# Synthesis of ROS scavenging microspheres from a dopamine containing poly(β-amino ester) for applications for neurodegenerative disorders

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## Materials and Methods

### Synthesis of dopamine-containing poly($\beta$-amino ester) (DPAE)

All reagents were purchased from Sigma Aldrich and used as received unless otherwise stated. DPAE was synthesized by mixing poly(ethylene glycol) diacrylate $(PEGDA)_{700}$ [678 mg] and dopamine hydrochloride [183.5 mg] in dimethylsulfoxide (DMSO) [678 µl], followed by 10 seconds of sonication to dissolve the dopamine. The 1.3:1 molar ratio of PEGDA to DOPA was used as reported previously, to ensure acrylate termination of the polymer chains [1]. The reaction was conducted at 90°C for 12 h with stirring at 700 rpm. The resulting macromers (hence forward termed pre-polymer) were precipitated in excess diethyl ether under vigorously stirring. Upon sedimentation the supernatant was aspirated and fresh diethyl ether added to repeat the process. The pre-polymer was dried in the presence of nitrogen and stored in the dark at 4°C prior to analysis. $^1$H NMR was utilized for chemical characterization of the synthesized DPAE. The $^1$H spectroscopy was performed in DMSO–d6 at 500 MHz using a NMR spectrometer Avance III (Bruker Biospin). The NMR data was analysed using ACD/NMR Processor Academic Edition. The molecular weight (number average molecular weight ($M_n$), weight average molecular weight ($M_w$), and polydispersity ($Đ$)) of DPAE was determined by gel permeation chromatography (Agilent 1260 Infinity Multi-Detector GPC) equipped with triple detectors (RI, viscometer and LS). The polymers were analyzed at a concentration of 5.0 mg/mL in DMF by the RI detector. Two 30 cm PLgel Mixed-C, columns in series were eluted with DMF (containing 0.1% LiBr) and calibrated using a series of 12 near-monodisperse PMMA standards (Mpfrom 690 to 1,944,000 g/mol). All calibrations and analyses were performed at 60oC and a flow rate of 1 ml/min.

### Synthesis of DPAE microspheres

The synthesis of the microspheres was carried out via adaptation of the "dual photoinitialor approach" described by Franco C.L. *et al.* [2]. 30 µL of the pre-polymer was added to 1 mL of mineral oil in a small glass vial, vortexed (Vortex Genie; VWR) at maximum speed for 5 seconds, and immediately exposed to UV light for 90 seconds at an intensity of ~8,000 mW/cm$^2$ (Delolux 04). The mineral oil (PlusOne Drystrip Cover Fluid) was supplemented with 1 % of the non-ionic detergent

sorbitane trioleate (SPAN® 85), and 2 % of polyethylene glycol sorbitan monolaurate (TWEEN® 20). In order to initiate a polymerization of the pre-polymer in the presence of UV light, the photoinitiator (PI) 2-hydroxy-2-methylpropiophenone, used previously for photoinitiated polymerization of acrylates [3], was added into the pre-polymer at 1 % (w/v). Furthermore, 2,2-dimethoxy-1,2-diphenyl acetophenone (Irgacure® 651, I651) dissolved in acetone [300 µg/mL] was added into the oil phase to act as a second PI at 10 µL $mL^{-1}$. The suspension containing the polymer microspheres was taken out and collected in a 15 mL falcon tube. The dispersed microspheres were separated from the mineral oil by addition of distilled water ($dH_2O$) and subsequent centrifugation at 6000 x g for 5 min. After the phase separation the oil layer was carefully aspirated and the procedure was repeated three times for additional wash steps with 3 mL of $dH_2O$. Finally, the microspheres were stored in sterile $dH_2O$ supplemented with penicillin [100 U/mL] and streptomycin [100 µg/mL]. A variation of the above protocol involved the addition of the cell-compatible surfactant Pluronic® F-68[114] to the pre-polymer at a final working concentration of 0.1% prior to applying the vortex.

**Synthesis of PEG microspheres**

The protocol described above was used for the synthesis of PEG microspheres for use as a non-catechol containing control for the subsequent experiments. 30 µL of PEGDA ($M_w$ = 700) was used instead of the DPAE but all other factors were kept the same.

