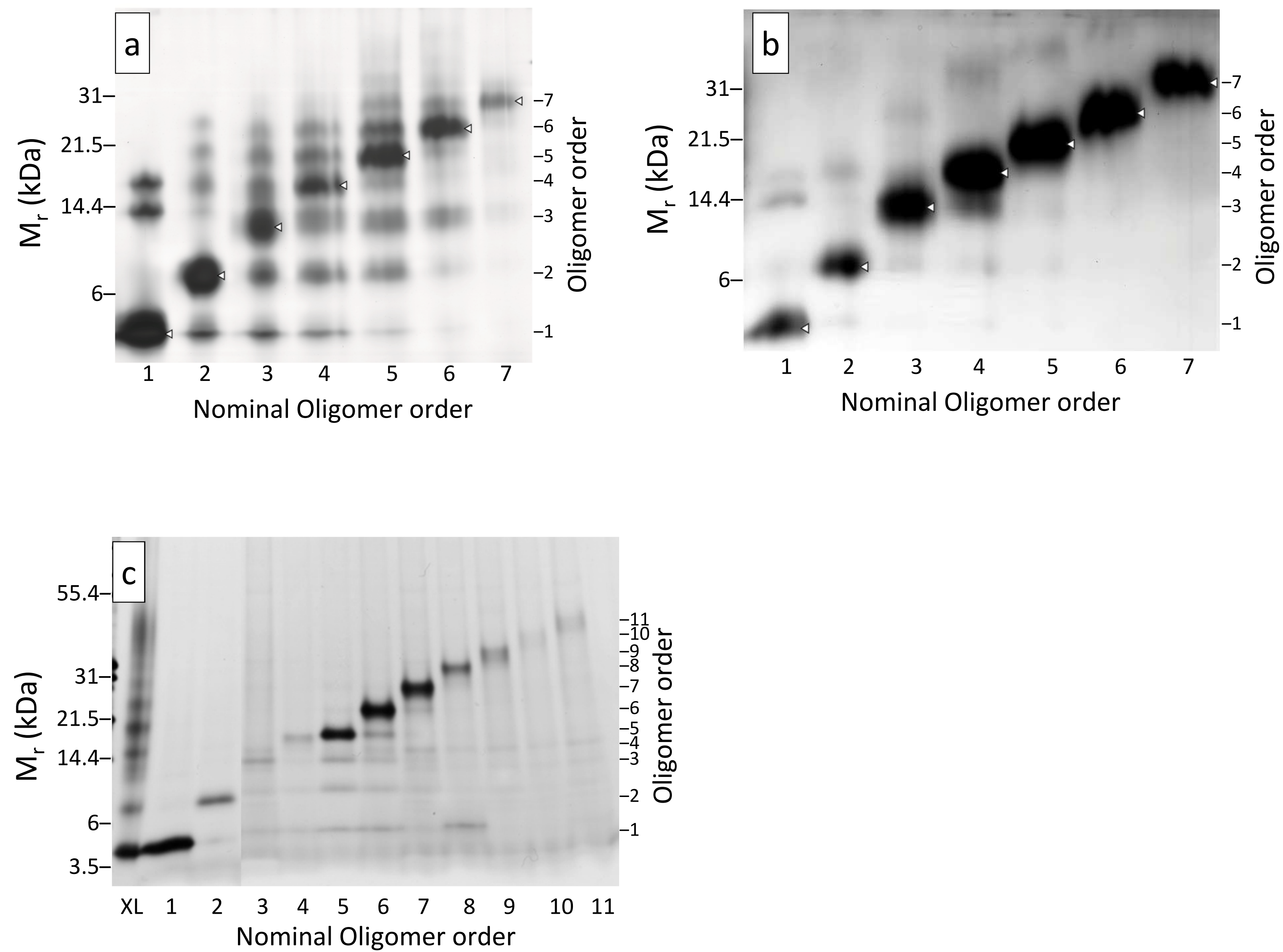
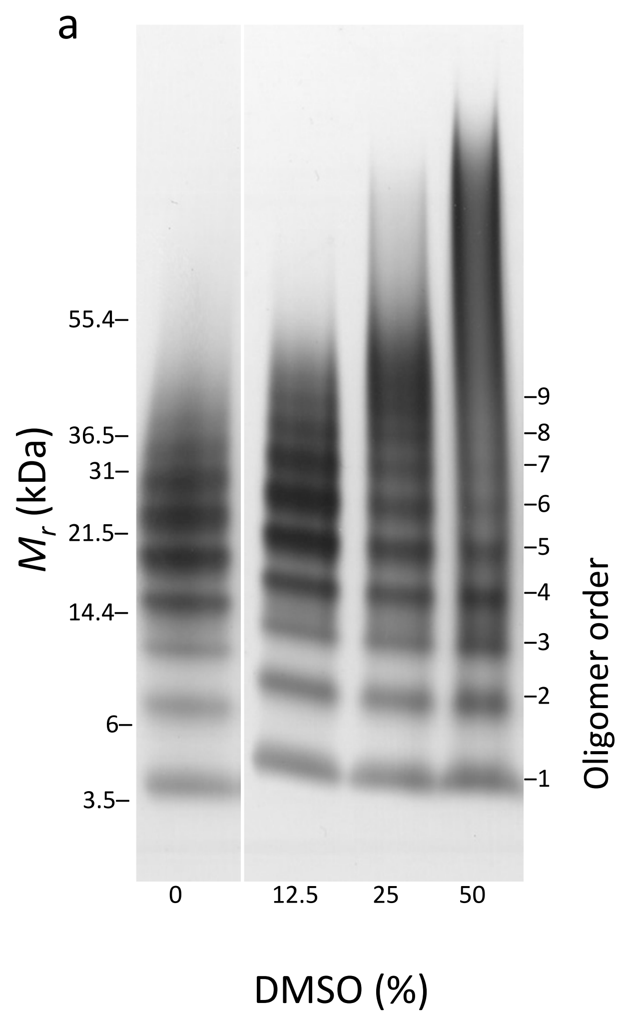


