

# Potential Inhibitor Against Phase Separation, 1,6-hexanediol Specifically Binds to Beta Actin in Nuclear Extract of Human Cell Line

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**Abstract:** RNA-binding protein (RBP) TLS/FUS plays a major role in induction of phase separation/phase transition and aggregation in relation to familial amyotrophic lateral sclerosis (ALS). Recently, organelles without lipid-bilayer membrane including stress granule, Cajal body, and nucleolus are found to be formed by the phase separation. The phase separation is an event that solutions with two solvents separate into two distinctive phases. The phase separation is prone to have solid phase and forms harmful precipitation or aggregation against living cells, indicating that the phase separation has both benefit and risk on cellular programs. Thus, it is essential for utilization of the phase separation in divergent cellular programs to control or inhibit the undesirable precipitation in living cells. Here, we analyze an inhibitory mechanism of the phase separation and precipitation. Inhibition of the phase separation is one of a critical regulatory step to prevent dysregulation of the phase separation and resulting deleterious precipitations. An inhibitory agent against the phase separation, amphiphilic alcohol, 1,6-hexanediol (1,6-HD) has been reported to examine function of phase separations. Thus, affinity chromatography of biotinylated 1,6-HD is employed to identify an initial event induced by 1,6-HD. Upon successful synthesis of biotinylated compounds of 1,6-HD (bio-1,6-HD), the affinity chromatography with bio-1,6-HD has been established at this study. The bio-1,6-HD captured protein bands on SDS-PAGE gel from HeLa cell nuclear extracts. The bands were analyzed with mass spectrometric analyses, showing that the proteins should be cytoskeleton related proteins. Further analysis using specific antibodies revealed one of the bands as human beta actin. It has been shown that beta actin is involved in divergent cellular activities including the phase separation and also neuronal functions like long-term potentiation. Therefore, beta actin might initiate the 1,6-HD-induced inhibition of the precipitation, although more experiments should be required to test whether it actually works in living cells.

**Keywords:** TLS, FUS, Phase Separation, Phase Transition, Aggregation, Precipitation, 1,6-hexanediol

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## 1. Introduction

RNA-binding protein TLS (translocated in liposarcoma)/FUS (fused in sarcoma) is a causative gene for amyotrophic lateral sclerosis (ALS) [1, 2] and frontotemporal lobar degeneration (FTLD) [3]. Clinical symptoms of ALS are characterized by the impairment of upper and lower motor neurons, indicate muscle atrophy, and gradually spreading to the muscle of whole body including respiratory

system. At the late stage of the ALS, patients suffering from the difficulty of speaking and breathing terminally die from the respiratory failure [4, 5]. More than 90% of ALS is sporadic disorder in which no genetic background is identified [4, 5]. Around 10% of ALS is familial disease. The causative genes of familial ALS reported are the superoxide dismutase-1 [6], TDP-43 [7], and TLS [1, 2]. We have been

focusing on TLS from a point of view of gene expression regulation. Mutated forms of TLS genes indicate the similar pathology with other ALS causative genes, which is dislocation into cytoplasm from nucleus, exhibiting the aberrant cytoplasmic inclusions. TLS binds RNA in specific and non-specific manners, and plays pivotal roles in RNA metabolisms in processing, and transport of RNA [8, 9]. Mutations in TLS might cause failure of these RNA metabolic functions, suggesting its function as a mediator for biologically active RNA molecules.

