Effect of Methionine Sulfoxide on the Synthesis and Purification of Aggregation-Prone Peptides

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A two-step synthesis for methionine-containing hydrophobic and/or aggregation-prone peptides is presented that takes advantage of the reversibility of methionine oxidation. The use of polar methionine sulfoxide as a building block in solid-phase peptide synthesis improves the synthesis quality and yields the crude peptide, with significantly improved solubility compared to the reduced species. This facilitates the otherwise often laborious peptide purification by high-performance liquid chromatography. The subsequent reduction proceeds quantitatively. This approach has been optimised with the methionine-rich Tar-DNA-binding protein 43 (307–347), but is also more generally applicable, as demonstrated by the syntheses of human calcitonin and two aggregation-prone peptides from the human prion protein.

Hydrophobic and aggregation-prone peptides are highly interesting drug targets due to their important physiological roles in cell communication, signal transduction or membrane transport and their pathological properties if mutated or overexpressed.[1,2] The latter include peptides, which aggregate upon membrane interaction and are therefore discussed as key players in a variety of neurodegenerative diseases and diabetes. Prominent examples include amyloid beta (Aβ), α-synuclein, and, more recently, the amyotrophic lateral sclerosis (ALS)- and frontotemporal dementia-related Tar-DNA-binding protein 43 (TDP-43).[2–4]

The prerequisite for in-depth studies of such peptides is good synthetic access. However, the production of "difficult peptides", which include hydrophobic and/or aggregation-prone sequences, is usually laborious, as customized synthesis and purification protocols are often required. Over the years, several synthesis strategies have been developed to reduce peptide aggregation during solid-phase peptide synthesis (SPPS).[5] These include the incorporation of backbone amide protecting groups,[6] pseudoprolines[7] or isopeptide building blocks.[8] Solubility and purification problems have been solved either by using special solvent mixtures and detergents[9] or by incorporating temporary or permanent solubilizing tags to the peptide backbone, side chains or termini.[10]

TDP-43 is a key player in ALS, as deposits are found in more than 97% of all ALS patients.[3,4] During our studies on the aggregation of TDP-43, we became interested in a hydrophobic segment within the *C*-terminal domain of the protein, more specifically in TDP-43 (307–347) (**1**; Figure 1A). This segment is part of a protein region that is discussed to be essential for the protein aggregation and liquid-liquid-phase separation.[11] To study the biophysical properties of TDP-43 (307–347) on a molecular level, an efficient synthetic access to this protein segment had to be developed. However, **1** is largely hydrophobic, aggregation-prone and contains seven methionine (Met, M) residues, which are sensitive to oxidation. Furthermore, the peptide composition reveals the lack of charged amino acid residues. Taken together, these features let us to expect a major synthetic challenge.

Indeed, microwave-assisted synthesis using a standard Fmoc/*t*Bu protocol resulted in a crude peptide of only moderate quality (Figure 1B) due to the formation of significant amounts of glycine and alanine addition sequences as well as truncation sequences (Figure S1 in the Supporting Information, Table S1). Additionally, purification by high-performance liquid chromatography (HPLC) became problematic because **1** was poorly soluble in water-acetonitrile mixtures and prone to aggregation. Even the use of denaturing solvents to suppress aggregation and improve solubility (Figure S3) did not facilitate the purification process because of the overall poor quality of the crude peptide material. The amphiphilic character of **1** and the resulting tendency for adsorption to the surfaces of plastic tubes and tips led to a further reduction in the synthesis yield. Nonetheless, we were able to obtain pure **1** in low yields (~ 1%).

As the synthesis of **1** by using standard methods takes so much time and effort, we sought an improved synthetic procedure towards **1** that not only provided crude **1** in a better quality but also facilitated the purification process. One of the particular features of TDP-43 (307–347) is the high content of Met residues (Figure 1A). The thioether moiety of the Met side chain is highly sensitive to oxidation; first, to the respective sulfoxide and, at high exposition to oxygen, to the sulfone.

