Article title: Hydrogel-based delivery of Tat-fused protein Hsp70 protects dopaminergic cells *in vitro* and in a mouse model of Parkinson's disease

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SUPPLEMENTARY MATERIAL S1 (Acrobat file)

Text summary: Supplementary Material S1 details the procedures to express, purify and characterize recombinant human Hsp70-1A and Tat-Hsp70-1A; characterize COLL-HA hydrogels, gelatin particles, composites and Tat-Hsp70-loaded composites; evaluate peripheral inflammatory response in naïve mice and the improvement of motor performance in 6-OHDA-induced mouse models of PD.

EXPERIMENTAL PROCEDURES

We purchased reagents for protein expression, purification and cell culture from Thermo Fisher Scientific, Waltham, MA, USA. We obtained reagents for bacterial fermentation, hydrogel and particle preparation and characterization, 6-hydroxydopamine (6-OHDA), H_2O_2, resazurin sodium salt, methylcellulose (M_w=41 kDa) and 3,3-diaminobenzidine (DAB) from Sigma-Aldrich, St. Louis, MO, USA. We purchased plasticware from Corning®, Corning, NY, USA.

We cultured SH-SY5Y cells (ATCC® CRL-2266™) as described[1], isolated mesencephalic dopaminergic neurons from CD-1 mice as reported[2], expressed and purified Tat-α-syn according to a previous publication[3] and performed silver staining according to ref.[4].

Expression and purification of Hsp70 and Tat-Hsp70

We designed the genes encoding human Hsp70-1A and Tat-Hsp70-1A by *in silico* back translation of the amino acid sequence. We optimized the codon usage for expression in *E. coli* (GenBank® accession numbers, respectively, KU894586 and KU894587), while the gene was produced by GeneArt (Thermo Fisher Scientific). To facilitate subcloning into the pRSET A plasmid (Invitrogen), we added *BamHI* and *HindIII* sites by PCR amplification at the 5'- and 3'-ends of the cDNAs, respectively. The resulting expression cassette comprised six histidine residues at the N-terminal.

We expressed recombinant proteins in *E. coli* BL21(DE3)pLysS cells grown at 37°C in LB medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol (+ 5 g/L NaCl for Hsp70), until OD_{600nm} ~ 0.6-0.8. For Hsp70, we added 0.1 mM IPTG and 25 g/L NaCl and collected the cells after 20h. For Tat-Hsp70, we added 1 mM IPTG, lowered temperature to 20°C, and collected the cells after 4h.

To improve the solubility of both Hsp70s, we removed IPTG and suspended cell pellets in 1/10 fresh medium supplemented with 30 µg/mL kanamycin, 100 µg/mL ampicillin and 34 µg/mL chloramphenicol for 2h at 20°C before harvesting[5]. We suspended cell pellets in 20 mM Tris-HCl buffer, pH 7.5, 2 mM $MgCl_2$, 150 mM NaCl, 1% glycerol, 0.7 µg/mL pepstatin, 0.19 mg/mL phenylmethylsulfonil fluoride and DNase, and disrupted by sonication. We removed the insoluble fraction by centrifugation at 39,000 x g for 1h at 4°C. We purified both proteins from soluble extracts using Ni^{2+}-affinity chromatography (HiTrap Chelating columns, GE Healthcare, Uppsala, Sweden) and equilibrated with 50 mM sodium pyrophosphate, pH 7.5, 1 M NaCl. We eluted the bound protein with 500 mM imidazole in 50 mM sodium pyrophosphate and 1% glycerol. We removed imidazole by dialysis at 4°C against 10 mM Tris-HCl pH 8.0 and 250 mM KCl. We performed size exclusion chromatography on a Superdex 200 Increase column (GE Healthcare), using 10 mM Tris-HCl, pH 8.0, and 250 mM KCl. We removed endotoxins[5].

Biochemical characterization of Hsp70 and Tat-Hsp70

Western blotting: after production, we confirmed the identity of purified recombinant Hsp70s by western blotting with anti-human Hsp70 antibody (1:500, Santa Cruz Biotechnology, Dallas, TX, USA).

Circular Dichroism: to assess whether the Tat sequence modifies the secondary structure of native Hsp70, we recorded circular dichroism spectra at 15°C in 10 mM Tris-HCl buffer, pH 8.0, 250 mM KCl, on a J-810 Jasco spectropolarimeter (0.1 mg protein/mL; cell path length was 0.1 cm for measurements in the 200-250 nm region).

ATPase activity: since the ability to bind and hydrolyze ATP are key features of a foldase chaperone, to confirm that Hsp70 and Tat-Hsp70 were active, we evaluated their ATPase activity as release of inorganic phosphate from ATP by measuring the absorbance at 660 nm[5,6].

Hydrogel characterization

Rheological characterization: we measured storage (G′) and loss modulus (G″) at 37°C using a rheometer (Bohlin Gemini, Malvern Instruments, Malvern, UK) with serrated parallel plates (15 mm diameter). We run small amplitude oscillatory shear tests from 0.01 to 2 Hz. To assess the viscosity as a function of shear rate, we performed steady shear measurements with shear rates from 0.01 to 100 s^{-1}. We investigated injectability at 37°C using a syringe (4.5 mm inner diameter, 30G needle) mounted on an INSTRON 5566 testing machine (Bucks, UK). We loaded 0.5 mL material into the syringe, then drove the piston at 40 mm/min and measured the load applied to inject the material into and through the needle. As control, we tested an empty syringe.