Chemical library screening of an agent to induce embryonic stem cells into myocardial cells provided isoxazole [10]. A biotinylated compound, biotinylated isoxazole (BISOX) was produced to capture binding molecules to isoxazole. Unexpectedly, BISOX induced precipitation of proteins from cultured cell lysates [11], and the precipitation contained more than hundreds of RBPs [11-14]. Many of these proteins are components of RNA-protein assemblies forming in nuclei of living cells, called RNA granules or membraneless organelles. Many proteins in these structures contain heterogeneous nuclear ribonucleoprotein K (hnRNP K) homology (KH) domain and RNA recognition motif (RRM) [15, 16] as well as low complexity sequences or intrinsically disordered regions (IDR) [14, 17]. One of these proteins turned out to be TLS. The IDRs in these proteins are susceptible for BISOX mediated aggregation, and later the IDRs are shown to undergo a phase separation or phase transition to a hydrogel-like state even without BISOX. X-ray crystallography and electron microscopic observation demonstrated that the hydrogels form with the amyloid-like fiber structures. Unlike pathogenic amyloid fibers, the IDR-based polymers shown are reversible structures. These observations present an outline for understanding the function of IDRs as well as an organizing principle for cellular structures without membrane, or, membraneless organelles [11]. These data present a forthcoming direction of phase separation towards biological investigations.

TLS forms liquid compartments, droplets or condensates in nuclei and also in the cytoplasm when exposure to divergent environment related to ALS [18-22]. In vitro reconstitution experiments indicated that the liquid droplets of TLS convert from a liquid state to an aggregated one over hours [2, 8, 9, 23]. This conversion is accelerated by ALS patient-derived mutations. These data confirm that the physiological role of TLS needs to form dynamic droplets [18]. It has been suggested that function of TLS as liquid-like compartments requires risk of accidental aggregation in cells [18]. Then, this aberrant phase transition of the droplets of TLS into aggregates is a potential cause for ALS.

Precipitation of proteins is recently emerging as a central concerns regarding various disorders related to neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) [1, 2, 8, 23-26]. It has been well recognized that precipitation of

proteins in living cells causes disturbance of biological processes and gives rise to neurodegenerative diseases in the neurons. More clinical impact to these neuronal disorders enhances investigation of precipitated RNA-binding proteins that intimately link to the diseases [27-30]. We have developed experimental systems to investigate TLS to analyze the precipitation with making a model system to assess the involvement of the precipitation into the neurodegenerative diseases. Published data from others and ours indicate that BISOX effectively precipitates divergent RNA-binding proteins including TLS [11, 12, 14].

The BISOX precipitation assay system works well to assess the phase separation and subsequent precipitation of TLS and also to examine functions of the precipitation of TLS. The phase separation has a beneficial aspect in biological programs, but also bears a harmful adverse effect suffering from subsequent precipitation. It is likely that any regulatory mechanism to suppress formation of the precipitation subsequent to the phase separation of TLS. We hypothesize an inhibitory system against the phase separation-induced precipitation functioning in living cells. Previous studies reported that 1,6-hexanediol (1,6-HD) melts the droplets forming by phase separation [31-34]. We take advantage of 1,6-HD to explore the inhibitory and regulatory system for phase separation and precipitation.

We initiate an experiment to detect any binding protein to 1,6-HD using its biotinylated compounds and found beta actin from the nuclear extract of cultured human cell lines. Based on the data shown here, we have discussed the possible roles of cytoskeleton molecule beta actin on the inhibitory mechanism and presented a model of the regulation of the phase separation and precipitation.

## 2. Materials and Methods

### 2.1. Antibodies and Reagents

Mouse anti-TLS/FUS antibody (611385, Lot no. 2209827) was purchased from BD Biosciences (New Jersey, USA). Rabbit anti-TLS/FUS antibody (11570-1-AP) was purchased from Protein Tech (Illinois, USA). Rabbit human beta actin antibody was purchased from St John's Laboratory (London, United Kingdom, STJ 91464, Lot. 1464901). Rabbit anti-mouse HRP conjugated IgG (P0161, 20017456) was purchased from Dako (Glostrup, Denmark). Goat anti-rabbit HRP-conjugated IgG (7074S, 25) was purchased from Cell Signaling Technology (Massachusetts, USA). Biotinylated isoxazole (BISOX), 6-(((5-(2-Thienyl) -1,2-oxazol-3 -yl) carbonyl)amino)hexyl,5-(2-oxohexahydro-1H-thieno [3,4-d] imidazol-4-yl) pentanoate (biotin-isoxazole; BISOX) was produced following the protocols [12]. HeLa cell nuclear extract (NE) was prepared as previously described protocol [35-38].