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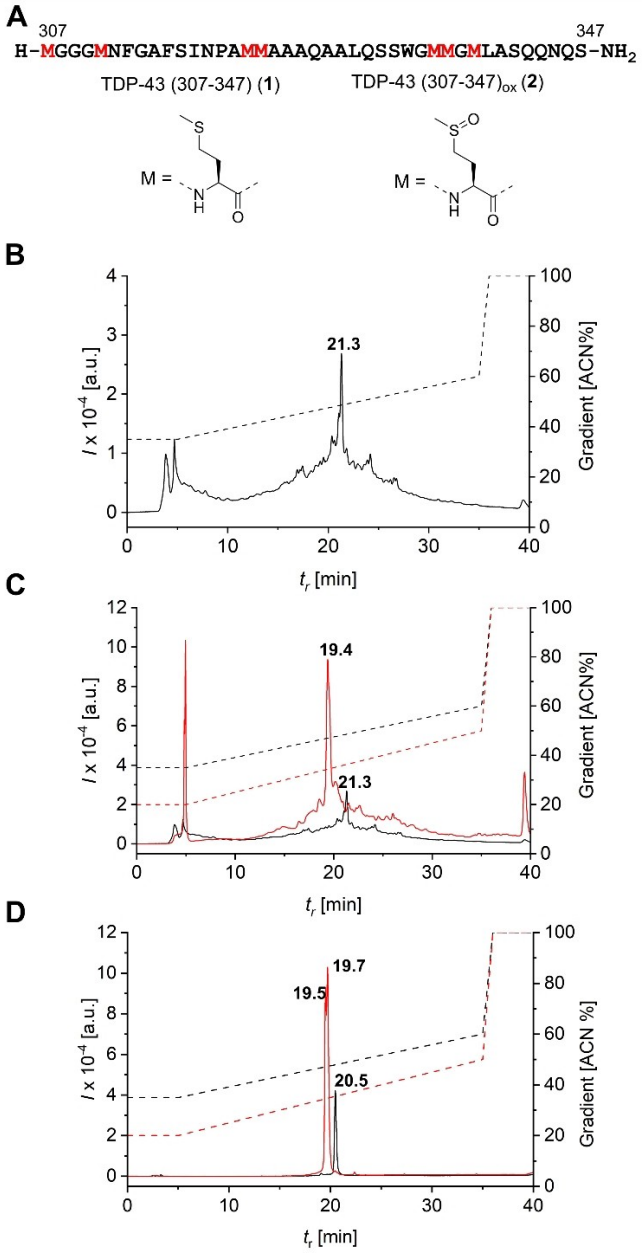

Figure 1. Synthesis of TDP-43 (307–347). A) Sequence of TDP-43 (307–347). B) HPLC trace of crude TDP-43 (307–347) (**1**). Overlay of HPLC traces of C) crude and D) purified TDP-43 (307–347)$_{ox}$ (**2**, red) and TDP-43 (307–347) (**1**, black). HPLC gradients are depicted as dashed lines. Sequence **2** gives a double peak due to the L-Met(O) diastereomers. (Samples were prepared from suspensions of 10 mg/mL crude peptide in 35% ACN (**1**) or 25% ACN (**2**) or 5 mg/mL pure peptide (**1** and **2**) in 20% ACN; HPLC-traces were monitored at 280 nm.)

Since oxidation to the sulfoxide is reversible, we decided to make a virtue out of necessity and use Met(O) building blocks in the SPPS. In the past, Met(O) has proven to be very versatile in many different applications. For instance, reversible methionine oxidation has been used as a protecting group strategy in chemical synthesis and especially chemical ligation to avoid undesired side reactions on the methionine side chain.[12] In addition, site-specific introduction of Met(O) into peptides has been used to study the effect of methionine oxidation on peptide aggregation and secondary structure or to design switch peptides that change their secondary structure upon oxidation.[13] Only recently, the use of Met(O) for site-specific peptide labelling *via* a Pummerer reaction was described.[14]

Our interest in Met(O) was piqued by a report by Janda et al. describing its use in a two-step synthesis of highly aggregation-prone Aβ (1–42) by SPPS followed by Met(O) reduction on-resin to reduce aggregation during the synthesis process.[15] Although this is also desirable in case of the synthesis of TDP-43 (307–347), we rather anticipated an improved solubility of the obtained all-Met(O) crude product (**2**) and thus a facilitated purification process. Subsequent reduction of purified **2** would provide desired **1**, which then should be easily purified in a second purification step. When we synthesized **2** using microwave-assisted SPPS, we obtained a crude product with an improved purity of about 40% and significantly higher polarity and reduced aggregation propensity. It should be noted that the product peak appears as a double peak. This is due to the use of the L-methionine-D,L-sulfoxide building block. Figure 1C shows the crude HPLC chromatograms of saturated solutions of **1** and **2** and clearly visualizes the improved solubility of **2** compared to **1**. Consequently, purification of **2** proceeded smoothly, and **2** was obtained in a yield of about 2–5%. Although the yield is significantly increased, it is still low, which we attribute to the general "stickiness" of this peptide even in the oxidized state. Finally, to assess the solubility of **1** and **2**, we prepared saturated solutions of the purified compounds and determined the concentration of the dissolved peptide fraction by UV spectroscopy. We found a fourfold improvement in solubility of fully oxidized **2** compared to unoxidized **1**, which is also evident from the HPLC chromatograms (Figure 1D).

