

Graphene Quantum Dots Attenuate TDP-43 Proteinopathy in Amyotrophic Lateral Sclerosis

Na Young Park,⁺ Yunseok Heo,⁺ Ji Won Yang, Je Min Yoo, Hye Ji Jang, Ju Hee Jo, Su Jeong Park, Yuxi Lin, Joonhyeok Choi, Hyeonjin Jeon, Sun Joo Cha, Gaeun Bae, Donghoon Kim, Juhee Kim, Wade Zeno, Jong Bo Park, Noriyoshi Isozumi, Tomohide Saio, Seung Hyun Kim, Hojae Lee, Byung Hee Hong,^{*} Minyeop Nahm,^{*} Young-Ho Lee,^{*} and Young Bin Hong^{*}



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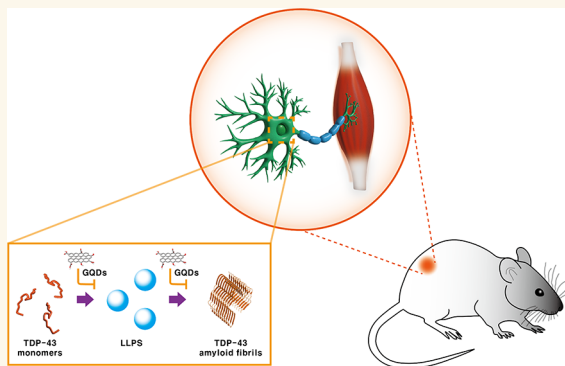
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ABSTRACT: Aberrant phase separation- and stress granule (SG)-mediated cytosolic aggregation of TDP-43 in motor neurons is the hallmark of amyotrophic lateral sclerosis (ALS). In this study, we found that graphene quantum dots (GQDs) potentially modulate TDP-43 aggregation during SG dynamics and phase separation. The intrinsically disordered region in the C-terminus of TDP-43 exhibited amyloid fibril formation; however, GQDs inhibited the formation of amyloid fibrils through direct intermolecular interactions with TDP-43. These effects were accompanied by attenuation of the ALS phenotype in animal models. Additionally, GQDs delayed the onset and survival of TDP-43 transgenic mouse models by enhancing motor neuron survival, reducing glial activation, and reducing the cytosolic aggregation of TDP-43 in motor neurons. In this research, we demonstrated the efficacy of GQDs on the SG-mediated aggregation of TDP-43 and the binding property of GQDs with TDP-43. Additionally, we demonstrated the clinical feasibility of GQDs using several animal models and other types of ALS caused by FUS and C9orf72. Therefore, GQDs could offer a new therapeutic approach for proteinopathy-associated ALS.

KEYWORDS: TDP-43, graphene quantum dots, liquid–liquid phase separation, stress granules



INTRODUCTION

Stress granules (SGs), which are membrane-less organelles, are formed by spontaneous liquid–liquid phase separation (LLPS) of initiation complexes and several RNA-binding proteins (RBPs), such as fused in sarcoma (FUS) and transactive response DNA-binding protein-43 (TDP-43), and their target RNAs, resulting in physicochemical separation from the environment.^{1–4} The transient formation of SGs is a protective mechanism against cellular stress. However, defective disassembly or clearance of SGs leads to the pathological irreversible phase transition in neurodegenerative diseases such as ALS and frontotemporal dementia (FTD).^{5,6} Notably, TDP-43 or FUS entrapped into LLPS might further undergo insoluble aggregates and pathogenic fibrillization,^{7,8} which exhibits toxic gain-of-function in the affected neurons.^{9,10} In addition, cytotoxic aggregation deprives those proteins of their nucleus, resulting in RNA processing defects. Therefore, the reversal of SG- or LLPS-mediated protein aggregation

dynamics might be an urgent issue in treating neurodegenerative diseases.

In amyotrophic lateral sclerosis (ALS), most patients (>97%) harbor cytoplasmic aggregates of TDP-43, regardless of etiology.¹¹ TDP-43, a predominant nuclear protein, is an RBP involved in alternative splicing and transcriptional regulation as well as the transport of mRNA into the cytoplasm.^{12–14} Loss of TDP-43 in the nucleus significantly affects RNA metabolism. Recent findings revealed that defects in the splicing activity of TDP-43 lead to the reduction of

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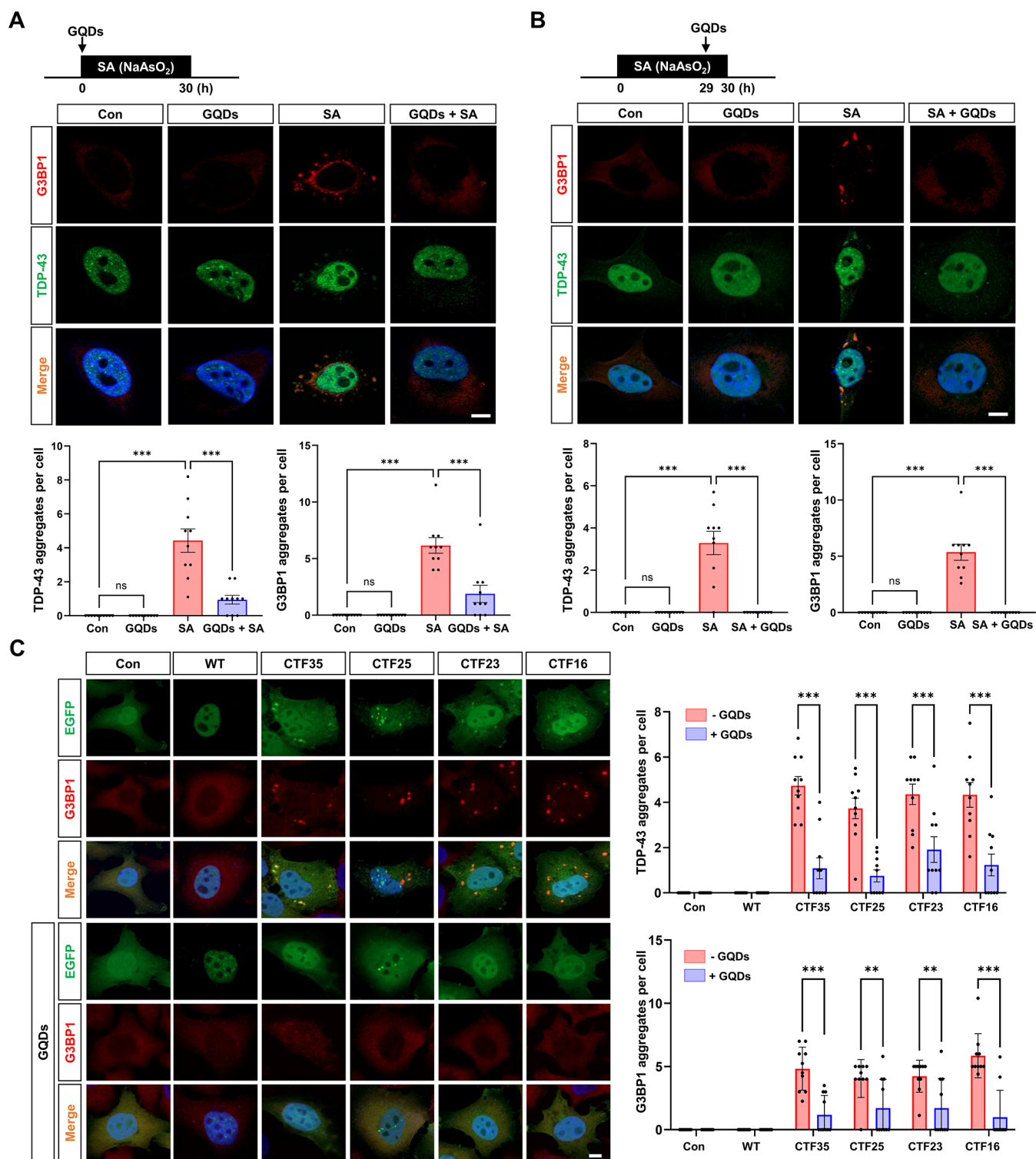


Figure 1. Prevention of stress-mediated TDP-43 aggregation by GQDs. (A) Immunocytochemical analyses revealed that a 30 h treatment with 10 μ M sodium arsenite (SA)-induced cytoplasmic aggregation of TDP-43 and coincubation of GQDs (1 μ g/mL) prevented aggregation in HeLa cells. The endogenous TDP-43 (green) and G3BP1 (red) aggregates per cell (blue for DAPI) were analyzed from each image ($n = 10$) and compared with untreated control cells (Con). Scale bar: 10 μ m. (B) Elimination of the aggregated TDP-43 by GQDs was analyzed. The cytosolic aggregation of TDP-43 was significantly reduced after 1 h of incubation of GQDs in the SA-pretreated HeLa cells. The endogenous TDP-43 (green) and G3BP1 (red) aggregates per cell were analyzed for each image ($n = 10$) and compared with untreated control cells (Con). Scale bar: 10 μ m. The significance was assessed by a one-way ANOVA followed by Tukey's posthoc test. (C) Aggregation of the C-terminal fragment (CTF) mutants of TDP-43 was prevented by treatment with GQDs. The HeLa cells transfected with EGFP-tagged full-length (WT) or each CTF for 48 h exhibited cytosolic aggregation without any stress, while the incubation of GQDs (1 μ g/mL) significantly reduced the aggregations. The EGFP-tagged TDP-43 (green) and G3BP1 (red) aggregates per cell (blue for DAPI) were analyzed from each image ($n = 10$) and compared with control (transfected with a vehicle vector, Con). Scale bar: 10 μ m. The significance was assessed by a two-way ANOVA followed by Sidák's posthoc test. ** $P < 0.01$ and *** $P < 0.001$.