Morphological characterization: we prepared the hydrogels (0.5 mL) in custom-made cylindrical molds (inner diameter 11.05 mm). We investigated their 3D organization by scanning electron microscopy (SEM, Quanta 200, FEI, Hillsboro, OR, USA). To have a better resolution than SEM, we also observed their morphology by transmission electron microscopy (TEM, 1010 EX, Jeol, Tokyo, Japan), as described[1]. One day after preparation, we fixed the hydrogels for 15 min in 4% p-formaldehyde in PBS, washed with PBS, embedded in Polyfreeze tissue freezing medium (Polysciences, Warrington, PA, USA) and frozen in liquid

nitrogen. We cut cryosections (7 μm thick) with a cryotome (CM 1850, Leica, Wien, Austria) before storing at -20°C.

We studied the arrangement of COLL fibers and HA chains by optical microscopy after staining hydrogel cryosections with Picrosirius red and Alcian blue.

To confirm that COLL had not loss its bioactivity during hydrogel preparation, we stained the slides with an anti-COLL type I antibody (1:100, Santa-Cruz Biotechnology) before incubation with Cy3-conjugated secondary antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA, USA), as described[1]. Finally, we mounted the slides with coverslips and examined with a fluorescence microscope (IX70, Olympus, Shinjuku, Tokyo, Japan) using 550/580 nm excitation/emission filters.

Cytocompatibility: we tested hydrogel cytocompatibility with SH-SY5Y cells. We prepared 3D samples (0.5 mL) by mixing 1 part (v/v) cell suspension ($6 \cdot 10^6$ cells/mL) to 9 parts (v/v) polymer solution, placed in 48-well plates, incubated for 1h at 37°C and flooded with 0.75 mL medium. On day 1, 4 and 7, we incubated (2h, 37°C) the samples with 10% (v/v) MTS (Promega Corporation, Madison, WI, USA) before measuring the absorbance at 490 nm.

Particle characterization

We examined particle morphology and size distribution by Nanoparticle Tracking Analysis (NTA, NanoSight LM10, Malvern). We investigated their biological effect on SH-SY5Y cells. We diluted the particles to 25, 50, 100, 250, 500 μg/mL. The day after plating, we incubated the cells ($93.75 \cdot 10^3/cm^2$) with 100 μL particle suspension. After 24h and 72h, we measured cell metabolic activity by resazurin assay. We normalized the results to controls in standard medium.

To reveal nuclei and cytoplasmic F-actin, we fixed the cells with p-formaldehyde, permeabilized with Triton X-100 and stained, respectively, with 1 μg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and 0.1 mg/mL phalloidin.

Composite characterization

We measured G′ and G″, viscosity as a function of shear rate and injectability, as explained and selected the most promising formulations (COLL-LMW HA and COLL-HMW HA semi-INPs, loaded with particles to a concentration of 25 μg/mL). We performed biological studies with SH-SY5Y cells, as described and selected COLL-LMW HA composites for further assessments.

After preparing Tat-Hsp70-loaded composites, we measured G′ and G″, viscosity as a function of shear rate and injectability, as described.

***In vivo* investigations**

Peripheral inflammatory response: we tested the effects of the basic components and the prototype on inflammatory response in a peripheral model (air pouch model[7]). On day 5 after pouch formation, we injected subcutaneously eight-week-old male CD-1 mice with 1 mL saline, materials (LMW HA solution 2.5 mg/mL, COLL or COLL-LMW HA gels, unloaded or Tat-Hsp70-loaded composites) or 0.5% methylcellulose containing 0.5 μg ultrapure lipopolysaccharide from *E. coli* 0111-B4 strain (InvivoGen, San Diego, CA, USA). After 4h, we recovered the exudates from ultrapure lipopolysaccharide, while after 24h we collected the remaining. We stained with Trypan blue dye and counted the total leukocytes.

Behavioral tests: as described, we injected the mice with Tat-Hsp70-loaded composites + 6-OHDA + Tat-Hsp70-loaded composites; free Tat-Hsp70 + 6-OHDA + free Tat-Hsp70; PBS + 6-OHDA + PBS; and PBS + PBS + PBS (control group) and performed behavioral experiments on day 10 after the injection of 6-OHDA. We assessed motor coordination and balance ability by Rotarod test and beam walk.

Rotarod test: we used an accelerating Rotarod apparatus (model 7650, Ugo Basile Biological Research, Varese, Italy) consisting of five rotating drums (diameter: 3 cm) divided by six gray Plexiglass disks. We positioned the mice on the rotating bar and accelerated from 4 to 30 rpm over 5 min at a constant velocity of 0.12 rpm/s. We repeated for four times (intertrial interval: 30 min). The equipment automatically recorded the latency period to fall. We considered two consecutive passive rotations without walking, but only accompanying the rod, as a fall. We calculated the average of the four trials and normalized the results to the control group (mice receiving three injections of PBS).

Before the first injection, we trained the mice on the Rotarod apparatus to acquire the capability to stay for 5 min on a diameter rod, rotating at 20 rpm. They learnt the task after a maximum of three trials.

Beam walk: before the first injection, we trained the mice to walk on an elevated and narrow (7 mm wide) wooden beam (100 cm long). When testing the mice (3 trials, intertrial interval: 2h), we recorded the number of foot faults. We calculated the average of the three trials and normalized the results to the control group.

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