### 2.2. Synthesis of Biotinylated Compounds.

#### 2.2.1. General Procedure

Reagents and solvents were purchased from standard

suppliers and used without further purification. HPLC analysis was performed on a Jasco Engineering PU-2089 plus series system using a COSMOSIL 150 × 4.6 mm 5C18-MS-II Packed Column (Nacalai Tesque, Inc., Kyoto, Japan) in 0.1% TFA in water with acetonitrile as the eluent at a flow rate of 1.0 mL/min and a linear gradient elution of 0-100% acetonitrile in 20 min with detection at 205 nm. Collected fractions were analyzed by MALDI-TOF-MS microflex-KS II (Bruker, Massachusetts, USA). Flash column purification was performed on CombiFlash Rf RFJ model (Teledyne Isco, Inc., Nebraska, USA) with RediSep® Rf 26g C18 Reverse phase column. 0.1% TFA in water with acetonitrile was used as the solvent (gradient/ acetonitrile 0%: 0-4 min, 0-30%: 4-45 min, 30%: 45-50 min, 100%: 50-55 min, 80%: 55-60 min). HPLC purification was carried out by Jasco engineering PU-2080 plus series using a COSMOSIL 150 × 10 mm 5C18-MS-II Packed Column (Nacalai Tesque, Inc.) in 0.1% TFA in water with acetonitrile as the eluent at a flow rate of 3.8 mL/min and a linear gradient elution of +1%/min acetonitrile for 20 min with detection at 205 nm. <sup>1</sup>H NMR spectra were recorded on JEOL JNM ECA-600 spectrometer (600 MHz for <sup>1</sup>H), with chemical shifts reported in parts per million relative to residual solvent and coupling constants in hertz. The following abbreviations were applied to spin multiplicity: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), quin (quintet) and m (multiplet).

### 2.2.2. Biotinylated 1,6-hexanediol (bio-1,6-HD).

(+)-Biotin (20.8 mg, 85.1 μmol) was dissolved in 3 mL of N,N-dimethylformamide (DMF) and 1,6-hexanediol (97.7 mg, 10 equiv.), 1- (3-Dimethylaminopropyl) -3-ethylcarbodiimide hydrochloride (EDCI, 31.4 mg, 2 equiv.), 4-Dimethylaminopyridine (DMAP, 10.3 mg, 1 equiv.), and N,N-diisopropylethylamine (DIEA, 83.5 μL, 6 equiv.) was added. The mixture was stirred at 60 °C for 26.5 hours. Then the solvent was evaporated and dried in vacuo. The remained oil was dissolved in H<sub>2</sub>O and lyophilized. Lyophilized sample was washed by diethyl ether (Et<sub>2</sub>O) and brown oil was precipitated. After Et<sub>2</sub>O was removed in vacuo, 93.2 mg of brown oil was obtained. This oil was purified by flash column. Collected fractions were lyophilized and 24.1 mg of target material was obtained (70.0 μmol, yield: 82%). <sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>): δ 6.44 (s, 1H), 6.39 (s, 1H), 4.31 (t, J=6.3 Hz, 1H), 4.14 (t, J=5.1 Hz, 1H), 3.70-3.63 (m, 5H), 3.24-3.17 (m, 2H), 3.10 (q, J=6.2 Hz, 1H), 3.05 (quin, J=7.5 Hz, 2H), 2.82 (dd, J=12.0 Hz, 4.8 Hz, 1H), 2.54 (d, J=7.8 Hz, 1H), 1.88-1.80 (m, 2H), 1.65-1.59 (m, 1H), 1.57-1.52 (m, 2H), 1.51-1.44 (m, 1H), 1.40-1.33 (2H, m), 1.09 (quin, J=6.6 Hz, 4H). Analytical HPLC: tR=8.4 min.

The related figure (Figure 5) is shown in Appendix.