**Microsphere characterization**

The concentration of microspheres within the suspensions was ascertained via counting of microsphere samples on micro grid dishes via bright field microscopy (Leica DM IL). Micro grid dishes (µ-Dish$^{35mm}$ Grid-500; Ibidi) featuring an imprinted 500 µm relocation grid were used for counting purposes. Counting of 10 µL samples in triplicate enabled the determination of the microsphere concentration within a suspension. For cell culture applications, the gained microsphere suspensions where initially sterilised via UV-C light exposure, and stored in sterile Milli-Q water supplemented with penicillin [100 U/mL] and streptomycin [100 µg/mL]. For size distribution analysis images of the microsphere samples were taken using an inverted microscope (Leica DM IL) with mounted camera (Leica DFC420 C), operated with the imaging software Leica Application Suite (LAS). Images were processed using the imaging software Fiji 1.47v for OS X. Statistical analysis were performed using Microsoft® Excel® 2011 for OS X v.14.5.2 and StatPlus®:mac v5 5.8.2.0., respectively. A minimum of 500+ particles were analysed per condition.

Scanning electron microscopy (SEM) was additionally used to image synthesized microspheres. Microspheres were dried on specimen stubs and sputter-coated with gold for 60 seconds (BALZERS SCD 050 Sputter Coater) before imaging. SEM studies were performed using a XL30 ESEM-FEG

microscope (Philips) in high vacuum mode using accelerating voltages of 5 kV.

**Measurement of the dissolved oxygen concentration**

The dissolved oxygen in PBS containing the microspheres was analysed using Presens non-invasive OXY4 mini equipment. Prior to measurements, cuvettes equipped with PSt3 oxygen sensors were washed with Milli-Q water, followed by methanol and subsequently dried. The chambers were then filled with 300 µL of PBS supplemented with penicillin [100 U/mL] and streptomycin [100 µg/mL]. Polymer samples were incubated in supplemented PBS under atmospheric conditions at room temperature for indicated times, respectively. This set-up allowed testing of four independent samples in parallel.

**Analysis of radical scavenging**

To investigate the radical scavenging properties of DPAE and PEG microspheres, DPPH was dissolved in ethanol to a final working concentration of [0.2 mM]. DPPH solutions were supplemented with the test samples in a 48 well plate, protected from light with aluminium foil and incubated at 37°C for the desired time period. After the set time of incubation the plates were put on a shaker for 15 min at 200 rpm. For the spectrophotometric analysis 3 x 50 µL of each well was transferred into a 96-well microtiter plate for absorbance measurements at $\lambda$ = 540 nm. Absorbance values were taken using a plate reader (Tecan GENios). Ascorbic acid (AA) [10 µM] was used as positive control of the assay. Four repetitions were performed per condition. DPPH [0.2 mM] served as 100 % radical control, in comparison to the test samples. The ratio of free radical scavenging was calculated via normalization of the test sample absorbance to the absorbance of the DPPH alone as reported by Guerrero, *et al.*:[115]

**Cell culture experimentation**

For cell culture experiments the human neuroblastoma cell line SH-SY5Y (Sigma-Aldrich) between passages 17-35, primary astrocytes for the newborn (P1-4) rat cortex, and primary neural cells from the midbrain of embryonic rats (E12-14) were used. The primary astrocytes were extracted as reported previously[4] and cultured in Dulbecco's Modified Eagles Medium (Sigma) supplemented with F12 Ham mixture (50%)(Sigma), fetal calf serum (10%) and penicillin/streptomycin (1%). The primary neural cells were a kind gift from Prof. Storch of the University Hospital Dresden. SH-SY5Y cells were cultured in Dulbecco´s Modified Eagle´s Medium with nutrient mixture F-12 (DMEM/F-12) and GlutaMAX™, supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), penicillin [100 U/mL] and streptomycin [100 µg/mL]. Cells were maintained in humidified atmosphere of 5% $CO_2$ at 37°C.

For *in vitro* experiments SH-SY5Y cells were seeded into 96-well plates in an appropriate density of 1 x $10^4$ per well 24 hours prior to experimentation.