To identify a suitable protocol for the reduction of **2**, three methods described in the literature were tested: a reduction procedure using ammonium iodide and dimethyl sulfide (Procedure 1),[16] a procedure described by Taboada et al. using tetrabutylammonium bromide and ethane-1,2-dithiol (Procedure 2)[17] and finally a protocol involving the use of trimethylsilyl bromide (TMSBr) and ethane-1,2-dithiol as a redox system (Procedure 3).[15] Met(O) reduction using ammonium iodide and dimethyl sulfide was described as a very mild procedure that also tolerates sensitive peptide modifications such as thioester moieties.[18] When testing this method on a small scale, we observed a relatively slow reduction over two hours, after which small amounts of partially oxidized **1** were still detected. Figure 2A shows the HPLC traces of a typical reaction monitoring, which depict the formation of various partly reduced species of **2** over time (see MALDI-monitoring of the formed intermediates in Figure S4) until fully reduced **2** becomes predominant. In contrast, Procedure 2 did not give satisfactory results as reduction led to an undesired and inseparable mixture of products (Figure S5). Procedure 3, using TMSBr and ethane-1,2-dithiol, yielded the fully reduced **2** in only five minutes without the formation of major by-products (Figures 2B and S6). When doubling the scale, only Procedure 3 resulted in a complete reduction of **2** in 15 min, while Procedure 1 resulted

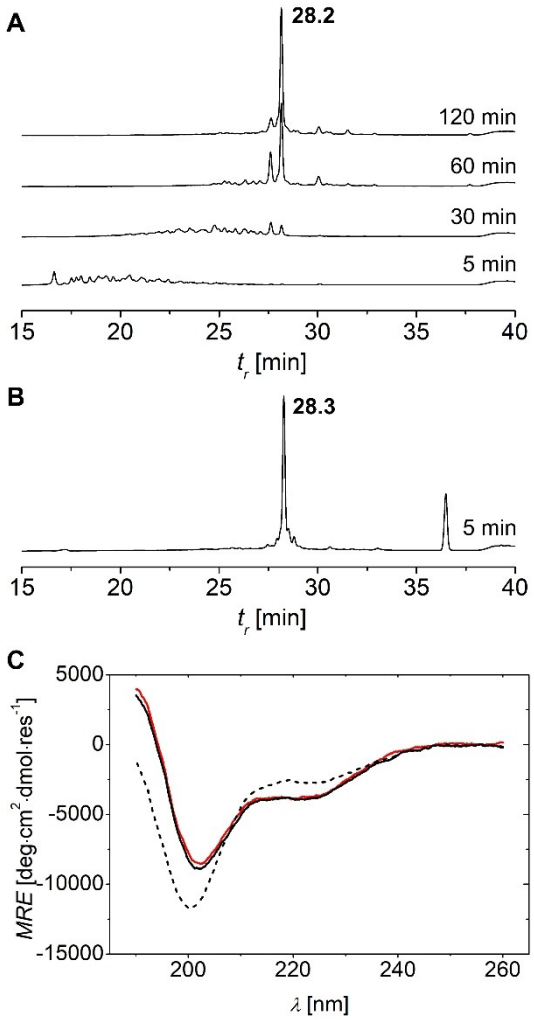

Figure 2. Reduction of TDP-43 $(307–347)_{ox}$. A) HPLC monitoring of the reduction of **2** with NH_4I and dimethyl sulfide (Procedure 1). B) HPLC trace of the reduction of **2** with TMSBr and ethane-1,2-dithiol (Procedure 3). Conditions: peptide concentration: 0.1 mM; HPLC gradient: 20–60% acetonitrile, 30 min, 280 nm. C) CD spectra of **1** synthesized by standard microwave-assisted Fmoc/*t*Bu SPPS (red line) or the oxidation–reduction protocol (solid black line) and **2** (dashed line; Conditions: 20 μM peptide concentration, 4 mM phosphate buffer, pH 6.8, 20 °C).

in a mostly incomplete reduction within the previously determined reaction time of two hours (Figure S7A). Therefore, only Procedure 3 was efficient enough to attempt a semi-preparative 1 μmol scale (Figure S7B). Although **2** was almost completely reduced after 10 min, extension of the reaction time to 60 min ensured complete conversion (Figure S7B).