### 2.2.3. Biotinylated PEG (polyethyleneglycol)12-NH-hexanol (bio-1,6-HD plus).

This compound has a spacer between biotin portion and hexanol (1,6-HD plus)

EZ-Link™ NHS-PEG12-Biotin 250 mM in DMF 21.25 μL (5.3 μmol) was mixed with 128.3 μL of DMF, 6-amino-1-hexanol (0.78 mg, 1.2 equiv.), and DIEA (1.08

μL, 1.2 equiv.) and then stirred at room temperature for 2.5 hours. The mixture was purified by reverse-phase HPLC and 3.2 mg of target material (colorless oil) was obtained (3.4 μmol, yield: 64%, analytical HPLC: tR=9.3 min. MALDI-TOF MS: m/z calcd for C<sub>43</sub>H<sub>83</sub>N<sub>4</sub>O<sub>16</sub>S+[M+H]<sup>+</sup> 943.54, found; 943.55).

The related figure (Figure 6) is shown in Appendix.

### 2.3. Affinity Chromatography with Biotinylated Compounds of 1,6-HD.

The biotinylated materials binding assays were performed as previously described [36-38]. Briefly, it has shown below. Dynabeads-M280 (Dynabeads: Thermo Fisher, Massachusetts, USA) was washed with PBS containing 0.02% Tween 20. Fifty μg of bio-1,6-HD was added to the beads and incubated for 15 min at room temperature with rotation. Subsequently, the beads were incubated with NE at 4°C with rotation for 1 to 5 h shown in text. The beads were then washed three times in 1 ml of WCE buffer and resuspended in SDS sample buffer and boiled at 100°C for 2 min. Finally, the beads were removed and supernatants were applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following for stain with coomassie brilliant blue (CBB) based staining reagent of the SimplyBlue™ SafeStain (Thermo Fisher) or Western blotting analyses.

The beads-prebound with biotinylated 1,6-HD compounds were incubated with bacterial lysates containing glutathione s-transferase (GST)-fused-human beta actin in 1 mM ATP, 50 mM KCl, 1 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub> at room temperature for five hours. After the incubation, the beads were washed three times with WCE buffer, and boiled in SDS-Sample buffer for 2 min. The samples were analyzed on SDS-PAGE above shown, following the Western blot with rabbit anti-human beta actin antibody.

### 2.4. Protein Analysis

SDS-polyacrylamide gel electrophoresis was performed with 10% gels following CBB staining [36-38]. Western blotting was done with anti-TLS monoclonal antibody with the dilution ratio 1:2000 using standard protocol shown previously [37].

The protein samples were separated by SDS-PAGE and identified with CBB staining. Protein bands were excised from the gel, digested with trypsin and subjected to nano-LC/MS/MS analysis by standard protocol [39] (Japan Proteomics Co.Ltd, Sendai, Japan).

## 3. Results

### 3.1. Inhibitory Activity of 1,6-hexanediol on the Phase Separation and Aggregation Induced by BISOX

To show inhibitory effect of 1,6-HD on phase separation and resulting aggregation or precipitation of TLS, we have exploited a BISOX-induced precipitation system. Treatment of NE with 100 μM BISOX effectively precipitated TLS at 4°C for 1 hour. The precipitates were detected by a Western

		HeLa Cell NE					
100uM BISOX	-	+	+	+	+	+	
	-	-	H <sub>2</sub> O 12.5ul	H <sub>2</sub> O 21.4ul	10% 1,6HD (12.5ul)	15% 1,6HD (21.4ul)	10% Input
(kDa)							
95-							
72-							
55-							
Lane:	1	2	3	4	5	6	7

### 3.2. Identification of Biotinylated 1,6-HD (bio-1,6-HD) Bound Proteins in NE with the Affinity Chromatography.

(A) 1,6-hexanediol (1,6-HD)

Bio-1,6-HD compounds

Bio-1,6-HD

hexanediol

biotin

Bio-1,6-HD plus spacer (PEG<sub>12</sub>)

hexanol

spacer (PEG<sub>12</sub>)

biotin

(B)