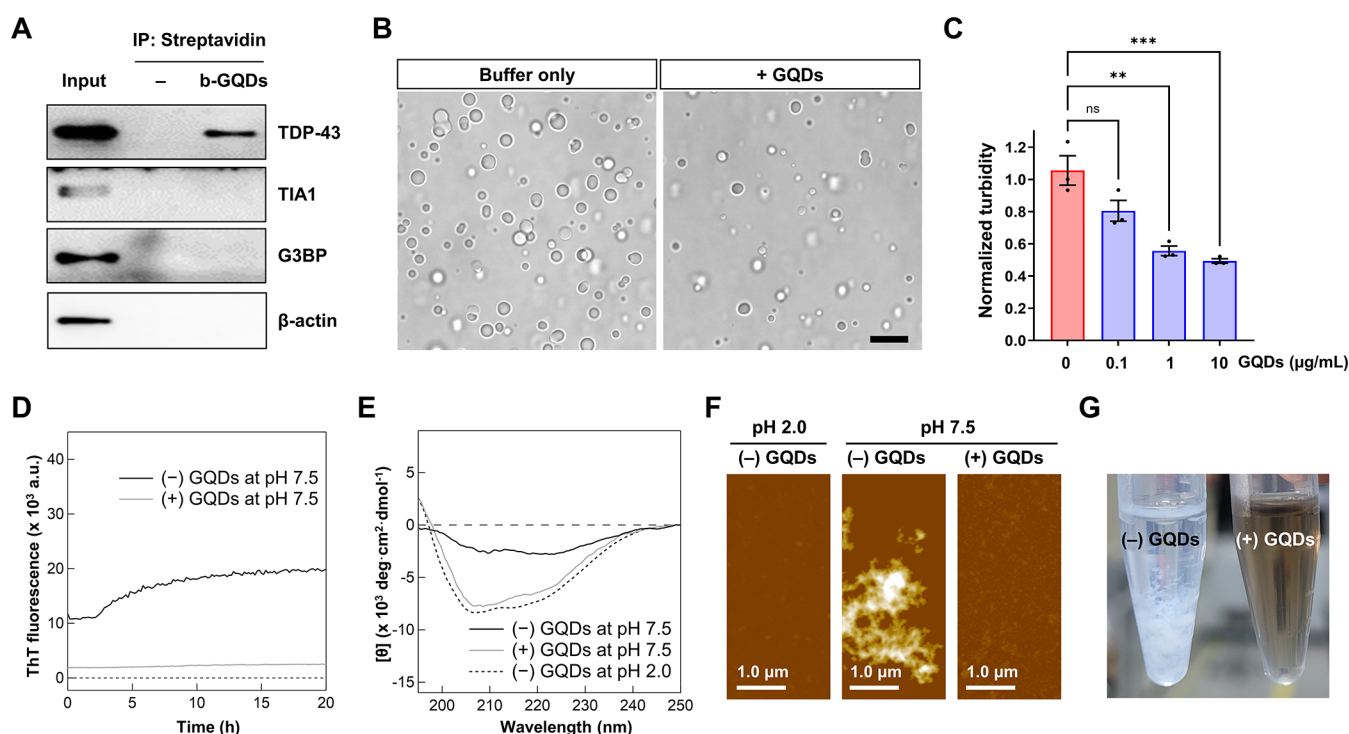


Figure 2. Effects of GQDs on the phase separation and amyloid formation of recombinant TDP-43. (A) Immunoprecipitation revealed that GQDs bind to TDP-43. The total HeLa cell lysates were incubated with 10 μg of biotinylated GQDs (b-GQDs). Then, immunoprecipitation was performed with streptavidin-conjugated agarose. Western blotting revealed that only TDP-43 was bound to b-GQDs. (B) Representative differential interference contrast images of TDP-43-MBP-His₆ (40 μM) droplets incubated with buffer or GQDs (1 $\mu\text{g}/\text{mL}$). Scale bar: 10 μm . (C) Liquid–liquid phase separation measured by turbidity (OD = 600 nm) in the same experimental conditions as (B) with indicated concentration of GQDs ($n = 3$). ns, nonsignificant; ** $P < 0.01$ and *** $P < 0.001$. (D) Amyloid fibrillation of full-length TDP-43 in the absence (black curve) and presence (gray curve) of GQDs at pH 7.5 was monitored using thioflavin T (ThT) fluorescence. (E) Far-UV CD spectra of full-length TDP-43 before (black dotted curve) and after (solid curves) the incubation. The two solid curves represent the CD spectra after the incubation with (gray solid curve) and without (black solid curve) GQDs. (F) AFM images of full-length TDP-43 obtained before the incubation (left) and after the incubation in the absence (middle) and presence (right) of GQDs. Scale bar: 1.0 μm . (G) Photograph of full-length TDP-43 in the absence (left) and presence (right) of GQDs at pH 7.5.

functional Stathmin 2 (STMN2) and Unc-13 homologue A (UNC13A) protein levels *via* inclusion of a cryptic exon of those genes.^{14–17} Under pathologic conditions, TDP-43 is often observed as hyperphosphorylated, ubiquitinated, and cleaved C-terminal fragments (CTFs) in the affected neurons of ALS patients.^{18,19} Notably, the C-terminal of TDP-43 harbors aggregation-prone, intrinsically disordered region (IDR); therefore, prion-like CTFs induce the seed-dependent formation of TDP-43 inclusions.^{20–22}

To modulate the TDP-43 aggregation-mediated proteinopathy in ALS, numerous approaches have been attempted including activation of degradation pathways using proteolysis-targeting chimera and viral delivery of antibody as well as prevention of its aggregation using oligonucleotides composed of TDP-43 target sequences.^{23–26} Recently, the application of nanoparticles to block the aggregation or fibrillation of amyloid proteins has been extensively studied.^{27,28} In this study, we assessed the potency of graphene quantum dots (GQDs) in attenuating the phenotype of ALS. Previously, GQDs have been reported to inhibit the fibrillization of α -synuclein through direct interaction with mature fibrils and facilitate their disaggregation.^{29,30} This efficacy was also observed in ALS causative genes, such as TDP-43 and FUS. GQDs can modulate the SG formation and phase transition of FUS,³¹ and modified GQDs exhibited TDP-43 phase separation and aggregation in the nucleus.³² In addition, GQDs can attenuate

excessive inflammation by regulating immune cells,³³ and the delivery through the blood–brain barrier (BBB) has raised the clinical applicability of GQDs in treating neurodegenerative diseases.^{29,34} We demonstrated the feasibility of GQDs in modulating SG formation and phase separation of TDP-43, identified the direct interaction of GQDs with TDP-43, and proved the efficacy of attenuating the phenotype of ALS mouse models.

RESULTS

Graphene Quantum Dots (GQDs) Prevent SG Formation and Block TDP-43 Aggregation. We generated the graphene quantum dots (GQDs) using carbon fibers with a 3:1 blend of sulfuric acid and nitric acid (Figure S1A–F).²⁹ The generated GQDs exhibited no cytotoxicity (up to 100 $\mu\text{g}/\text{mL}$) to a mouse motor-neuron-like cell line, NSC-34 cells. The effective delivery of GQDs into the cytosol of NSC-34 cells was also monitored using biotinylated GQDs (b-GQDs) (Figure S1G–I). Then, we determined whether GQDs have potency in preventing the formation of stress granules (SGs) using TIA1 cytotoxic granule-associated RNA binding protein (TIA1) and G3BP stress granule assembly factor 1 (G3BP1) as SG markers.³⁵ Treatment with sodium arsenite (SA) induced the formation of SGs; however, pre- and postincubation of GQDs significantly blocked the formation of the aggregates in a dose-dependent manner (Figures S2 and S3).

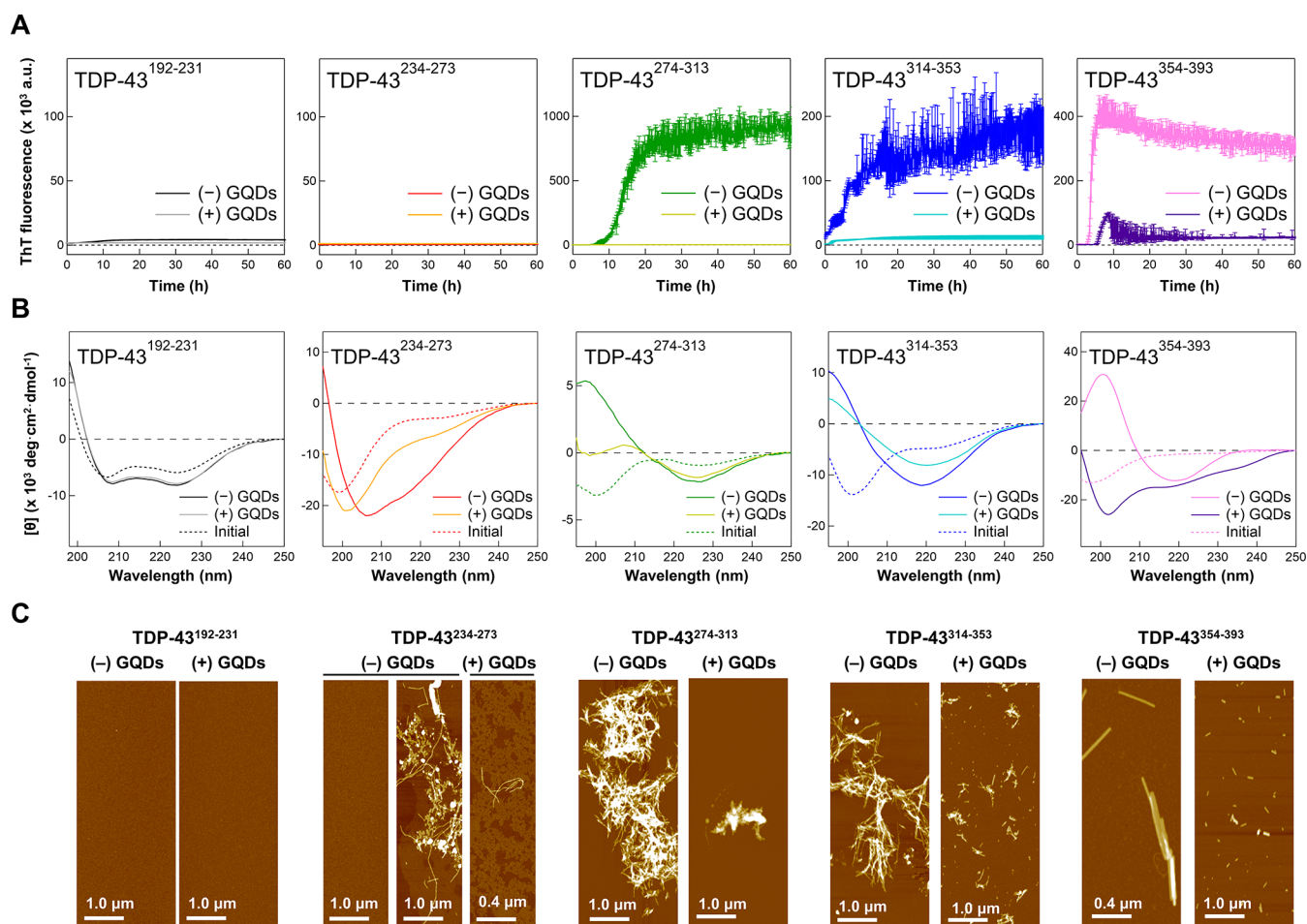


Figure 3. Effects of GQDs on amyloid formation of TDP-43 peptides. (A) Amyloid fibrillation of the five peptides of TDP-43 in the absence and presence of GQDs was monitored through thioflavin T (ThT) fluorescence. Each point is presented as the mean value $\pm$ SD of three independent experiments. (B) Far-UV CD spectra of the TDP-43 peptides before (dotted line) and after (solid line) the incubation. The two solid lines for each peptide present the CD spectra after the amyloid fibrillation with and without GQDs. The CD measurements were performed immediately after the incubation, except for TDP-43^{234–273}, which was measured after further incubation for 2 weeks. (C) AFM micrographs of the TDP-43 peptides obtained immediately after the incubation. For TDP-43^{234–273}, the second and third micrographs were obtained after an additional incubation for 2 weeks.

Next, we investigated whether TDP-43 aggregation during SG formation can be blocked by incubation with GQDs. Under oxidative stress, endogenous TDP-43 can be aggregated with G3BP1-positive SGs; however, the preincubation of GQDs significantly reduced the SA-induced aggregation of TDP-43 and G3BP1 (Figure 1A). On the other hand, the addition of GQDs during the oxidative stress also dissociated the aggregated TDP-43 (Figure 1B). The suppression of TDP-43 aggregation by GQDs was also observed in a dose-dependent manner (Figures S4 and S5). Then we determined whether the modulation of the TDP-43 aggregation by GQDs is effective for the ALS-associated mutations. As C-terminal fragments (CTFs) of TDP-43 such as CTF35 and CTF25 have been detected in the nerve tissues of ALS patients, and several point mutations in TDP-43 are involved in the familial type of ALS, we generated four CTFs of TDP-43 (CTF35, CTF25, CTF23, and CTF16) as well as two point mutants (A315T and Q331K) (Figure S6A). Overexpression of all of the mutants, as well as wild-type (WT) TDP-43, significantly reduced the viability of NSC-34 cells, but those were restored by the incubation of GQDs, which was also in a dose-dependent manner to WT of TDP-43 or CTF35 transfected cells (Figure

S6B–D). In the point mutants (A315T and Q331K) of TDP-43 overexpressing cells, osmotic and oxidative stress using 0.4 M sorbitol (SO) induced SG formation as well as coaggregation of transiently expressed TDP-43 mutant proteins, which were significantly blocked by GQD treatments (Figure S6E). On the other hand, the overexpressed CTF proteins aggregated with G3BP1 without any oxidative stress, while the treatment of GQDs effectively eliminated the preformed aggregates of the G3BP1-positive CTFs (Figure 1C). Additionally, b-GQDs also exhibit similar cytoprotective and TDP-43 aggregation-preventing effects (Figure S7).

GQDs Inhibit Phase Separation and Amyloid Fibrillation of Recombinant TDP-43 *In Vitro*. To address whether GQDs can directly interact with TDP-43, we performed an immunoprecipitation assay using biotinylated GQDs. Total lysates of HeLa cells were harvested, and then the biotinylated GQDs and streptavidin-conjugated agarose were incubated. Immunoblotting with specific antibodies revealed that GQDs specifically interact with TDP-43 rather than TIA1 or G3BP1 (Figure 2A). ALS-linked RNA binding proteins such as TDP-43 and FUS are known to incorporate into SGs through liquid–liquid phase separation (LLPS). Based on our data

showing that GQDs are closely associated with SG dynamics, we proceeded to investigate whether GQDs also inhibit the phase separation of TDP-43. Under *in vitro* conditions using 5% PEG as a crowding agent, purified recombinant TDP-43-MBP-His₆ spontaneously formed liquid-like droplets. We found that GQDs are capable of effectively inhibiting the formation of TDP-43 liquid droplets (Figure 2B). Turbidity measurement under the same conditions also revealed that GQDs inhibit LLPS of TDP-43 in a dose-dependent manner (Figure 2C). These data indicate that GQDs can modulate the phase separation properties of TDP-43, thereby inhibiting its aggregation.