Circular dichroism (CD) spectroscopy was used to study the structural integrity of **1** synthesized by the oxidation-reduction protocol using Procedure 3. The structure of TDP-43 $(307–347)_{ox}$ (**2**) is almost entirely a random coil (Figure 2C, black dotted line). After reduction, the obtained CD spectrum (Figure 2C, black solid line) compares well with the reference spectrum of **1** previously synthesized by standard SPPS (Figure 2C, red solid line) and is consistent with the literature.[3,19]

As the incorporation of Met(O) instead of Met not only improved the solubility but also the quality of the peptide raw material and reduced the tendency to aggregate, we were interested to see whether this trend would also be confirmed in the synthesis of other aggregation-prone peptides. We tested three peptides: two peptide segments from the human prion protein hPrP (125–155) (**3**) and hPrP (109–135) (**7**) and the 32 residue thyroid hormon peptide human calcitonin hCT (**5**; Table 1).

Aggregates of misfolded hPrP isoforms are found in prion diseases such as Creutzfeldt-Jakob disease, bovine spongiform encephalopathy (BSE) or Scrapie.[20] hPrP (125–155) (**3**) is the N-terminal peptide segment of the prion protein domain of hPrP.

The peptide sequence is not as hydrophobic as that of TDP-43 (307–347), but it contains three Met residues and was expected to be prone to aggregation. The synthesis of **3** using non-oxidized Met building blocks resulted in a crude material of moderate quality (Figure 3A, top). Furthermore, the purification of **3** was hampered by the aggregation of this peptide. Hence, **3** was obtained in only 2% yield. Using Met(O) instead of Met in SPPS gave a crude peptide **4** of significantly improved quality and solubility (Figure 3A, bottom). HPLC purification and subsequent reduction yielded **3** in 7%. It should be noted that we also attempted direct reduction of **4** during cleavage from the resin. Crude hPrP (125–155) (**3**) was obtained in remarkably better purity (Figure S8), showing that incorporation of Met(O) instead of Met has a significant effect on the synthesis result, probably due to reduced aggregation during synthesis.

The peptide hormon hCT (**5**) is a regulator of calcium and phosphate levels in the blood. However, it has also been found that hCT is aggregation-prone and is suspected to cause amyloidosis-related diseases such as thyroid carcinoma.[21] As hCT contains only one Met residue, it proved to be ideal for studying the influence of Met oxidation on peptide solubility,

Table 1. Sequences and synthesis yields of the methionine containing and aggregation-prone peptides explored in this study.

Peptide	Sequence		Yield [%] Method A[a]	Method B[b]
TDP-43 (307–347)	H-MGGGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQS-NH_2	**1**	0.7–1.2[c]	2–4[d]
hPrP (125–155)	H-LGGYMLGSAMSRPIIHFGSDYEDRYYRENMH-NH_2	**3**	2	7
hCT[c]	H-CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH_2	**5**	5	10
hPrP (109–135)	H-MKHMAGAAAAGAVVGGLGGYMLGSAMS-NH_2	**7**	7	14

[a] Standard Fmoc/*t*Bu SPPS using non-oxidized Met. [b] Two-step oxidation-reduction synthesis approach. [c] Sequence **5** was isolated as reduced peptide. [d] The range of yields of TDP-43 (307–347) was determined from four independent syntheses, which were carried out over the period of one year. Yields refer to isolated compounds.