Fig. 2a



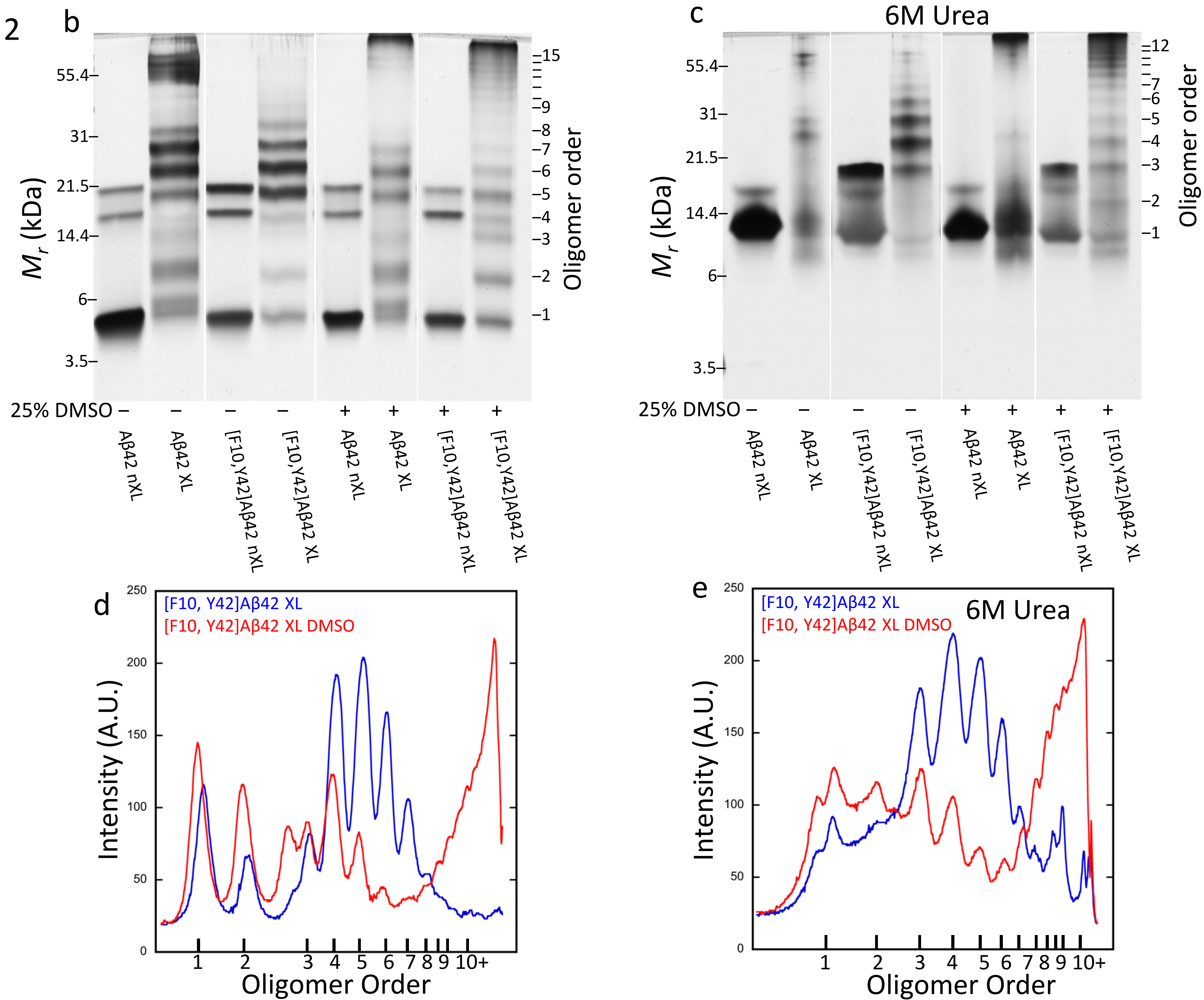


Fig. 2
b
c
6M Urea
M_r (kDa)
Oligomer order
25% DMSO
Aβ42 nXL
Aβ42 XL
[F10,Y42]Aβ42 nXL
[F10,Y42]Aβ42 XL
d
e
[F10, Y42]Aβ42 XL
[F10, Y42]Aβ42 XL DMSO
Intensity (A.U.)
Oligomer Order