To examine the inhibitory effects of GQDs on full-length TDP-43, we performed ThT fluorescence assays, CD spectra analyses, and AFM imaging on full-length TDP-43. We first prepared full-length TDP-43 with the MBP tag and TEV cleavage site in *Escherichia coli*. To solubilize full-length TDP-43 after TEV cleavage at pH 7.5, we used an acidic solution at pH 2.0. The amyloid formation was initiated using a pH jump from 2.0 to 7.5. The fast amyloid formation of full-length TDP-43 at pH 7.5 was traced (Figure 2D). The far-UV CD spectra showed the structural transition of full-length TDP-43 monomers to β -structured amyloid fibrils with a minimum CD intensity of 222 nm (Figure 2E). The fibrillar aggregates were observed in the AFM image (Figure 2F). All of these results revealed amyloidogenesis of TDP-43 at neutral pH. The addition of GQDs significantly decreased ThT fluorescence intensity at pH 7.5 (Figure 2D). Notably, GQDs possess broad absorption bands that overlap entirely with the absorption spectrum of ThT. Such an overlap can cause significant inner filter effects, leading to the quenching of the ThT fluorescence, as previously reported.^{36,37} The far-UV CD spectrum of TDP-43 solution with GQDs after incubation at pH 7.5, showing the two minima at 209 and 218 nm, was very similar to that of TDP-43 solution without GQDs at pH 2.0 where full-length TDP-43 exists as soluble monomers (Figure 2E). We could not find any fibrillar aggregates in the AFM image of full-length TDP-43 samples in the presence of GQDs (Figure 2F). The rapid aggregation and precipitation of TDP-43 without GQDs were observed; however, TDP-43 solution with GQDs was transparent even after incubation (Figure 2G). Taken together, GQDs inhibit the amyloid aggregation of full-length TDP-43.

GQDs Inhibit Amyloid Formation of the C-Terminal Intrinsically Disordered Region (IDR). Since the antiaggregating effects of GQDs toward TDP-43 were observed in all CTFs, the binding targets of GQDs might be located in the intrinsically disordered region (IDR) of TDP-43. To gain deeper insights into the effects of GQDs on TDP-43 aggregation, we carefully selected the four peptides (TDP-43^{234–273}, TDP-43^{274–313}, TDP-43^{314–353}, and TDP-43^{354–393}) based on the atomistic structure of TDP-43 (Figure S8A–D) and previous study.³⁸ We added one more peptide in RNA recognition motif 2 (RRM2) for a control (TDP-43^{192–231}). Among them, TDP-43^{192–231} and TDP-43^{234–273} are located in RNA recognition motif 2 (RRM2). TDP-43^{274–313}, TDP-43^{314–353}, and TDP-43^{354–393} are situated in IDR, which constitutes the core region of full-length TDP-43 amyloid fibrils. The structural prediction using AlphaFold2 revealed that TDP-43^{192–231} and TDP-43^{234–273} adopt α -helices and β -strands, while others are predominantly disordered (Figure S8B–G).

To study the aggregation kinetics within a time frame suitable for analyses, thioflavin T (ThT) fluorescence assays of

five TDP-43 peptides were applied. Based on the previous reports,^{39–42} we performed ThT assay under four distinct conditions in variations of the addition of beads or NaCl, and the condition including 100 mM NaCl without beads was selected for further investigation (Figure S9 and Tables S1 and S2).

In the absence of GQDs, TDP-43^{192–231}, and TDP-43^{234–273} did not exhibit a significant increase in the ThT fluorescence for 60 h incubation (Figure 3A). To further characterize the end state of the samples, circular dichroism (CD) and atomic force microscopy (AFM) were performed. The CD spectrum and AFM image of TDP-43^{192–231} revealed that it maintained predominantly helical conformations without significant aggregation (Figure 3B,C), while fit analyses of the cooperative transition curve indicated that TDP-43^{192–231} is three-dimensionally folded (Figure S10). In the presence of GQDs, the ThT fluorescence of TDP-43^{192–231} remained consistently low during incubation (Figure 3A). TDP-43^{234–273} also did not exhibit a significant increase in the ThT fluorescence for 60 h incubation (Figure 3A); however, the CD spectrum showed a significant alteration, characterized by an enhanced signal at 218 nm after further incubation for 2 weeks. The AFM images revealed a significant number of amyloid fibrils with extended incubation for 2 weeks (Figure 3C). In the presence of GQDs, the ThT fluorescence of TDP-43^{234–273} remained consistently low during incubation (Figure 3A). Although the AFM images of TDP-43^{234–273} presented small fibrillar aggregates, the postincubation CD spectra exhibited a single negative band at $\sim$ 200 nm in the presence of GQDs (Figure 3B,C).

On the other hand, the ThT fluorescence of TDP-43^{274–313} and TDP-43^{354–393} increased after a time lag of $\sim$ 9 and $\sim$ 3 h in the absence of GQDs and reached a plateau at $\sim$ 20 and $\sim$ 8 h, indicating the nucleation-dependent amyloid formation, which has been commonly observed in previous amyloidogenesis studies. TDP-43^{314–353} exhibited a gradual increase in the ThT fluorescence without a time lag, possibly due to the rapid formation of numerous nuclei (Figure 3A and Table S1). Far-UV CD spectra also revealed that TDP-43^{274–313}, TDP-43^{314–353}, and TDP-43^{354–393} without GQDs exhibited a single minimum at $\sim$ 218 or $\sim$ 225 nm, suggesting the presence of β -strand-rich structures of the amyloid fibrils (Figure 3B). β -Structure-rich proteins typically exhibit a minimum at $\sim$ 218 nm, while proteins with antiparallel β -strand-rich structures show a minimum around 220–230 nm.⁴³ Furthermore, the AFM results revealed the formation of aggregates with a fibrillar morphology (Figure 3C). The presence of GQDs significantly suppressed the increase in the ThT fluorescence of all of the three peptides, which also accounts for the quenching effect of GQDs as we mentioned earlier. Further assessments using CD revealed a single negative band of TDP-43^{354–393} at $\sim$ 200 nm, suggesting that the majority of this peptide still maintained its disordered structure. On the other hand, the CD spectra of TDP-43^{274–313} and TDP-43^{314–353} showed the existence of fibrillar aggregates with the β -structures. However, the signal intensity at 218 nm was lower than that of the spectra obtained without GQDs, possibly indicating a reduction in the number of fibrils. AFM further verified this finding by displaying significantly fewer amyloid fibrils than in the samples without GQDs (Figure 3C).

In addition, one-dimensional proton nuclear magnetic resonance (1D-¹H NMR) spectra showed that the intensity of the four peptides except TDP-43^{192–231} decreased significantly after the incubation due to the formation of

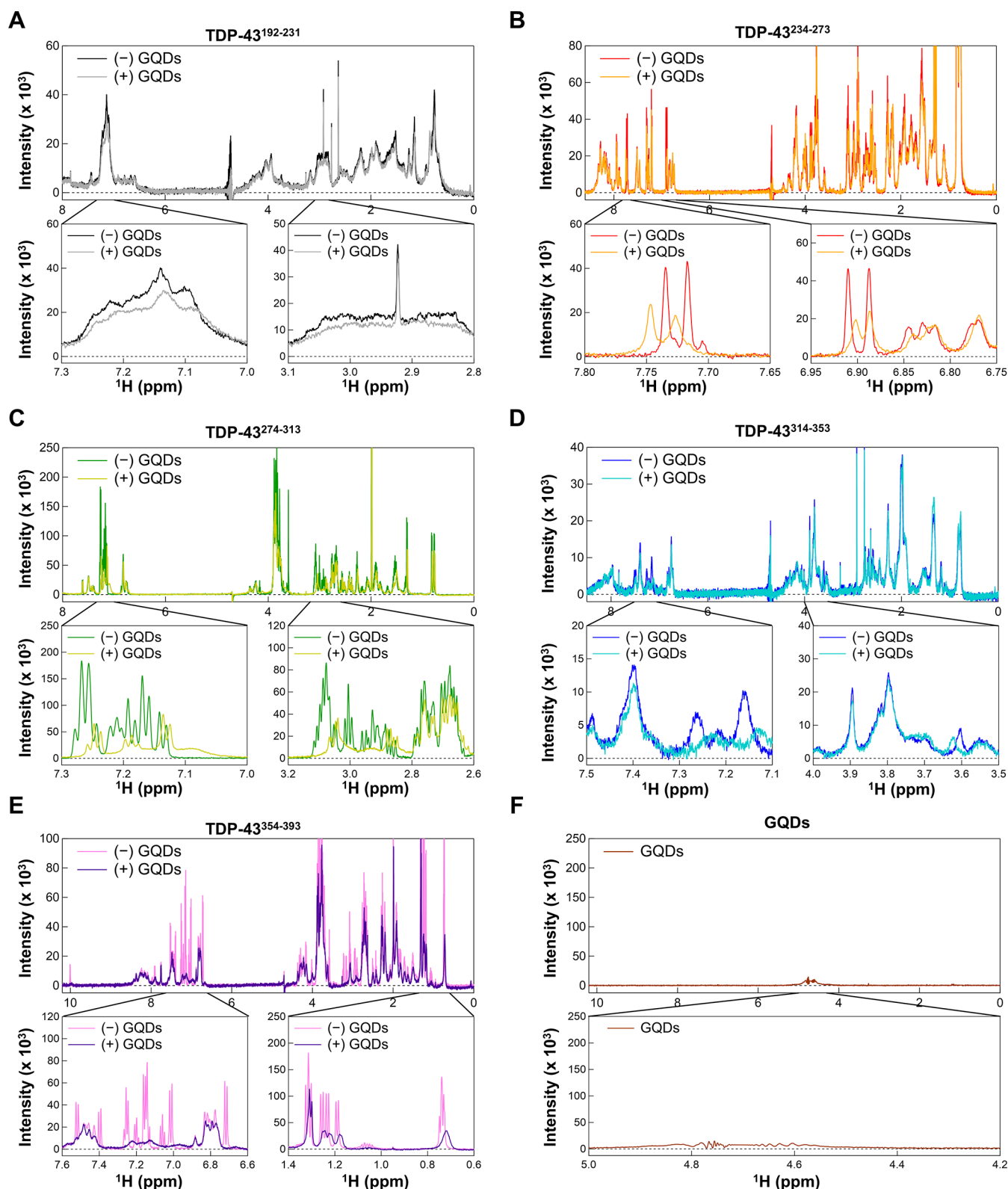


Figure 4. Interaction of GQDs with the TDP-43 peptides. (A–E) 1D-¹H NMR spectra of TDP-43^{192–231} (A), TDP-43^{234–273} (B), TDP-43^{274–313} (C), TDP-43^{314–353} (D), and TDP-43^{354–393} (E) in the absence and presence of GQDs. The magnified regions highlight the GQD-induced differences in the low- and high-field regions, respectively. (F) 1D-¹H NMR spectra of GQDs. The region where peaks are present was magnified.

large molecules such as amyloid fibrils (Figure S11). Collectively, the amyloid formations of TDP-43 peptides

except for TDP-43^{192–231} were detected, and GQDs can inhibit the amyloid formation of those TDP-43 peptides.