HeLa Cell NE

None Biotin Bio-1,6-HD Bio-1,6-HD plus

(kDa)

250

130

95

72

55

43

36

24

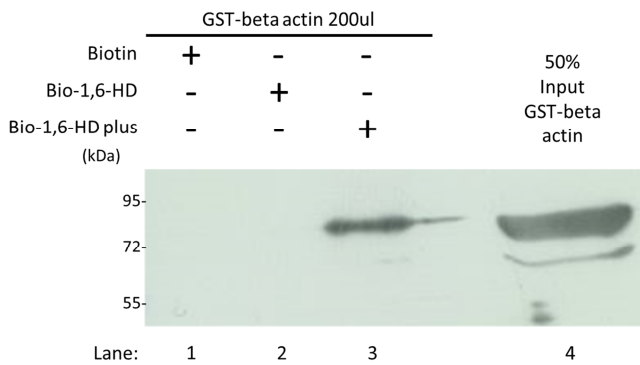
← Specific Band

Lane: 1 2 3 4

### 3.3. Confirmation of Beta Actin Binding to Bio-1,6-HD Compounds with Specific Antibody Against TLS of a Western Blot

HeLa Cell NE					
	None	Biotin	Bio-1,6-HD	Bio-1,6-HD plus	10% Input
(kDa)					
55					
43				Strong band	Weak band
32					
Lane:	1	2	3	4	5

We designed bacterial expressed beta actin binding assay with GST-human beta actin. GST-beta actin in reactions was incubated with bio-1,6-HD compounds. After the incubation, the Western blot detected specific signal on the lane of bio-1,6-HD plus, indicating that GST-beta actin binds to bio-1,6-HD plus (Figure 4, lane 4). These data confirm that the interaction of bio-1,6-HD plus with beta actin in NE should be functional, although we need to have more experiments to examine biological significance of binding of 1,6-HD to beta actin to know physiology of beta actin in living cells. This will prompt us to obtain a whole figure of contribution of beta actin to regulation of phase separation in cells.



**Figure 4.** Confirmation of the GST-beta actin binding to bio-1,6-HD compounds with Western blot using beta actin antibody. Fifty  $\mu$ g of biotinylated compounds was added to each reaction.

## 4. Discussion

Recent studies have been revealed significant impact of phase separation on biological programs rapidly [40-45]. Phase separation has been found to be involved in divergent cellular functions including formation of various membrane-less organelles [46-49], cellular mobility [50, 51], and transcriptional regulation [52-54]. Contrarily, negative side of phase separations is derived from consequential precipitation of RBPs including TLS. One of major concerning is a cause for neurodegenerative disease like Alzheimer disease [55-57], frontotemporal dementia [3, 58], and ALS [1, 2, 20, 59, 60]. Dysregulation of phase separation has harmful influences [61] on many cellular programs also regarding neuronal functions causing these neurodegenerative diseases. Therefore, it is likely that safety systems against dysregulation of phase separation should work in living cells. It might be inhibitory mechanism for phase separation. We have initiated experiments to identify molecules targeted by 1,6-HD, putative inhibitor against phase separation [31, 32, 34]. In this study, we have demonstrated evidence regarding regulatory systems based upon inhibition of the precipitation induced by phase separation. Unexpectedly, we detected specific binding of 1,6-HD to beta actin using affinity chromatography experiments. This presents a hypothesis regarding divergent functions of beta actin and regulation of phase separation. We discuss this unanticipated relationship between beta actin and 1,6-HD later in this section.