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Fig. 3

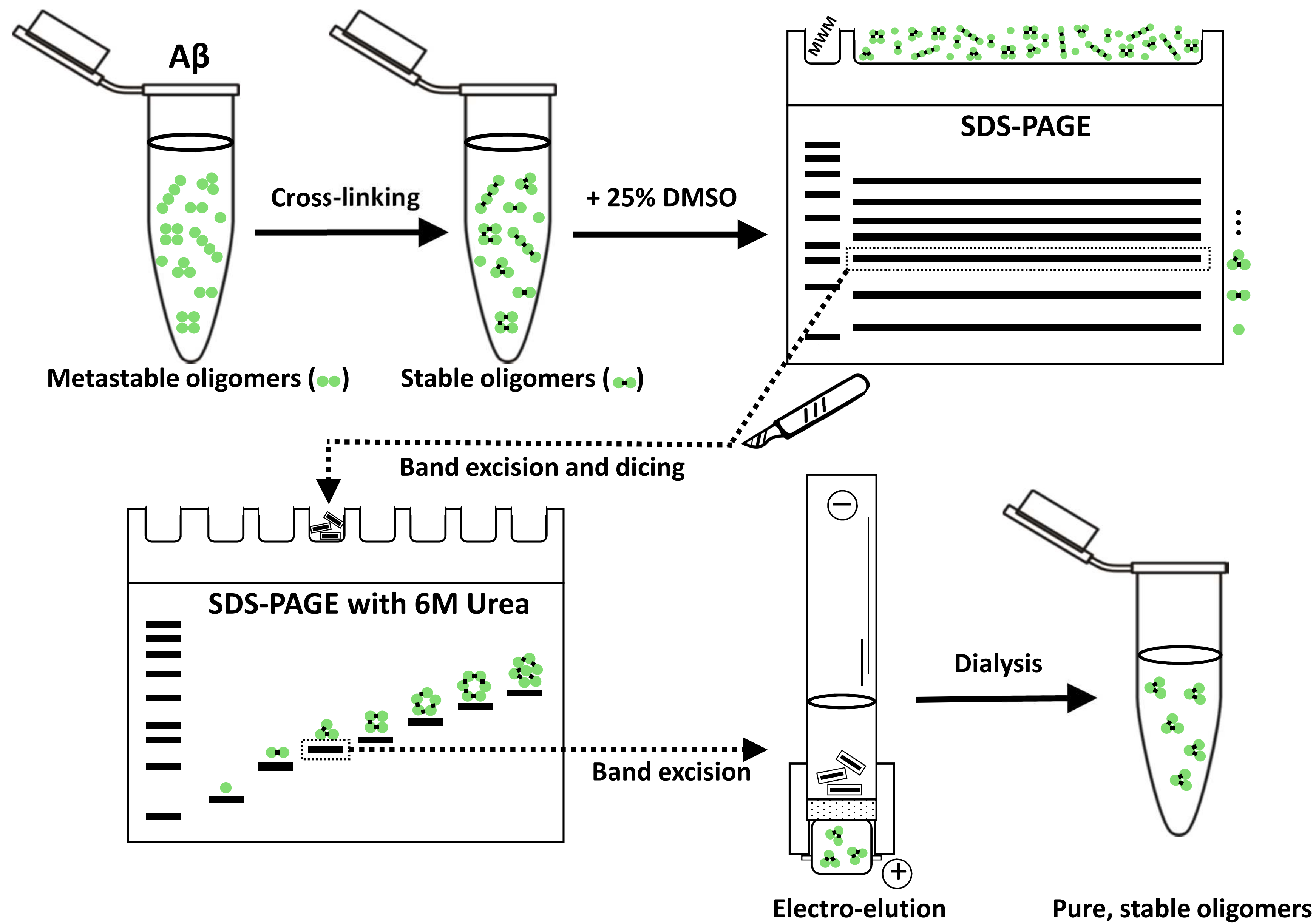
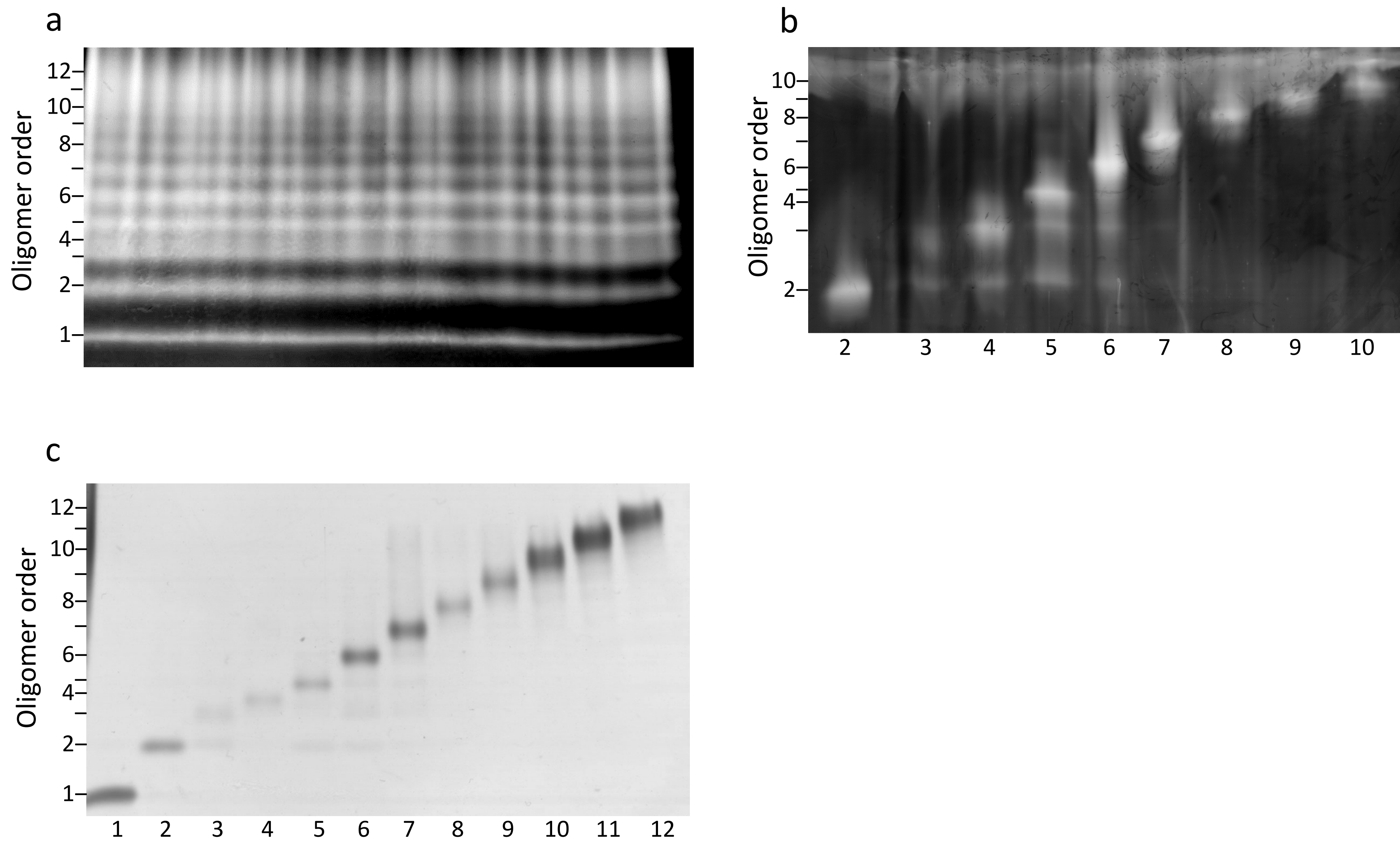
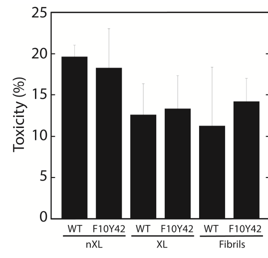


Fig. 4





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