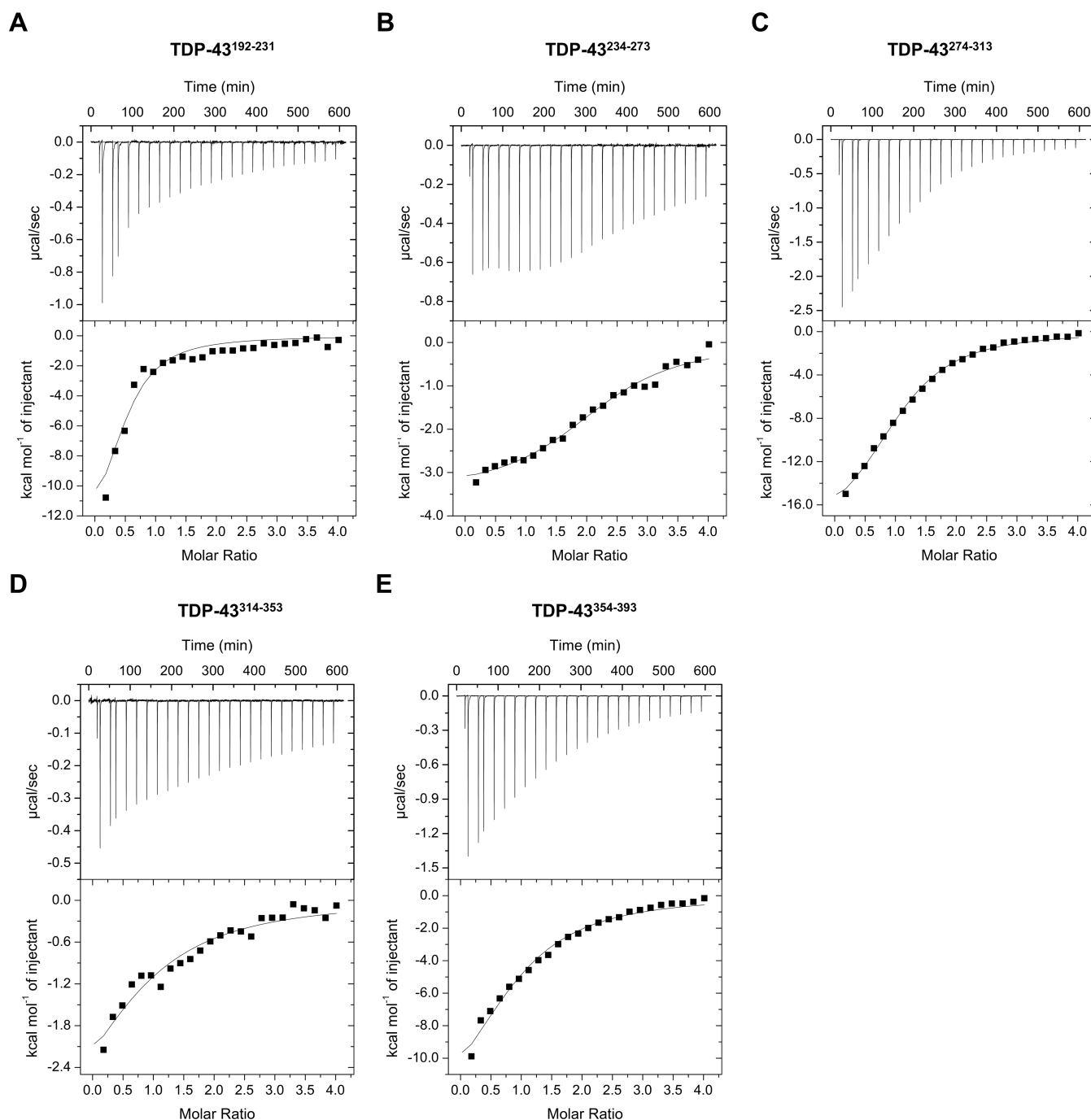


Figure 5. Isothermal titration calorimetry (ITC)-based characterization of the intermolecular interaction between TDP-43 peptides and GQDs. The ITC thermogram (top) and binding isotherm (bottom) resulting from titrating GQDs to each peptide are shown: (A) TDP-43^{192–231}, (B) TDP-43^{234–273}, (C) TDP-43^{274–313}, (D) TDP-43^{314–353}, and (E) TDP-43^{354–393}. The solid lines in the binding isotherms represent the fitting curves based on a one-set-of-sites binding model.

GQDs Directly Bind to IDR of TDP-43. To probe whether the inhibitory effect of GQDs is linked to their interactions with the TDP-43 peptides, we recorded the 1D-¹H NMR spectra of five different TDP-43 peptides in the presence or absence of GQDs. Upon the addition of GQDs, significant alterations were observed in the NMR spectra. The intensity of several peaks of TDP-43^{192–231} was diminished by ~20% when GQDs were added (Figure 4A). Moreover, the NMR spectra for TDP-43^{234–273}, TDP-43^{274–313}, TDP-43^{314–353}, and TDP-43^{354–393} exhibited substantial chemical shift perturbations and reduced the peak intensities in the chemical shift regions of

7.7–7.8, 7.1–7.3, 7.1–7.5, and 6.6–7.6 ppm, respectively (Figure 4B–E). The 1D-¹H NMR spectra of GQDs exhibited extremely weak intensity in the ranges of 0–4 ppm and 6–10 ppm (Figure 4F). Thus, GQDs had a negligible influence on the NMR spectra of each TDP-43 peptide. The surface of GQDs used in this study consists of the hydrophobic backbone and two carboxyl groups as characterized in our previous report.²⁹ Both hydrophobic and charged regions showed the importance of interacting with proteins and aggregates.^{29,44} To thermodynamically explore the intermolecular interactions between TDP-43 peptides and GQDs, we conducted

Table 1. Thermodynamic Parameters for the Binding of GQDs to each TDP-43 Peptide^a

	TDP-43 ^{192–231}	TDP-43 ^{234–273}	TDP-43 ^{274–313}	TDP-43 ^{314–353}	TDP-43 ^{354–393}
ΔH (kcal/mol)	-15.5 ± 4.2	-3.4 ± 0.1	-19.3 ± 0.6	-4.5 ± 1.9	-15.9 ± 2.0
$T\Delta S$ (kcal/mol)	-8.3 ± 5.9	3.6 ± 0.2	-12.3 ± 0.8	1.8 ± 2.7	-9.4 ± 2.8
ΔG (kcal/mol)	-7.1 ± 4.2	-7.0 ± 0.1	-7.0 ± 0.6	-6.3 ± 1.9	-6.6 ± 2.0
K_D (μM)	6.1 ± 2.4	6.8 ± 1.1	7.5 ± 0.6	25.2 ± 9.6	14.9 ± 2.5

^a ΔH , change in enthalpy; T , absolute temperature; ΔS , change in entropy; ΔG , Gibbs free energy change; and K_D , dissociation constant.

isothermal titration calorimetry (ITC) (Figure 5). Titration of GQDs with each of the TDP-43 peptides exhibited exothermic heat in the ITC thermograms, indicating intermolecular interactions. The dissociation constant (K_D) values ranged from 6.1 ± 2.4 to $25.2 \pm 9.6 \mu\text{M}$, corresponding to the Gibbs free energy change (ΔG) values from -7.1 ± 4.2 to -6.3 ± 1.9 kcal/mol. The binding of TDP-43^{192–231}, TDP-43^{234–273}, and TDP-43^{274–313} was highly strong, and TDP-43^{314–353} and TDP-43^{354–393} also showed relatively strong binding affinities for GQDs. All complexation was driven by an energetically favorable negative change in enthalpy (ΔH). Change in entropy (ΔS) assisted the intermolecular binding with the positive sign for TDP-43^{234–273} and TDP-43^{314–353} while the loss of entropy gain hampered the intermolecular interactions of GQDs for TDP-43^{192–231}, TDP-43^{274–313}, and TDP-43^{354–393} (Table 1). In conclusion, these data provided compelling evidence of a direct interaction between GQDs and the five TDP-43 peptides.

Amelioration of the ALS Phenotype by GQDs in the TDP-43 Mouse Models. Next, we investigated whether GQDs can ameliorate the ALS phenotype. Before the evaluation, we observed that the biotinylated GQDs could be delivered into the ventral horn of the spinal cord and layer V of the motor cortex (Figure S12). Then we evaluated the efficacy of GQDs with two independent experiments by administering GQDs to the TDP-43^{A315T} mice (Figure 6A).⁴⁵ This administration of GQDs drastically delayed the onset (61.8 ± 3.1 days in male and 86.7 ± 2.2 days in female) compared with the control TDP-43^{A315T} mice (54.6 ± 2.7 days in male and 74.4 ± 3.6 days in female) in both genders. Additionally, GQDs prolonged the survival (117.6 ± 7.3 days in male and 218.6 ± 8.3 days in female) of the TDP-43^{A315T} mice compared with the control TDP-43^{A315T} mice (100.5 ± 6.5 days in male and 193.2 ± 4.5 days in female) (Figure 6B,C). Further, improvement of the disease phenotype by GQDs was observed through the rotarod performance and grip strength (Figure 6D,E).

Next, we analyzed the integrity of the motor neurons in both the spinal cord and the motor cortex. In the control TDP-43^{A315T} female mice, a reduced number of healthy motor neurons (larger than $25 \mu\text{m}$) and increased dysmorphic neurons were observed in the ventral horn of the spinal cord compared with the WT mice. However, the integrity of the motor neurons was significantly protected by GQDs (Figure 6F). The activation of astrocytes and microglia in the tissues of TDP-43^{A315T} was also reduced after administration of GQDs. The protection of the motor neurons and inhibition of glial activation by GQDs were also observed in the motor cortex (Figure S13). To analyze the correlation between the histological improvements by GQDs and the attenuation of TDP-43 aggregation, we examined TDP-43 aggregation in the motor neurons. Compared with the WT control, most motor neurons in the ventral horn of the TDP-43^{A315T} mice exhibited

cytosolic aggregation of TDP-43. However, administration of GQDs significantly reduced TDP-43 aggregation (Figure 6G).

To ascertain our findings, we evaluated the efficacy of GQDs using another TDP-43 mouse model, TDP-43^{WT} mouse.⁴⁵ As the mouse model harbors the WT human TDP-43 in the X-chromosome, we could obtain only female mice from *in vitro* fertilization. The administration of GQDs significantly delayed the disease onset and prolonged survival in the TDP-43^{WT} mouse (Figure S14). The protection of motor neurons was also observed in the TDP-43^{WT} mouse. The increase of motor neurons by GQD administration was observed in the spinal cord of the TDP-43^{WT} mouse. Additionally, the cytosolic aggregation of TDP-43 in the ventral horn was significantly reduced by GQDs compared with control TDP-43^{WT} mice (Figure S15). Increased numbers of activated astrocytes and microglia were also found in the motor cortex of TDP-43^{WT} mice, while GQD administration attenuated their activation (Figure S16). Collectively, these data suggested that the TDP-43 aggregation-mediated toxicity in motor neurons might be alleviated by administering GQDs in the TDP-43 mouse models.

GQDs Modulate TDP-43 Aggregation in Induced Pluripotent Stem Cell (iPSC)-Derived Motor Neurons (MNs). To investigate whether the effects of GQDs on TDP-43 aggregation are consistent in neuronal cells, which are mainly affected in ALS, we assessed the cytoplasmic aggregation of TDP-43 in induced pluripotent stem cell (iPSC)-derived motor neurons (MNs) under stress conditions. For generation of heterozygous TDP-43 mutation-harboring MNs, we used TDP-43^{M337V} iPSC line that was genetically edited from its isogenic control (TDP-43^{WT}) iPSC line. After differentiation into MNs, we confirmed that both iPSC-MNs expressed neuronal (tubulin β 3 class III, TUBB3) and motor neuronal (ISL LIM homeobox 1, ISL-1) markers (Figure 7A). The osmotic and oxidative stressor sorbitol has been used to incorporate TDP-43 into SGs. Treatment of sorbitol (0.4 M, SO) for 60 min induced the cytoplasmic localization and aggregate formation of TDP-43 in both iPSC-MNs. Intriguingly, TDP-43^{M337V} MNs exhibited significantly higher levels of TDP-43 aggregation compared with isogenic control (TDP-43^{WT}, Con) MNs. Then we analyzed the effect of GQDs on blocking TDP-43 aggregation in iPSC-MNs. Similar to data observed in HeLa cells, preincubation with GQDs significantly inhibited the stress-induced formation of cytoplasmic aggregates of TDP-43 in both iPSC-MNs with a heterozygous TDP-43^{M337V} mutation and its isogenic control (Con). Moreover, the addition of GQDs during the sorbitol treatment also significantly reduced the level of aggregation of TDP-43 (Figure 7B,C). Thus, GQDs prevent the stress-induced formation of TDP-43 aggregates or dissolve the preformed TDP-43 aggregates in the iPSC-MNs.