To validate regulatory systems of phase separation in cells, 1,6-HD is employed to assess inhibitory mechanism of phase separations. This reagent functions as a deterrent to lysis cellular membranes. Permeability of lipid bilayer membranes of 1,6-HD has been documented already by Finkelstein in 1976 [62]. It was expected that 1,6-HD should permeate and melt cellular membranes of eukaryotic cells. The McKnight group has focused this permeability of 1,6-HD to disassemble IDR polymer of RBPs [34]. The toxic, C9orf72 gene-encoded Proline Arginine (PRn) poly-dipeptide binds divergent intracellular targets mainly consisting of the IDR polymers of RBPs [27, 28]. Actually, 1,6-HD melted the IDR polymers and intracellular puncta, assemblies of the PRn

poly-dipeptides with intermediate Filaments. The PRn-poly-dipeptides which are encoded by C9orf72 hexanucleotide expansions, has been identified in the lesion sites of ALS. The PRn poly-dipeptides bind to polymer of IDR proteins like TLS, and might cause ALS.

At neutral pH and physiological ionic strength TLS prompts its IDR to polymerize into labile, cross-beta fibers to form amyloid-like polymers, hydrogel droplets. Treatment of the TLS hydrogels with 1,6-HD resulted in melting them down. The same result was obtained also with another RBP, hnRNPA2. Previous studies have indicated that the PRn and Glycine-Arginine (GRn) poly-dipeptides encoded by C9orf72 repeat expansion bind to hydrogels composed of amyloid-like polymers of the IDRs of various RBP including hnRNPA2 and TLS [63]. The PRn exhibits more enhanced interaction with polymeric forms of IDRs than their unfolded, monomers. This suggests that the PRn interaction should be a cause for neurodegenerative disease ALS. On the other hands, 1,6-HD breaks the interaction of PRn and IDRs of RBPs. This implies that 1,6-HD could be a seed for drug development against ALS.

Incubation of the HA-tagged PR20 peptides with the 20 kDa IDR of hnRNPA2 resulted in immunoprecipitation with a HA antibody. Addition of 1,6-HD at 6% reduced interaction of HA-tagged PR20 with the IDR of hnRNPA2. This clearly indicates that 1,6-HD should block interaction of PR20 with hnRNPA2, and work as an inhibitor against phase separation and aggregation. These data suggest that 1,6-HD could interact with PRn peptides and/or the IDR of RBPs. On the other hands, present data of ours show that 1,6-HD binds to beta actin in nuclear extract of HeLa cells, suggesting that the beta actin is novel target molecule of 1,6-HD other than PRn peptides and the IDR of RBPs.

1,6-HD disrupts various cellular structures of membrane-less organelles which are based upon phase separations, in vitro and in vivo [31-33, 61, 64], while 1,6-HD has no effect on Endoplasmic Reticulum and mitochondria [65]. Furthermore, 1,6-HD has been reported to destroy the actin assembly of cytoskeleton organization [65], suggesting relation between inhibition of phase separation by 1,6-HD and function of actin. Our observation on the binding of 1,6-HD to beta actin might represent the data showing its disruption of the beta actin assembly in yeast and human HeLa cells [65]. Actin assembly is involved in phase separation in relation to cytoskeleton dynamics. It is possible that binding of 1,6-HD to beta actin might contribute to regulation of phase separation.

The actin cytoskeleton is a critical regulator of cytoplasmic architecture and mechanics, essential in numerous physiological processes. Assemblies of actin are regulated by phase separation in vitro [50] and in vivo systems [51]. Microscopic observation with cross-linked actin bundles revealed a liquid phase of actin filaments in the presence of the physiological cross-linker, filamin [50]. Filamin condenses short actin filaments into spindle-shaped droplets termed tactoids. The cross-linker filamin density controls the droplet shape and deformation timescales, consistent with a



variable interfacial tension and viscosity. These observations show a conceivable mechanism to control organization, morphology, and dynamics of the actin cytoskeleton through phase separation. Then, it is likely that actin works as a regulator for phase separation and resultant precipitation formation, confirming that the binding of 1,6-HD to beta actin should play a role in its inhibition to phase separation in cellular programs.

