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

Journal name: NPG Asia Materials

Author names: Marta Tunesi, Ilaria Raimondi, Teresa Russo, Laura Colombo, Edoardo Micotti, Edoardo Brandi, Pamela Cappelletti, Alberto Cigada, Alessandro Negro, Luigi Ambrosio, Gianluigi Forloni, Loredano Pollegioni, Antonio Gloria, Carmen Giordano and Diego Albani

Correspondence to: Prof. Carmen Giordano, Department of Chemistry, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milan, Italy

Phone: +39.02.2399.3122

Fax: +39.02.2399.3280

E-mail: carmen.giordano@polimi.it

SUPPLEMENTARY MATERIAL S4 (Acrobat file)

Text summary: Supplementary Material S4 details the experimental procedure to obtain Tat-Hsp70-loaded particles and reports the preliminary data related to the release kinetics.

1. Preparation of Tat-Hsp70-loaded particles

We adapted a previous two-step desolvation method[1] to produce gelatin nanoparticles encapsulating Tat-Hsp70. We prepared a 5% (w/v) solution of gelatin (200 mg) in deionized water, and then we added an equal volume of acetone to separate the high-molecular weight gelatin. After exactly 2 min, a white colored supernatant was discharged. We freeze-dried overnight the gel-like precipitate and used the powder to obtain a 5% (w/v) solution of gelatin in deionized water (pH 3.5). After stirring for 10 min, we added Tat-Hsp70 (2% w/w) and 40 mL of acetone dropwise (less than 3 mL/min). The mixture was stirred for another 10 min. To form stable gelatin nano-colloids, we crosslinked with 100 µL of glutaraldehyde solution (25% w/w in

water) and stirred overnight. We separated the solid gelatin particles by centrifuging (5,800 rpm for 10 min) and washing with water/acetone (70/30), followed by sonication and centrifugation. We repeated this procedure for three times. Finally, we freeze-dried Tat-Hsp70-loaded particles overnight and stored as a powder.

2. *In vitro* protein release

By using a 2% w/w theoretical drug loading, we produced particles with a diameter around 295 nm (mean value, data from dynamic light scattering) and entrapment efficiency (EE) of about 79.3%. We calculated the EE by dividing the difference between the total mass of Tat-Hsp70 and the mass of Tat-Hsp70 in solution for the total mass of Tat-Hsp70.

We performed *in vitro* release experiments at 37°C with an orbital shaker. We suspended Tat-Hsp70-loaded particles in PBS. At predetermined time intervals, we withdrew 1 mL supernatant and replaced with 1 mL fresh PBS before returning the tube to the orbital shaker. As control, we used unloaded gelatin particles. Finally, we measured the absorbance of the supernatants at 280 nm by a UV spectrophotometer and subtracted the background from unloaded particles. We calculated the amount of Tat-Hsp70 released thanks to a calibration curve based on known amounts of Tat-Hsp70. We performed the experiment in triplicate and plotted the results as a function of time (mean ± standard deviation, SD).

Fig.1 shows the release profile of Tat-Hsp70 from gelatin particles.

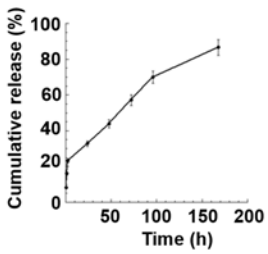

Fig.1: Release profile of Tat-Hsp70 from gelatin particles

We performed the experiment at 37°C on an orbital shaker. We reported the results as cumulative protein release (%) over time (mean ± SD, 3 replicates).

Over 60% of Tat-Hsp70 was released in less than 80h, and then the release rate decreased. The initial burst phase may be due to the protein absorbed on the surface of the gelatin particles, while the reduction of the release rate may be attributed to the reduced concentration of Tat-Hsp70 within the gelatin particles.

Our results agree with previous work on the release of bovine serum albumin from gelatin particles obtained with the same preparation method[2].

References

1. Coester, C. J., Langer, K., van Briesen, H. & Kreuter, J. Gelatin nanoparticles by two step desolvation-a new preparation method, surface modifications and cell uptake. *J Microencapsul*. **17**, 187-193 (2000).
2. Foox, M & Zilberman, M. Drug delivery from gelatin-based systems. *Expert Opin Drug Deliv*. **12**, 1547-1563 (2015).