Extensive research has demonstrated that the depletion or ALS-linked mutations of TDP-43 can lead to a reduction in the expression of the Stathmin 2 (STMN2) mRNA by disturbing

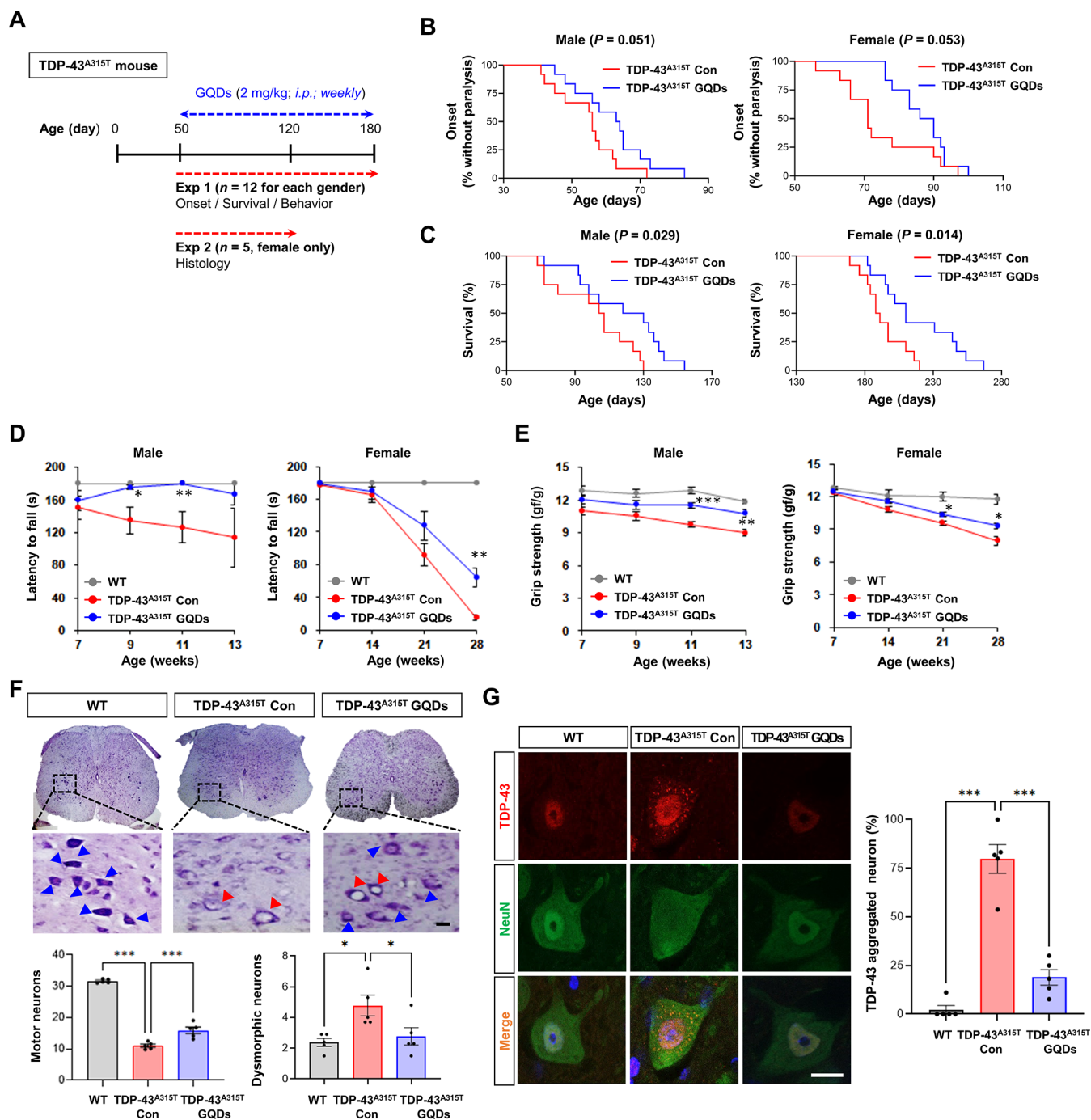


Figure 6. Attenuated ALS symptoms of the TDP-43^{A315T} mice after GQDs administration. (A) A schematic protocol of an *in vivo* experiment for GQD administration. The TDP-43^{A315T} mice were intraperitoneally injected with GQDs (2 mg/kg) weekly from postnatal day 50. The first experiment group (Exp 1, *n* = 12 for each gender) was monitored for neurological scores and behavioral changes. The second group (Exp 2, *n* = 5) was administered GQDs for 10 weeks, followed by histological analyses. (B) The disease onset was delayed in the GQD-treated TDP-43^{A315T} mice. The significance was evaluated with a log-rank test. (C) Survival was significantly enhanced in the GQD-treated TDP-43^{A315T} mice. The significance was assessed by the Kaplan–Meier log-rank test. (D) Rotarod performance was significantly enhanced in the GQD-administered TDP-43^{A315T} mice compared with the control TDP-43^{A315T} mice. (E) Grip strength [grams force/gram (body weight)] was significantly enhanced in both the male and the female GQD-administered TDP-43^{A315T} mice. (F) Reduced numbers of motor neurons (>25 μ m) were observed in the spinal cord of the TDP-43^{A315T} mice compared with the WT mice. However, the numbers were significantly elevated in the ventral horn of the GQD-administered TDP-43^{A315T} mice. Normal (blue arrowhead) and dysmorphic (red arrowhead) motor neurons in each section of the spinal cord were calculated (*n* = 5). Scale bar: 100 μ m. (G) The ratio of neurons (NeuN, green) containing the TDP-43 aggregation (red) was calculated in the TDP-43^{A315T} mice from each image (*n* = 5). DAPI (blue) was used for nucleus detection. The significance was assessed by a one-way ANOVA followed by Tukey's posthoc test. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

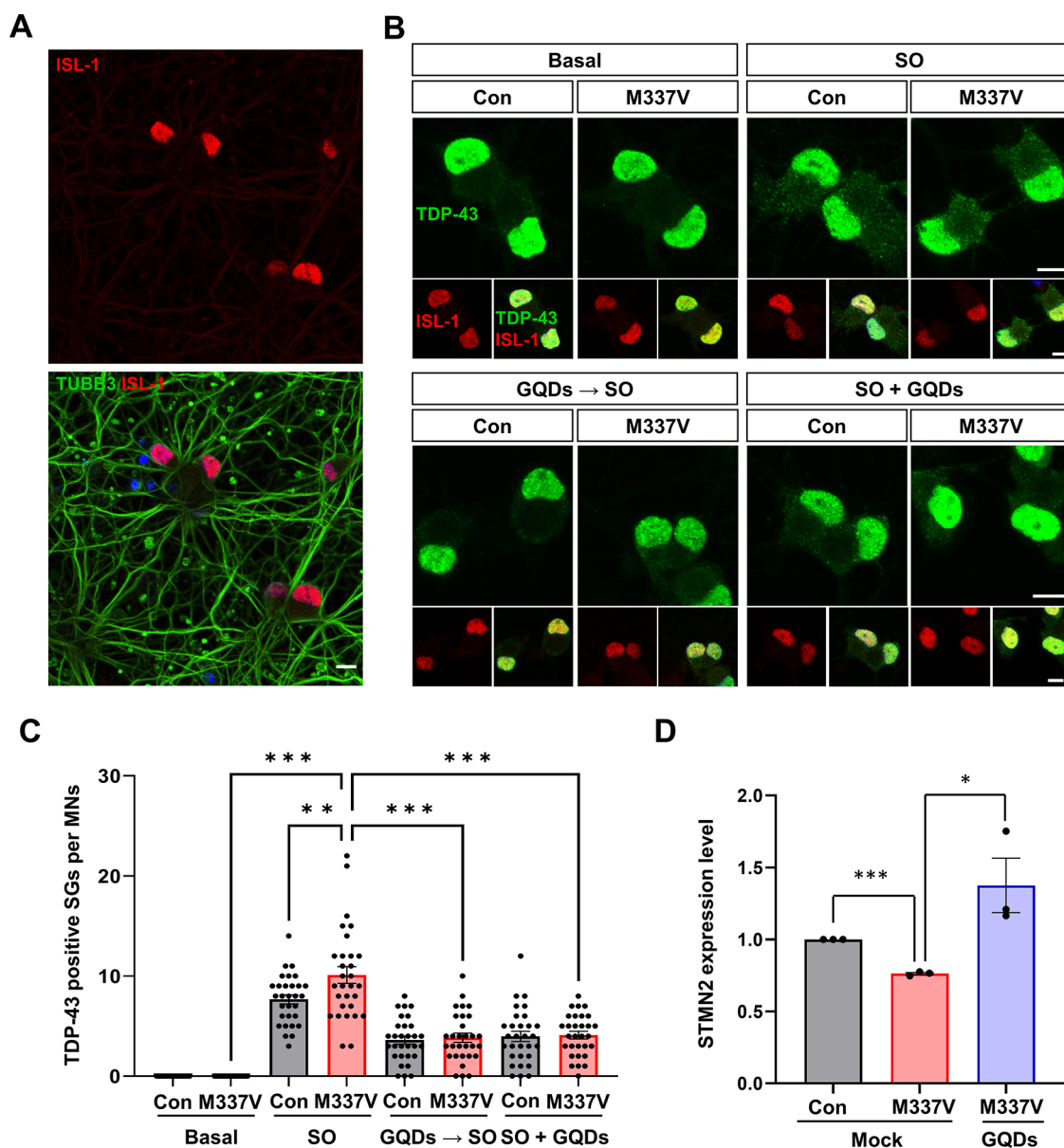


Figure 7. GQDs modulate stress-induced TDP-43 aggregation in iPSC-derived motor neurons (iPSC-MNs). (A) Representative image of iPSC-MNs (Con, isogenic control TDP-43^{WT} and M337V, TDP-43^{M337V}) after 30 days of differentiation. TUBB3 (green), a neuronal marker, shows the soma and neuron processes. ISL-1 (red), an MNs marker exhibits the differentiation. Scale bar: 10 μ m. (B) Immunocytochemical analyses revealed that 60 min treatment of 0.4 M sorbitol (SO) induced cytoplasmic aggregation of TDP-43 and pre- (GQDs $\rightarrow$ SO) or coincubation (SO + GQDs) of GQDs (1 μ g/mL) prevented the TDP-43 aggregation in iPSC-MNs. Scale bar: 10 μ m. (C) The number of TDP-43 aggregates per ISL-1 positive cells was counted in each experimental condition of (a) ($n = 30$). (D) Quantitative real-time PCR analysis showed a significant reduction in STMN2 mRNA levels in TDP-43^{M337V} iPSC-MNs (M337V) compared with isogenic control MNs (Con, TDP-43^{WT}). GQD treatment restored STMN2 mRNA expression levels to those of control. Expression of GAPDH mRNAs was used as endogenous controls ($n = 3$). The significance was assessed by a one-way ANOVA followed by Tukey's posthoc test. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

the regulation of pre-mRNA splicing.¹⁵ Thus, we investigated whether GQD treatment could restore STMN2 expression in iPSC-MNs with a heterozygous TDP-43^{M337V} mutation. As expected, STMN2 mRNA was significantly downregulated in TDP-43^{M337V} iPSC-MNs relative to the isogenic control iPSC-MNs despite primarily nuclear localization of TDP-43 protein in both control and mutant iPSC-MNs. Importantly, treatment with GQDs was effective in restoring STMN2 mRNA expression levels to those of the control (Figure 7D).

GQDs Improve the Phenotype of Other ALS Models. We further investigated whether GQDs can target other major

aggregates in ALS. First, we observed that FUS aggregation was modulated by GQDs. Osmotic stress (sorbitol) induced the aggregation of endogenous FUS, which overlapped with G3BP1. However, pretreatment of GQDs inhibited the formation of either SGs or FUS aggregation. On the other hand, GQDs also disaggregated the preformed SGs and FUS aggregation (Figure S17). Then, we further investigated using mutant FUS (G540Wfs and R495X); however, coincubation of GQDs prohibited these aggregations. These findings are also correlated with the viability of the FUS overexpressing cells.