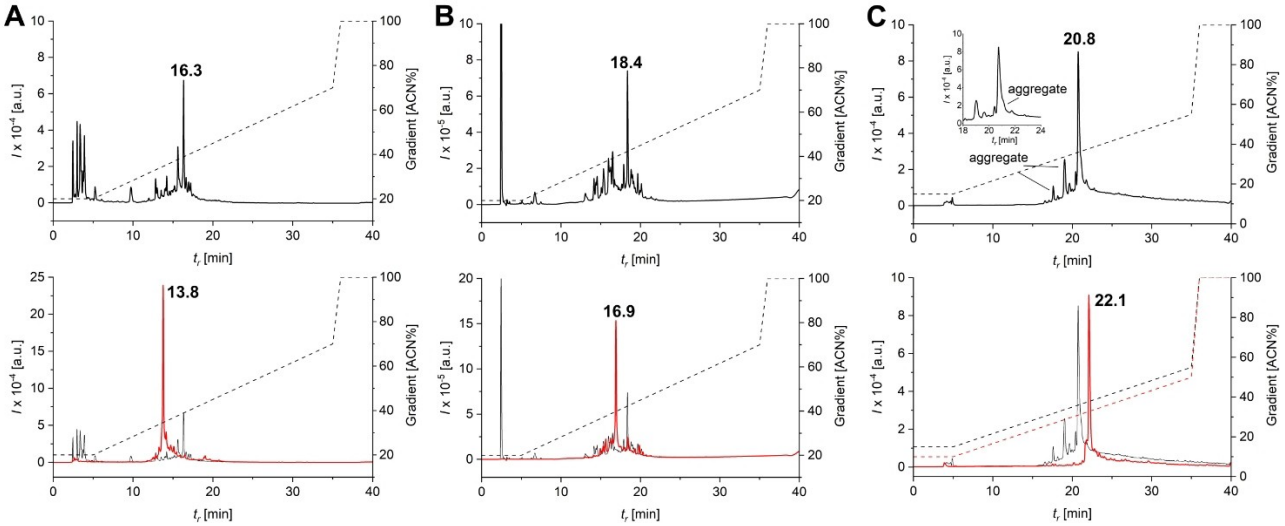

Figure 3. Synthesis of methionine-containing aggregation-prone peptides. Top: HPLC traces of A) crude hPrP (125–155) (**3**), B) crude hCT (**5**) and C) crude hPrP (109–135) (**7**, semi-preparative HPLC). Bottom: Overlay of HPLC traces of A) crude hPrP (125–155)$_{ox}$ (**4**, red) and hPrP (125–155) (**3**, black); B) crude hCT$_{ox}$ (**6**, red) and hCT (**5**, black), and C) crude hPrP (109–135)$_{ox}$ (**8**, red) and hPrP (109–135) (**7**, black): semi-preparative HPLC. Samples were prepared from saturated peptide solutions (suspensions of 10 mg/mL crude peptide in 20% ACN; injection volume peptides **3**–**7**: 1 mL, peptide **8**: 0.5 mL; HPLC traces were recorded at A), C) 280 nm and B) 220 nm.

aggregation and synthesis. As shown in Figure 3B (top) SPPS of non-oxidized **5** yielded a crude peptide material, in which many by-products were present. Consequently, **5** was isolated in 5% yield. Using Met(O) instead of Met in SPPS greatly improved the synthetic outcome (Figure 3B, bottom). Although only one Met(O) was incorporated, the solubility of **6** improved compared to **5**, and the amount of by-products was significantly reduced. Reduction of purified **6** to **5** resulted in overall 10% yield.

As a third example, we chose a peptide, hPrP (109–135) (**7**), which did not present a particular synthetic challenge, but tended to aggregate. The yield after direct synthesis of **7** was relatively low at 7%, considering the quality of synthesis. The problem was that **7** could not be completely dissolved due to the formation of insoluble aggregates. Additionally, aggregation also complicated the purification process as can be seen from the chromatogram of the semi-preparative HPLC (Figure 3C, top), in which the peaks next to the main peak originate mostly from non-covalent aggregates. Using the two-step oxidation-reduction approach did partly solve the solubility problem. Oxidized **8** was easier to handle as the aggregation-propensity was reduced. Consequently, after reduction, **7** was isolated in 14% yield. Interestingly and in contrast to peptide **2**, none of the oxidized peptides **4**, **6**, **8** give a double peak in the HPLC, although diastereomeric L-methionine-D,L-sulfoxide was used. We assume that this is due to the lower methionine content and that the diastereomers are not resolved.

In summary, we have presented a two-step protocol for the synthesis of hydrophobic and aggregation-prone peptides that exploits the higher polarity of Met(O) compared to the reduced amino acid residue. Our approach involves the incorporation of Met(O) instead of Met, resulting in crude peptides with significantly improved quality and solubility compared to the non-oxidized variants. We could show that the reduction to the desired non-oxidized peptides is most efficient when using TMSBr and ethane-1,2-dithiol as redox system. However, Procedure 1, which uses NH_4I and dimethyl sulfide as redox system, is a mild alternative for peptides with sensitive modifications such as thioester moieties.

The synthesis of hydrophobic peptides is generally challenging. Many strategies have been reported to facilitate synthetic access by reversibly introducing more or less structurally complex solubility tags and backbone modifications. In comparison, Met(O) is a small, simple and reversible peptide modification and stable at the high temperatures applied in microwave-assisted SPPS. Moreover, the corresponding SPPS building block is commercially available and less costly compared to isopeptide fragments. Although its proteomic abundance is relatively low,[22] Met is overrepresented in peripheral membrane- and membrane-interacting proteins and peptides, many of which are interesting drug targets.[23] Therefore, we believe that the method described has broad applicability and is a valuable addition to established peptide synthesis protocols.

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Conflict of Interest

The authors declare no conflict of interest.

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