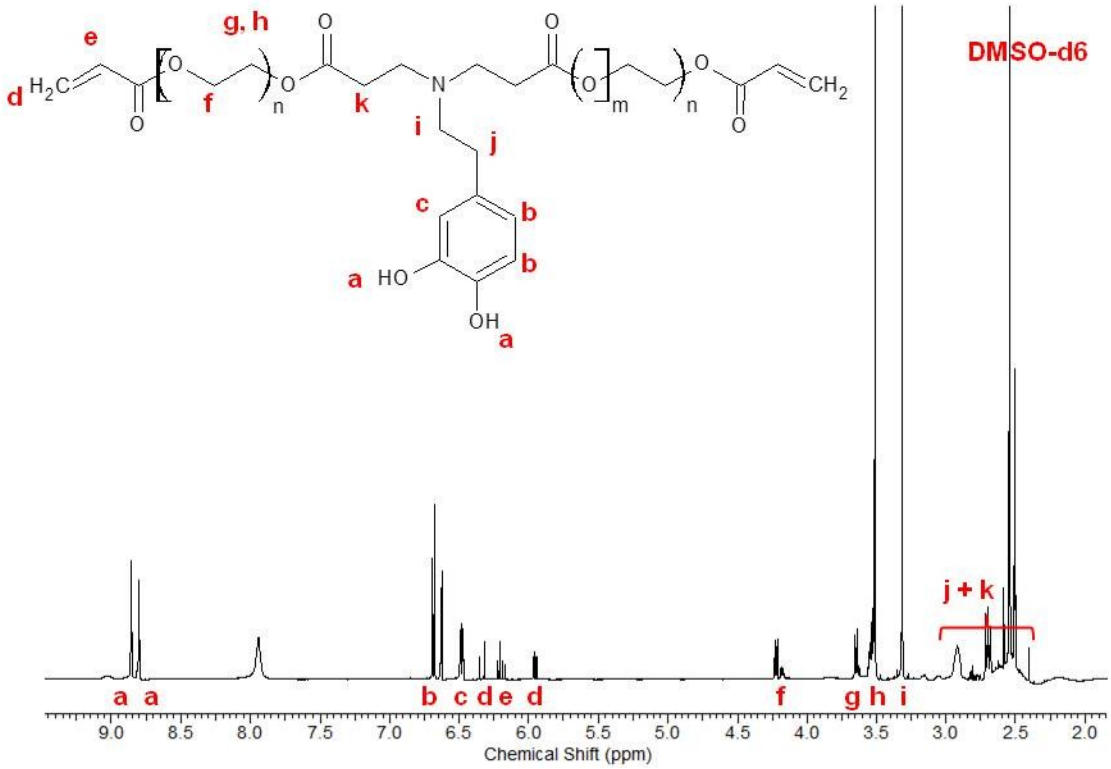
Primary neural cells from the midbrain were cultured in DMEM (high glucose) supplemented with GlutaMAX™ [4 mM], 10 % (v/v) heat-inactivated fetal bovine serum (FBS), penicillin [100 U/mL], streptomycin [100 µg/mL], and HEPES [10 mM]. Culture flasks and test wells were pre-coated with poly-L-lysine (PLL) beforehand. Primary cells were seeded in a density of 3 x $10^4$ per well in 96-well plates 24 hours prior to experimentation.