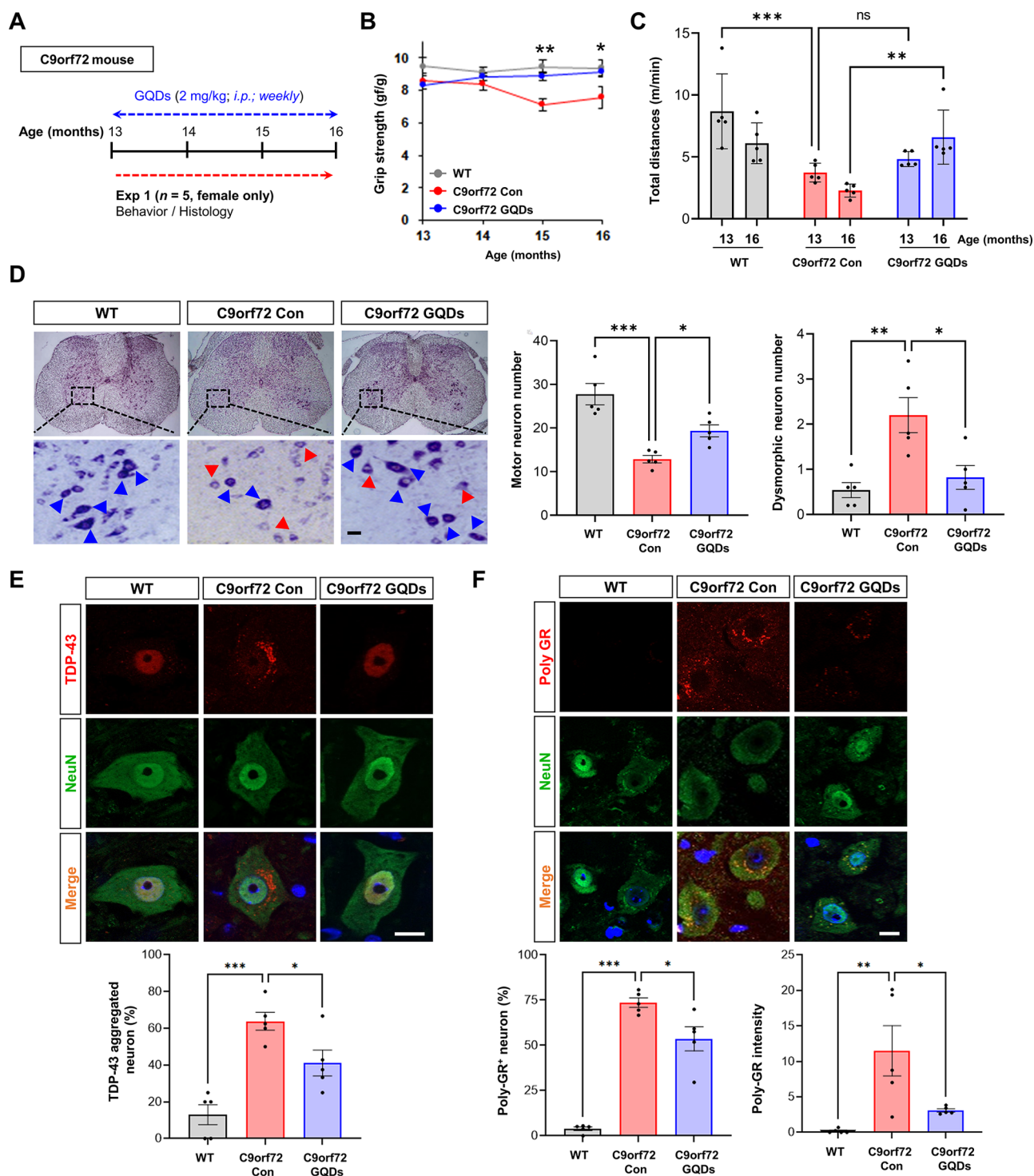


Figure 8. Attenuated phenotype of the C9orf72 mice by GQDs. (A) A schematic protocol of the GQD administration to the C9orf72 mice. The female C9orf72 mice were intraperitoneally injected with GQDs (2 mg/kg) weekly for 3 months from 13 months of age ($n = 5$). (B) Grip strength was significantly enhanced after 2 months of GQD administration. The significance was assessed by a two-way ANOVA followed by Sidák's posthoc test. (C) The open field test revealed that the total distance (meters per minute) was significantly improved in the GQD-treated C9orf72 mice ($n = 5$). (D) Reduced numbers of motor neurons ($>25 \mu\text{m}$) in the spinal cord of the C9orf72 mice were significantly elevated in the ventral horn of the GQD-administered C9orf72 mice. Normal (blue arrowhead) and dysmorphic (red arrowhead) motor neurons in each section of the spinal cord were calculated from each image ($n = 5$). Scale bar: 100 μm . (E) The aggregation of TDP-43 in the spinal cord of the C9orf72 mice was significantly lowered by the GQD treatment. The ratio of the neurons (NeuN, green) with the TDP-43 aggregation (red) was measured ($n = 5$). Scale bar: 20 μm . (F) Aggregated poly-GR (red) in the spinal cord of the C9orf72 mice was reduced by treatment with GQDs. The ratio of the poly-GR⁺ neurons to the poly-GR intensity in each neuron (NeuN, green) was measured from each image ($n = 5$). DAPI was used for the detection of the nucleus. Scale bar: 10 μm . The significance was assessed by a one-way ANOVA followed by Tukey's posthoc test. ns, nonsignificant; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

Transfection of either WT or mutant FUS reduced the viability; however, the GQD treatment significantly restored the viability (Figure S18).

Next, we addressed whether GQDs exhibited potency toward another ALS model, C9orf72 mice. As previously reported, the mutations in C9orf72 result in the TDP-43 aggregation in ALS patients.⁴⁶ With this notion, we hypothesized that the disease phenotype of the C9orf72 mice might be partially ameliorated by disaggregating TDP-43. C9orf72 transgenic mice⁴⁷ were administered 2 mg/kg of GQDs every week for 3 months (Figure 8A). The mice exhibited a mild decrease in muscle strength. However, the GQD administration reversed the reduction of the grip strength (Figure 8B). This behavioral improvement by GQDs was also observed in the open field test. The significantly reduced total distance in the C9orf72 mice was increased by 3 months of GQD administration (Figure 8C).

Histologically, the GQD administration protected the motor neurons in the ventral horn of the spinal cord and motor cortex (Figure 8D), which were also correlated with the inhibition of glial activation in both tissues (Figure S19). In the motor neurons of the spinal cord, the majority of the C9orf72 mice exhibited cytosolic aggregation of TDP-43 compared with that of the WT mice; however, it was significantly reduced by GQDs (Figure 8E). Intriguingly, the polyglycine-arginine (GR)-positive neurons in the spinal cord of the C9orf72 mice were also significantly reduced by GQDs (Figure 8F).

Collectively, these results indicate that GQDs can ameliorate TDP-43 aggregation under stress or disease conditions. Phenotypic improvements were additionally observed in the genetic mutations of FUS and C9orf72; therefore, GQDs could be broadly applied to most ALS cases.

DISCUSSION

The cytosolic aggregation of TDP-43 is a key feature of ALS patients. TDP-43 proteinopathy is also commonly observed in frontotemporal dementia (FTD) and Alzheimer's disease.^{48,49} TDP-43, like other ALS-associated RNA-binding proteins, is an aggregation-prone protein with the intrinsically disordered region (IDR) in the C-terminus and is incorporated into the stress granules (SGs) by the liquid–liquid phase separation (LLPS).⁵⁰ Chronic stress and/or ALS-linked mutations in TDP-43 result in irreversible phase transitions and impaired SG dynamics, leading to the formation of pathogenic aggregates.^{50,51} Studies on cultured cells and patient tissues have reported that cytoplasmic TDP-43 aggregates colocalize with the SG markers, suggesting that SGs or LLPS contribute to the ALS pathology. However, whether the SG dynamics are responsible for the formation of pathogenic TDP-43 aggregates in neurons is still under debate.^{26,52}

Extensive investigations have been performed to isolate the pathogenic aggregation and fibrillization domain of TDP-43. IDR of TDP-43 is critical in the formation of amyloid fibrils or TDP-43 aggregation, as revealed through structural analyses. According to the cryoelectron microscopic structure, the fibril core of TDP-43 consists of two distinguishing parts. The anterior part of IDR (residues 276–343), mainly containing short β -strands, is stabilized with plenty of hydrophobic amino acids, while the posterior part of IDR (residues 344–414) is stabilized by the steric zipper interactions of relatively long β -strand packs.⁵³ A nuclear magnetic resonance (NMR) analysis also revealed that the β -strand segments in residues 365–400 involve the formation of liquid droplets and amyloid fibrils,

while another report indicated that residues 368–414 are crucial for the fibril formation.^{54,55} Experimentally, residues 316–346 with a propensity to form α -helices are reported as critical for LLPS,^{56,57} and glycine repeats containing the anterior part of IDR (residues 287–322) have proven to be significant in fiber formation.⁵⁸

In this study, all of the tested TDP-43 peptides manifested dynamic conversions in the secondary structure, as revealed in our previous study on amyloid- β aggregation in Alzheimer's disease.³⁹ TDP-43^{192–231} maintained helical structures with different contents after the incubation. We speculate that the fragmentation of α -helical TDP-43^{192–231} from full-length TDP-43 in the native state might induce a transient intermediate. This intermediate might be partially unfolded with a random-coil (RC) structure due to the loss of the interactions which stabilized the native structure. The rapid association between the sticky TDP-43^{192–231} with a partial helical structure might form small, soluble oligomers, i.e., low-order oligomers with helical structures through binding-coupled helical folding.⁵⁹ We previously suggested partial helical structures of amyloid- β and α -synuclein are sticky and transient intermediates.^{39,60,61} Therefore, TDP-43^{192–231} is most likely to exhibit an α -partial RC- α transition. In contrast, TDP-43^{234–273} exhibited an RC-RC- β transition. TDP-43^{234–273} in the native structure of TDP-43 exhibited a folded structure with both α -helices and β -strands; however, TDP-43^{234–273} in the aqueous solution presented unfolded conformations (i.e., RC), likely due to the loss of noncovalent interactions present in the original three-dimensional (3D) structure. Sequentially, the RC structures of TDP-43^{234–273} were converted to the cross- β structures of the amyloid fibrils in the end. TDP-43^{274–313} and TDP-43^{354–393} showed similar conformational conversion (the RC-RC- β transition). The two peptides were located in the RC regions of native TDP-43. The fragmentation did not cause changes in secondary structures and maintained an RC structure; however, both peptides were finally converted to cross- β -structured amyloid fibrils over time. TDP-43^{314–353} showed the α -helical and RC structures in the native TDP-43 and mostly RC structures in solution. The rapid conversion to cross- β amyloid fibrils was eventually observed (the α /RC-(α /)RC- β transition). In summary, four TDP-43 peptides, except TDP-43^{192–231}, formed β -structured amyloid fibrils. The ability of amyloid formation decreased in the following order: TDP-43^{314–353} $\approx$ TDP-43^{354–393} > TDP-43^{274–313} $\gg$ TDP-43^{234–273}. Solubility, which is one of the most important factors for amyloidogenesis, manifested a negative correlation with the amyloidogenicity of each peptide. TDP-43^{192–231} and TDP-43^{234–273} were predicted to have a higher solubility and exhibited a lower propensity for amyloid generation than the other three peptides. Thus, low solubility is a determinant to accelerate the amyloid generation of a TDP-43 peptide as well as nucleation.

Previously, we have elucidated the significance of structural change and destabilization for amyloidogenesis in various amyloid peptides and fibrils.^{43,62–64} Amyloid- β peptide showed distinct aggregation pathways during the accumulation of amyloid fibrils in mouse brains,³⁹ and the structural transition of intrinsically disordered α -synuclein to an amyloidogenic helical structure is critical to the generation of β -structured amyloid fibrils.^{60,61,65} Based on these experiences, we examined the structural information on the amyloid fibrils formed from the core region residues of amyloid fibrils of TDP-43 (Table S3). Recent structural studies revealed that the residues from

R272 to Q360 are important for the stable core regions of TDP-43 amyloid fibrils. According to the AlphaFold2 structure, TDP-43^{274–313}, TDP-43^{314–353}, and TDP-43^{354–393} are mostly unfolded. On the other hand, the other TDP-43 peptides showed different behaviors. TDP-43^{192–231} consisting of the three-dimensionally folded structure did not induce amyloid formation and formed low-order oligomers. TDP-43^{234–273} partly accommodating the three-dimensionally folded structure showed highly slow amyloid aggregation. Therefore, there is a correlation between the AlphaFold2 structure and the amyloid fibril structure: the regions that are unfolded in the AlphaFold2 structure are more likely to convert into the β -structure of the amyloid fibrils.