## 5. Conclusion

In order to explore a regulatory mechanism of phase separation and resultant aggregation that causes neurodegenerative diseases including ALS, we have employed affinity chromatography using a phase separation inhibitor, 1,6-HD. These experiments performed to search the initial event of inhibition of phase separation. We demonstrated binding of 1,6-HD to beta actin in NE. This finding indicates that interaction of 1,6-HD with beta actin could initiate inhibition of phase separation. Previous observation that 1,6-HD disrupts actin assemblies in the cytoskeleton of eukaryotic cells presents possibility of active control of actin cellular functions by the inhibition of phase separation prompted by 1,6-HD.

In the dendritic spine, actin is involved in neuronal functions. The actin assemblies play a pivotal role in long-term potentiation in the human central nervous system

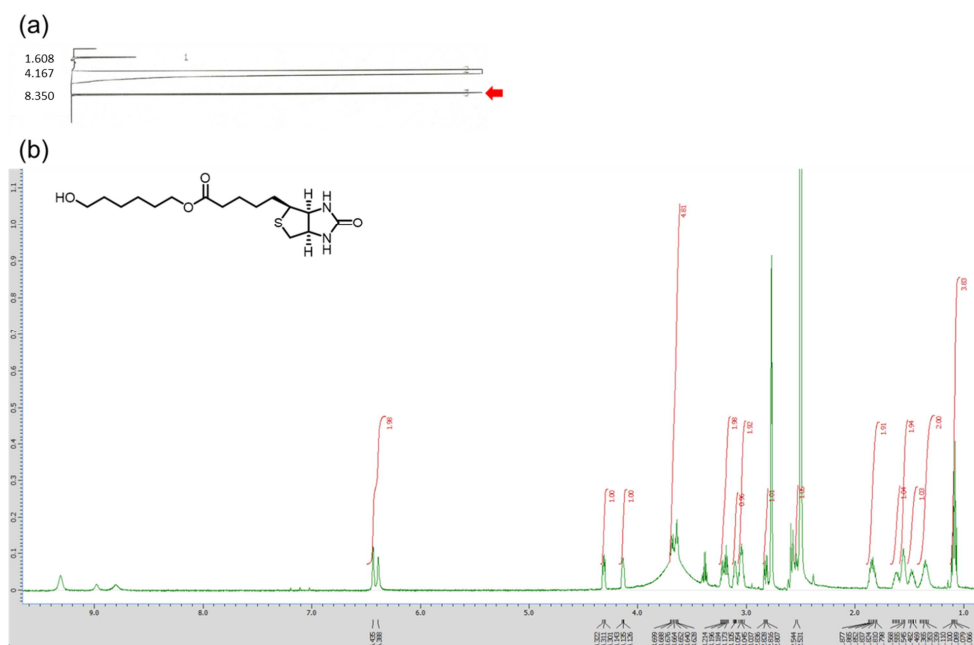
upon morphological alteration of the dendritic spine [66], suggesting that 1,6-HD could control memory formation in the human brain. Phase separation and precipitation has harmful effect on the motor neuron and cause neuronal death related to ALS. Inhibition of the precipitation or formation of inclusion bodies in the motor neuron of ALS patients will provide potential therapeutics with a seed for a drug against ALS.

Phase separation and precipitation elicited by the IDP conserved in the evolution over eukaryotic cells. The harmful precipitation should be restrained by the inhibitory systems of phase separation. The prevention of the precipitation would serve as a risk management action to maintain it for efficient biological activities. These prospects could contribute to future investigation of phase separation in divergent biological programs. This will enhance elucidation of open questions to be addressed in the future endeavors.