**Analysis of the effect of microspheres on cell metabolic activity**

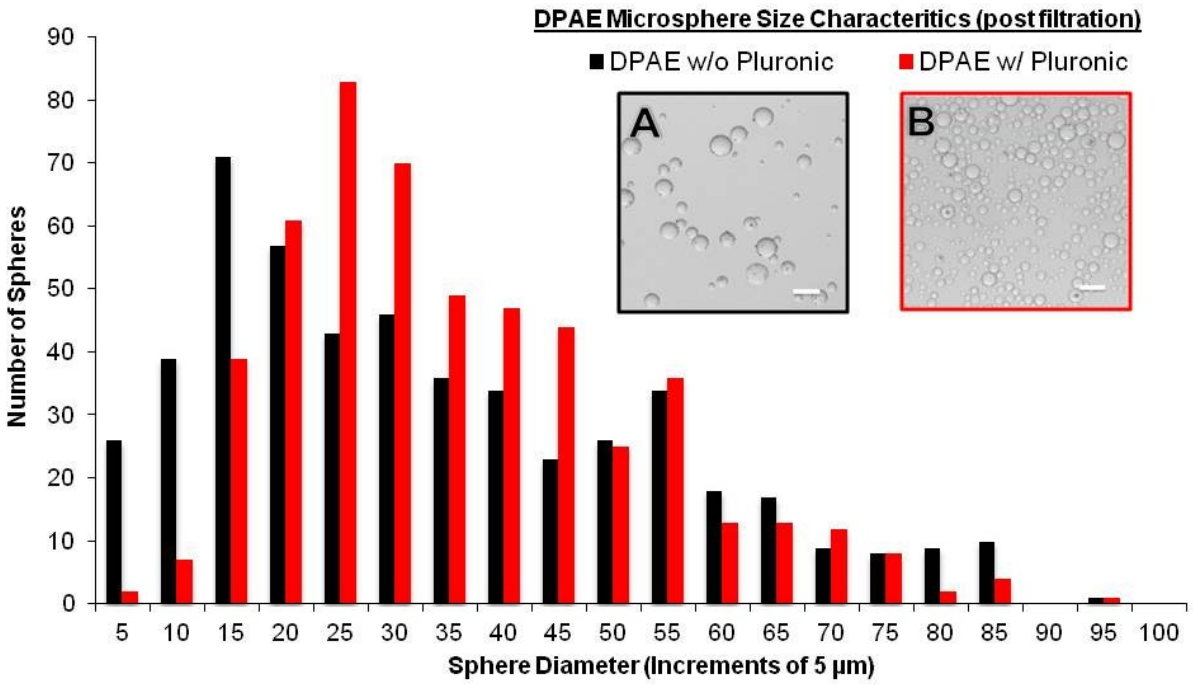
Cytotoxicity analysis of the PEG and DPAE microspheres was carried out using SHSY-5Y and two primary cultures using the PrestoBlue® cell viability assay which measures the cell metabolic activity. Two concentrations of microspheres were incubated with the cells which had previously been seeded in a 96 well-plate. After the desired incubation period the cell media was removed and replaced with media containing 10% PrestoBlue®. After 30 minutes the fluorescence was measured using a plate reader (TECAN) according to manufacturers protocol. Cell viability was normalized to cells that received no treatment (100% viable), subtracting the blank reading from PrestoBlue® solution alone (n= 5), average and standard deviation calculated). The positive control group was treated with 10% v/v dimethyl sulfoxide (DMSO) to ensure killing of the cells. To analyse whether the microspheres themselves interacted with the assay, two cell free control groups were included which composed of PEG spheres and DPAE spheres at 7000 spheres/mL. No enhancement or reduction in the fluorescent read-out was observed.