As an emerging nanomaterial, the applicability of GQDs in treating numerous diseases has been evaluated. Since GQDs can be delivered through the blood–brain barrier (BBB),^{29,34} the efficacy of GQDs has been demonstrated in neurodegenerative diseases such as Parkinson's disease and ALS. GQDs can modulate fibrillization of α -synuclein as well as phase separation of TDP-43 and FUS.^{29–32} In addition, GQDs can be used as an anti-inflammatory therapy for colitis by modulating regulating immune cells.³³ Besides, carbon quantum dots (CQDs) have also received remarkable interest for clinical application as a tool for drug delivery.⁶⁶ CQD-loaded apoptotic vesicle alleviates sepsis by improving the antibacterial effect of Kupffer cell,⁶⁷ and CQD-mediated nanocarrier also improves the cytotoxicity of doxorubicin against breast cancer cells.⁶⁸ To improve the clinical application, the modifications of GQDs and CQDs have been extensively studied.⁶⁹ For example, halogen-doped GQDs can penetrate the nuclear envelope and inhibit the assembly of TDP-43 nuclear bodies.³² In addition, the design of a synthesis strategy for GQDs has been raised as an imperative issue for future applications. Recently, a machine learning-based strategy was demonstrated for optimization of CQD synthesis.⁷⁰

GQDs have a hydrophobic nature due to the presence of carbon networks, while they also have hydrophilic and electrostatic properties of the carboxyl groups, which make them soluble and biocompatible. The carboxyl groups of GQDs confer hydrophilicity, while the graphene core region exhibits hydrophobicity. These properties may confer with GQDs to interact with both the hydrophilic and the hydrophobic regions of TDP-43. We previously demonstrated the attractive charge–charge interactions between the positive charges of α -synuclein, a causative protein of Parkinson's disease, and the negative charges in GQDs using multidimensional solution NMR spectroscopy and molecular dynamics simulations.²⁹ In addition, the interaction of the modified GQDs with FUS, another ALS causative gene, indicated hydrophobic interactions between the modified GQDs and IDR of FUS.³¹ In this study, we also demonstrated the molecular interactions between the unmodified GQDs and TDP-43 as well as the identification of the putative binding site using several experiments. First, the direct intermolecular interactions between individual TDP-43 peptides and GQDs were elucidated using the chemical shift and peak intensity in the 1D-¹H NMR spectra. Although detailed information on the intermolecular interactions at the level of amino acids was not available, it is most likely that GQDs use both the hydrophobic and the hydrophilic properties for contacting the hydrophobic and hydrophilic parts in TDP-43. Second, isothermal titration calorimetry (ITC) experiments revealed diverse thermody-

namic parameters between the TDP-43 peptides and GQDs. The binding affinities of the five TDP-43 peptides with GQDs were strong ($K_D = 6.1$ – $25.2 \mu\text{M}$). Particularly, the binding affinities of TDP-43^{192–231}, TDP-43^{234–273}, and TDP-43^{274–313} to GQDs were stronger ($K_D = 6.1$ – $7.5 \mu\text{M}$) than those of the other peptides. Third, the immunoprecipitation assay also supported the idea that full-length TDP-43 could interact with GQDs. Intriguingly, GQDs effectively bind to TDP-43 rather than other SG-forming proteins with IDR, such as G3BP1 or TIA1. These data infer that the interaction of GQDs with IDR-containing and aggregation-prone proteins might require more specificity. Because TDP-43 is important in the formation of SGs,^{71,72} we postulate that prevention of SG formation by GQDs might be primarily derived from the direct inhibition of the TDP-43 aggregation.

In this study, we revealed that GQDs can effectively modulate the formation of SGs and TDP-43 aggregation. Additionally, GQDs can inhibit the phase separation of recombinant TDP-43. The modulation of TDP-43 aggregation by GQDs was positively correlated with the enhanced cell viability under overexpression of the mutant TDP-43 proteins. The efficacy of GQDs in preventing TDP-43 aggregation was also validated in the induced pluripotent stem cell (iPSC)-derived motor neurons (MNs). As loss of TDP-43 in the nucleus significantly affects splicing activity,^{15–17} we also demonstrated that the treatment of GQDs restores the expression level of functional Stathmin 2 (STMN2).

Going further from a previous report on modulation of TDP-43 aggregation by modified GQDs,³² we demonstrated the feasibility of unmodified GQDs toward future clinical trials using *in vivo* animal studies. Previously, intraperitoneal administrations of GQDs were successfully delivered into the neurons in the central nervous systems *via* penetrating BBB.^{29,34} Specifically, the permeability of GQDs in the *in vitro* model of brain microvascular endothelial cells (BMECs) was almost 100%.³⁴ In this study, we also validated the delivery of GQDs into the spinal cord and motor cortex after the intraperitoneal administration of biotinylated-GQDs (b-GQDs). Although there is a common concern that intraperitoneal administration in rodent models has a limitation to future clinical translation, the route has several advantages in early preclinical studies by ensuring safety on small rodent models, reproducibility of the experiments, and bioavailability of drugs regardless of formula *via* rapid absorption from the peritoneal cavity and following systemic circulation.⁷³ In our results, administration of GQDs attenuated the disease phenotype of the TDP-43^{A315T} and TDP-43^{WT} mouse models. The efficacy could be due to the protection of the motor neurons and suppression of glial activation through the reduction of the TDP-43 aggregation in the motor neurons. The feasibility of GQDs toward other genetic causes of ALS, such as FUS and C9orf72, was also observed. In particular, GQDs reduced the aggregation of TDP-43 as well as poly-GR in the spinal cord of the C9orf72 mice. Previously, the correlation of the TDP-43 aggregation with the dipeptide repeats of C9orf72 had been clearly manifested.^{74–76} Since arginine-rich poly-GR or poly-PR binds to TDP-43, TIA1, and G3BP1, thereby impairing the SG dynamics,^{75,77,78} the reduction of the poly-GR aggregation by GQDs could be derived from the dissociation of TDP-43 in SGs.

CONCLUSIONS

In summary, we provided a series of evidence that GQDs possess the potency of modulating TDP-43 aggregation *via* direct interaction, thereby protecting neurons from neuronal death and alleviating the phenotype of ALS mouse models. Further development of GQD-based nanomedicine may provide a new therapeutic strategy for proteinopathy-associated ALS/FTD as the effects of GQDs are extended to other genetic causes of ALS.

MATERIALS AND METHODS

Preparation of GQDs. GQDs were synthesized using a thermooxidative cutting method in Graphene Square Chemical Inc. (Pohang, Korea). The carbon fibers (0.9 g, 99.999%, Toray) were placed in the acid mixture and heated at 80 °C for 24 h. Then, the acidic mixture of GQDs was continuously diluted by DI water flow for 3 days, followed by vacuum-filtering to achieve the desired sizes of GQDs. The resulting solution was then rotary-evaporated to obtain GQD powders. To analyze the morphology and size distribution of GQDs, a 10 μ L solution (10 μ g/mL) was dropped onto a CVD graphene-covered TEM grid. High-resolution TEM imaging was performed using an FEI Talos F200C microscope. The atomic force microscope (AFM) (Park Systems, Suwon, Korea) was used to measure the height of GQDs spin-coated on a SiO₂/Si substrate. The UV–vis absorption of GQD solution (0.1 mg/mL) was conducted using a UV–visible spectrophotometer (GENESYS 150, Thermo Fisher Scientific, Waltham, MA). The binding energy of GQD powder was detected by X-ray photoelectron spectroscopy (XPS). The GQD solution (1 mg/mL) was dropped on a SiO₂/Si wafer, and the dried sample was analyzed by XPS (VersaProbe III, UIVAC-PHI, Inc., Kanagawa, Japan). The analysis of functional groups present in GQDs was conducted using Fourier transform infrared (FT-IR) spectroscopy. The samples were prepared using the conventional KBr pellet method. The analysis was performed with a Cary 630 FTIR Spectrometer (Agilent Technologies, Palo Alto, CA) with specific parameters including 32 scans, a resolution of 4, and a wavenumber range of 4000–40 cm^{−1}. Biotinylated GQDs (b-GQDs) were generated by the following process. To activate the carboxyl groups on GQDs, 50 mg of GQDs was dissolved in 1 mL of BupH MES-buffered saline (pH 4.7; Thermo Fisher Scientific), and 12.5 mg of EDC reagent (Sigma-Aldrich, St. Louis, MO) was added. After 1 h of vigorous stirring, 25 mg of EZ-Link amine-PEG3-biotin (Thermo Fisher Scientific) was introduced to the EDC-activated GQDs. The solution was then dialyzed, and the amount of biotin conjugation was measured using a Quant Tag Biotin Kit (Vector Laboratories, Burlingame, CA). The concentration of biotin was quantified by plotting the standard curve for the concentration vs 535 nm absorbance measured with a calibration kit and the absorbance of the sample. Then the molar ratio of GQDs to biotin was determined and revealed that each GQD was conjugated with 1–2 biotin labels.

Immunocytochemistry for SG Formation and TDP-43 Aggregation. To induce SG formation and TDP-43 aggregation, HeLa cells were treated with either SA (0.5 mM, 1 h or 10 μ M, 30 h) or D-sorbitol (0.4 M, 2 h) (Sigma-Aldrich). The cells grown on coverslips in 12-well plates were fixed in 4% paraformaldehyde (PFA) for 10 min and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS; HyClone, Logan, UT). They were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA). Following the incubation of primary antibodies followed by secondary antibodies, the cells were mounted using Vectashield Antifade Mounting Medium containing DAPI (Vector Laboratories). Fluorescence analysis was performed using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Jena, Germany) located at the Neuroscience Translational Research Solution Center of Dong-A University, and the intensity of each fluorescence was measured using Zeiss Zen software (version 3.3).

Recombinant Protein Purification, Liquid Droplet Formation, and Turbidity Assay. An expression plasmid for MBP-TDP-

43, containing a TEV protease-cleavable site between them, was constructed using the pMAL vector.⁷⁹ MBP-TDP-43 was expressed in *E. coli* BL21(DE3) with 0.5 mM IPTG at 20 °C overnight. Cells were lysed in a buffer (50 mM HEPES, pH 7.4, 1.5 M NaCl, 10% glycerol, 2 mM DTT, 0.5 mM EDTA, protease inhibitor), sonicated, and centrifuged at 12,000g for 30 min. MBP-TDP-43 was purified using Amylose Resin (New England Biolabs, Ipswich, MA) and gel filtration (Superdex 200pg, Cytiva, Chicago, IL) with a buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, and 2 mM DTT). Fractions containing MBP-TDP-43 were collected and concentrated using an Amicon Ultra (Merck Millipore, Burlington, MA). Purified MBP-TDP-43 was cleaved by TEV protease at 25 °C. After 4 h, precipitated TDP-43 was collected by centrifugation at 16,000g for 30 min. The pellet was dissolved in 10 mM HCl (pH 2.0) and used for further experiments. Droplet formation and turbidity assay were performed previously described with minor modification.³² After removing preformed aggregates by centrifugation at 16,100g for 10 min, a phase separation reaction of MBP-TDP-43 (40 μ M) was induced in LLPS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% PEG) with or without indicated concentration of GQDs. Previously, MBP tag is reported to have a minimal impact on the interaction between TDP-43 and GQDs.³² Droplet formation was imaged by differential interference contrast (DIC) microscopy within 10 min of reaction initiation. Turbidity (optical density at 600 nm) measurements of phase-separated proteins were performed using a Tecan infinite M200 plate reader.

Peptide Synthesis. Peptides with the following partial sequences of TDP-43 were synthesized using the solid-phase peptide synthesis method with FMOC-Phe-Wang resin LL (0.32 mmol/g): TDP-43^{192–231}, KVFVGRCTEDMTEDELREFFSQYGDVMDVFIPKP-FRAFAF; TDP-43^{234–273}, FADDQIAQSLCGEDLIIKGISVHIS-NAEPKHNNSNRQLERS; TDP-43^{274–313}, GRFGGNP GGFGNQGGFGNSRGGGAGLGNNQGSNMGGGMNF; TDP-43^{314–353}, GAFSINPAMMAA AQAALQSSWGMMGM-LASQQNQSGPSGNN; TDP-43^{354–393}, QNQGNNMQREPNQAFGS GNNSYSGSNSGAAIGWGSASNAGS. A SYMPHONY X Multiplex peptide synthesizer (Gyros Protein Technologies AB, Uppsala, Sweden) was used for the synthesis. After being cleaved from the resin using trifluoroacetic acid/1,2 ethanedithiol/thioanisole/H₂O, crude peptides were purified to >98% purity using high-performance liquid chromatography (Shimadzu, Kyoto, Japan).