## Acknowledgements

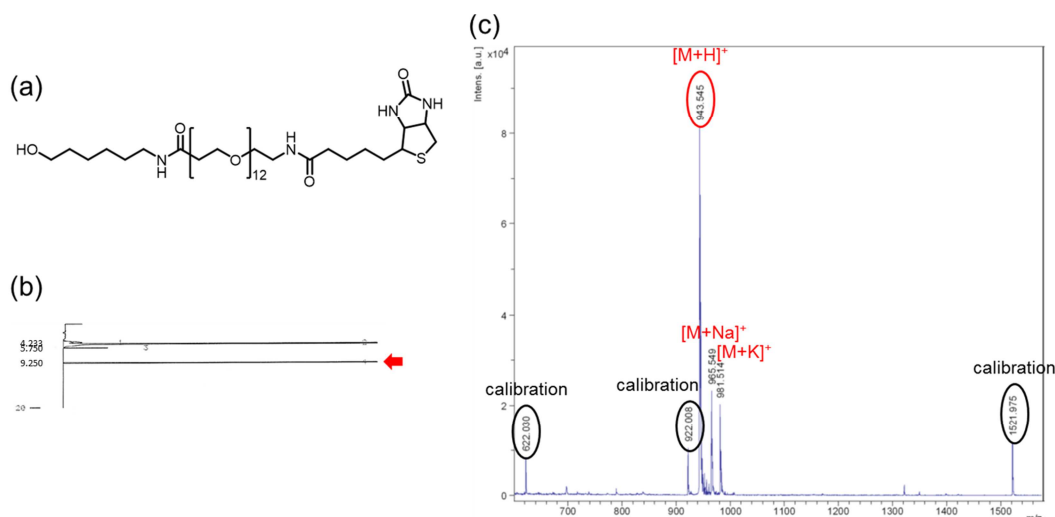
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## Appendix



**Figure 5.** Biotinylated 1,6-hexanediol (bio-1,6-HD).

(a) The HPLC spectrum of Biotinylated 1,6-hexanediol. Conditions: 0.1% TFA containing 0–100% acetonitrile over a linear gradient for 20 min at a flow rate of 1.0 mL/min detected at 205 nm. The retention time was 8.350 min. (b) The chemical structure of Biotinylated 1,6-hexanediol and the <sup>1</sup>H-NMR (600 MHz, DMSO-*d*<sub>6</sub>) spectrum of Biotinylated 1,6-hexanediol.  $\delta$  6.44 (s, 1H, H<sub>a</sub>), 6.39 (s, 1H, H<sub>a</sub>), 4.31 (t,  $J$ =6.3 Hz, 1H, H<sub>b</sub>), 4.14 (t,  $J$ =5.1 Hz, 1H, H<sub>b</sub>), 3.70–3.63 (m, 5H, H<sub>f</sub>), 3.24–3.17 (m, 2H, H<sub>c</sub>), 3.10 (q,  $J$ =6.2 Hz, 1H, H<sub>c</sub>), 3.05 (quin,  $J$ =7.5 Hz, 2H, H<sub>c</sub>), 2.82 (dd,  $J$ =12.0 Hz, 4.8 Hz, 1H, H<sub>d</sub>), 2.54 (d,  $J$ =7.8 Hz, 1H, H<sub>d</sub>), 1.88–1.80 (m, 2H, H<sub>e</sub>), 1.65–1.59 (m, 1H, H<sub>e</sub>), 1.57–1.52 (m, 2H, H<sub>e</sub>), 1.51–1.44 (m, 1H, H<sub>e</sub>), 1.40–1.33 (2H, m, H<sub>e</sub>), 1.09 (quin,  $J$ =6.6 Hz, 4H, H<sub>e</sub>).



**Figure 6.** Biotinylated PEG<sub>12</sub>-NH-hexanol (bio-1,6-HD plus).

EZ-Link™ NHS-PEG<sub>12</sub>-Biotin 250 mM in DMF 21.25 μL (5.3 μmol) was mixed with 128.3 μL of DMF, 6-amino-1-hexanol (0.78 mg, 1.2 equiv.), and DIEA (1.08 μL, 1.2 equiv.) and then stirred at room temperature for 2.5 hours. The mixture was purified by reverse-phase HPLC and 3.2 mg of target material (colorless oil) was obtained (3.4 μmol, yield: 64%, analytical HPLC: tR=9.3 min. MALDI-TOF MS: m/z calcd for C<sub>43</sub>H<sub>83</sub>N<sub>4</sub>O<sub>16</sub>S<sup>+</sup>[M+H]<sup>+</sup> 943.54, found; 943.55).

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