ThT Fluorescence Assay. The amyloid formation of TDP-43 peptides was traced in a ThT fluorescence assay at 37 °C. We prepared 100 μ M TDP-43 peptides in 20 mM sodium phosphate buffer (pH 7.5), 0 or 100 mM NaCl, 0 or 0.045 mg/mL GQDs, and 5 μ M ThT. Full-length TDP-43 was prepared at 12 μ M in 20 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl, 0 or 0.045 mg/mL GQDs, and 5 μ M ThT, and was monitored at 27 °C. The amyloid formation of full-length TDP-43 or TDP-43 peptides was monitored using a Synergy H1 plate reader (BioTek Instruments, Winooski, VT). Triplicates of each sample (100 μ L per well) were loaded into a 96-well plate with a nonbinding surface (Corning, Inc., Corning, NY). A sealing film (Power Seal Crystal View, Greiner-Bio-One, Kremsmünster, Austria) was affixed to the plates to prevent sample evaporation. The TDP-43 peptides were continuously shaken with or without 3 mm stainless-steel beads. In general, beads accelerate the formation of amyloid fibrils and have been extensively used as an amyloid inducer. Thus, we utilized beads to efficiently reveal the aggregation propensities of each peptide based on the bead-generating cavitation effects. Of note, the stainless-steel beads providing high reproducibility due to their smooth surface was used.⁴² The fluorescence intensity of ThT was recorded from the top of the plate at excitation and emission wavelengths of 445 and 490 nm, respectively. After the data acquisition, kinetic analyses of TDP-43 peptides were carried out using the following equation as the previous study⁵⁹

$$Y = y_i + m_i t + \frac{y_f + m_i t}{1 + e^{-k(t-t_0)}}$$

where $y_i + m_i t$ and $y_f + m_f t$ are the initial and final baselines, respectively. t_0 is the half-time at which ThT fluorescence reaches 50% of its maximum amplitude. k represents the elongation rate constant. The lag time was obtained based on the following equation: lag time = $t_0 - 2(1/k)$.⁸⁰ y_i and y_f were fixed to the values of initial and final ThT fluorescence intensities obtained from measurements. ThT data were fitted with variations in m_i , m_f , k , and t_0 . The average and error values of the lag time and elongation rate constant were calculated from the three independent samples.

Isothermal Titration Calorimetry (ITC). ITC experiments for TDP-43 peptides and GQDs at 25 °C were performed with a MicroCal VP-ITC instrument (Malvern Panalytical Ltd., Malvern, United Kingdom). The concentration of GQDs in the ITC syringes was 500 μ M. The concentration of each TDP-43 peptide in the ITC cell was 25 μ M. The TDP-43 peptides were dissolved in 20 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl. The reference power was set to 10 μ cal/s, and the initial delay was 1200 s. Titration experiments were composed of 25 injections, and the injection volume was 2 μ L for the first injection and 11 μ L for the other injections. The stirring speed was 307 rpm. Data were analyzed with a one-set-of-sites binding model using the MicroCal Analysis Software (Malvern Panalytical Ltd.) as in the previous study.⁶⁰

CD Spectroscopy. CD measurements were performed on a J720 spectrophotometer (Jasco, Tokyo, Japan) using a quartz cuvette with a 1 mm path length at room temperature. TDP-43 peptides were prepared in 20 mM sodium phosphate buffer (pH 7.5), 0 or 100 mM NaCl, 0 or 0.045 mg/mL GQDs, and 5 μ M ThT. The far-UV CD spectra of TDP-43 peptides without GQDs were promptly recorded after sample preparation. Subsequently, after the ThT fluorescence assay of TDP-43 peptides in the absence or presence of GQDs, the far-UV CD spectra of the samples were immediately recorded again. Full-length TDP-43 was prepared in either 20 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl, 0 or 0.045 mg/mL GQDs, and 5 μ M ThT, or 10 mM HCl (pH 2.0). The bandwidth and scan rate were 1 and 100 nm min⁻¹, respectively, and CD signals are expressed as the mean residue ellipticity (deg cm² dmol⁻¹).

NMR Spectroscopy. Nuclear magnetic resonance (NMR) spectroscopy of the TDP-43 peptides was performed using a Bruker Avance II 700 MHz spectrometer equipped with a cryogenic probe (Bruker BioSpin, Billerica, MA) at 15 °C. The peptides were prepared in 20 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl and 10% (v/v) D₂O for one-dimensional (1D) ¹H NMR measurements in the absence and presence of 0.045 mg/mL GQDs. The data were processed and analyzed using NMRPipe and Sparky.

AFM Sample Preparation and Measurement. For sample preparation, 20 μ L aliquots of the sample, post-ThT fluorescence assay completion, were deposited onto freshly cleaved mica. Then, the aliquots were incubated for 5 min, rinsed with 200 μ L of deionized water, and dried. Park NX10 atomic force microscopy (Park Systems) was used to obtain micrographs of the TDP-43 samples.

Animal Experiments. TDP-43A^{315T} [B6SJL-Tg(Prnp-TARDBP*A315T)23Jle/J, Strain #016143], TDP-43^{WT} [Tg(Prnp-TARDBP)4Jle/J, Strain #016144], and C9orf72 [FVB/NJ-Tg(C9orf72)500Lpwr/J, Strain #029099] were purchased from Jackson Laboratory (Bar Harbor, ME) and used after *in vitro* fertilization by MacroGen, Inc. (Seoul, Korea). Briefly, GQDs (2 mg/kg) dissolved in PBS containing 10% ethanol or vehicle (PBS containing 10% ethanol) were orally administered every week into mice. The onset and survival of the mice were determined by monitoring the neurological disease progression according to the guidelines for preclinical animal research on ALS/MND⁸¹ and motor coordination and muscle integrity were assessed weekly. Spinal cord and brain tissue analyses were performed after the mice were anesthetized and perfused with 4% paraformaldehyde in PBS. The number of motor neurons with a diameter >25 μ m was calculated after staining with a 0.1% (w/v) cresyl violet stain solution. Activated astrocytes and microglia were determined using anti-GFAP (Abcam, Cambridge, U.K.) and anti-Iba1 Ab (Wako, Osaka, Japan). The aggregation of TDP-43 in the spinal cord was detected using anti-TDP-43 (Abcam) and anti-NeuN Ab (Sigma-

Aldrich). The detailed experimental information is described in the [Supporting Information](#).

Statistical Analyses. All statistical analyses and data visualizations were performed using Prism 8.1.0 (GraphPad Software, San Diego, CA). The significance of the data was assessed by either a one-way ANOVA with a posthoc Tukey's multiple comparison test or a two-way ANOVA followed by Sidák's posthoc test. The *P* values of disease onset and survival were derived using the Kaplan–Meier log-rank test.

Study Approval. All animal experiments were performed according to the approved protocol provided by the Institutional Animal Care and Use Committee of Dong-A University with the Association for Assessment and Accreditation of Laboratory Animal Care International regulations.

ASSOCIATED CONTENT

Data Availability Statement

All data are available in the main text or the [Supporting Information](#).

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.4c15283>.

Results, Methods, and additional data for characterization of GQDs, dose responses of protein aggregates by GQDs or b-GQDs, characterization of TDP-43 peptides, and *in vivo* study using TDP-43^{A315T}, TDP-43^{WT}, and C9orf72 mice ([PDF](#))

AUTHOR INFORMATION

Corresponding Authors

Byung Hee Hong – Department of Chemistry and Advanced Institute of Convergence Technology, Seoul National University, Seoul 08826, Korea; orcid.org/0000-0001-8355-8875; Phone: +82-2-882-6569; Email: byunghhee@snu.ac.kr

Minyeop Nahm – Dementia Research Group, Korea Brain Research Institute, Daegu 41062, Korea; Phone: +82-53-980-8333; Email: nmy92@kbri.re.kr

Young-Ho Lee – Biopharmaceutical Research Center, Korea Basic Science Institute, Cheongju 28119, Korea; Bio-Analytical Science, University of Science and Technology, Daejeon 34113, Korea; Graduate School of Analytical Science and Technology, Chungnam National University, Daejeon 34134, Korea; Department of Systems Biotechnology, Chung-Ang University, Gyeonggi 17546, Korea; Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Miyagi 980-8578, Japan; orcid.org/0000-0002-8441-5814; Phone: +82-43-240-5071; Email: mr0505@kbsi.re.kr

Young Bin Hong – Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea; Departments of Biochemistry, College of Medicine, Dong-A University, Busan 49201, Korea; orcid.org/0000-0002-4333-3847; Phone: +82-51-240-2762; Email: ybhong@dau.ac.kr

Authors

Na Young Park – Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea

Yunseok Heo – Biopharmaceutical Research Center, Korea Basic Science Institute, Cheongju 28119, Korea; orcid.org/0000-0002-2233-4306

Ji Won Yang – Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea

Je Min Yoo – Chaperone Ventures, LLC., Los Angeles, California 90006, United States; orcid.org/0000-0003-3074-859X

Hye Ji Jang – Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea

Ju Hee Jo – Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea

Su Jeong Park – Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea

Yuxi Lin – Biopharmaceutical Research Center, Korea Basic Science Institute, Cheongju 28119, Korea

Joonhyeok Choi – Biopharmaceutical Research Center, Korea Basic Science Institute, Cheongju 28119, Korea; Chemical Analysis Team, Korea Basic Science Institute, Cheongju 28119, Korea

Hyeonjin Jeon – Dementia Research Group, Korea Brain Research Institute, Daegu 41062, Korea

Sun Joo Cha – Dementia Research Group, Korea Brain Research Institute, Daegu 41062, Korea

Gaeun Bae – Department of Chemistry and Advanced Institute of Convergence Technology, Seoul National University, Seoul 08826, Korea

Donghoon Kim – Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea; Department of Pharmacology, College of Medicine, Dong-A University, Busan 49201, Korea

Juhee Kim – Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, California 90089, United States

Wade Zeno – Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, California 90089, United States; orcid.org/0000-0002-2341-8923

Jong Bo Park – Graphene Square Chemical Inc., Pohang 37673, Korea

Noriyoshi Isozumi – Department of Future Basic Medicine, Nara Medical University, Nara 634-8521, Japan

Tomohide Saio – Institute of Advanced Medical Sciences, Tokushima University, Tokushima 770-0855, Japan; orcid.org/0000-0003-3639-7399

Seung Hyun Kim – Department of Neurology, College of Medicine, Hanyang University, Seoul 04763, Korea

Hojae Lee – Biomanufacturing Center, Cedars-Sinai Medical Center, West Hollywood, California 90048, United States

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acsnano.4c15283>

Author Contributions

*N.Y.P. and Y.H. contributed equally to this work. Conceptualization: B.H.H., M.N., Y.H.L., Y.B.H. Methodology: N.Y.P., Y.H., Y.H.L., J.M.Y., J.W.Y., M.N., D.K., J.B.P., S.H.K., H.L., J.K., W.Z., N.I., T.S. Investigation: N.Y.P., Y.H., J.W.Y., H.J.J., J.H.J., S.J.P., Y.L., J.C., H.J., S.J.C., G.B., J.K., W.Z. Supervision: B.H.H., M.N., Y.H.L., Y.B.H. Writing (review and editing): N.Y.P., Y.H., J.M.Y., W.Z., Y.H.L., Y.B.H.

Notes

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ABBREVIATIONS

AFM, atomic force microscopy; ALS, amyotrophic lateral sclerosis; BBB, blood–brain barrier; CD, circular dichroism; CTFs, C-terminal fragments; ΔG , Gibbs free energy change; ΔH , change in enthalpy; ΔS , change in entropy; $1D-^1H$ NMR, one-dimensional proton nuclear magnetic resonance; FTD, frontotemporal dementia; FUS, fused in sarcoma; GQDs, graphene quantum dots; G3BP1, G3BP stress granule assembly factor 1; IDR, intrinsically disordered region; iPSC, induced pluripotent stem cell; ITC, isothermal titration calorimetry; K_D , dissociation constant; LLPS, liquid–liquid phase separation; MNs, motor neurons; RBPs, RNA-binding proteins; RRM, RNA recognition motif; SA, sodium arsenite; SG, stress granules; SO, sorbitol; STMN2, stathmin 2; TDP-43, transactive response DNA-binding protein-43; ThT, thioflavin T; TIA1, TIA1 cytotoxic granule-associated RNA-binding protein; UNC13A, Unc-13 homologue A

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