**Analysis of the effect of microspheres on cell membrane integrity**

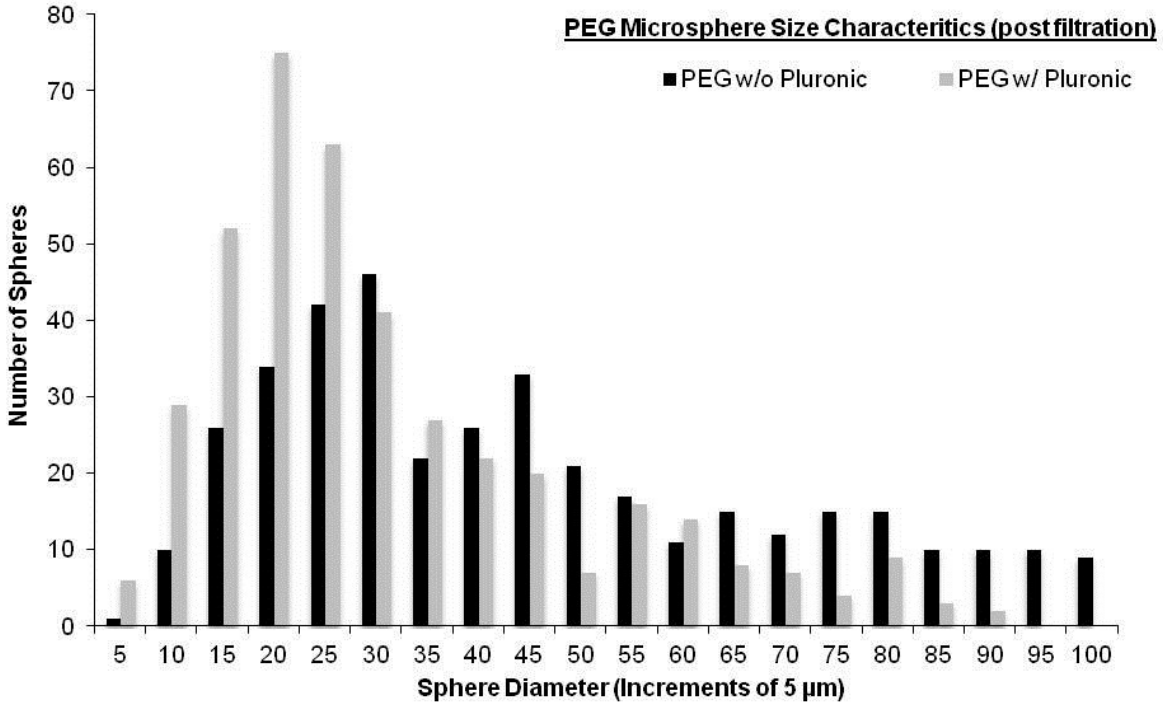
To analyse the effect of the microspheres on lactose dehydrogenase (LDH) release, astrocytes and SH-SY5Y cells were subjected to the same treatment of microspheres or control groups as described above. After the desired incubation period, the supernatant was carefully removed from the cells for analysis via the LDH assay (Takara) according to the manufacturer's instructions using a TECAN plate reader (absorbance wavelength = 492nm). Again, cell free controls (as above) were analysed and found to have no effect on the absorbance.



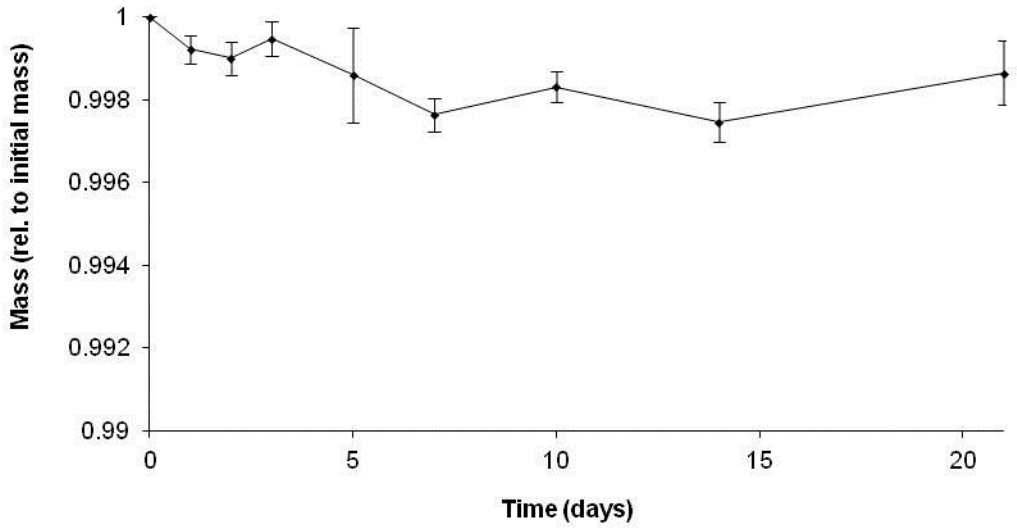

*Supplementary Information Figure S1 - $^{1}H$ NMR spectroscopy of the DPAE polymer with subsequent peak allocation. Note the presence of a peak at 7.96ppm indicating chain end dopamine or dopamine impurity.*



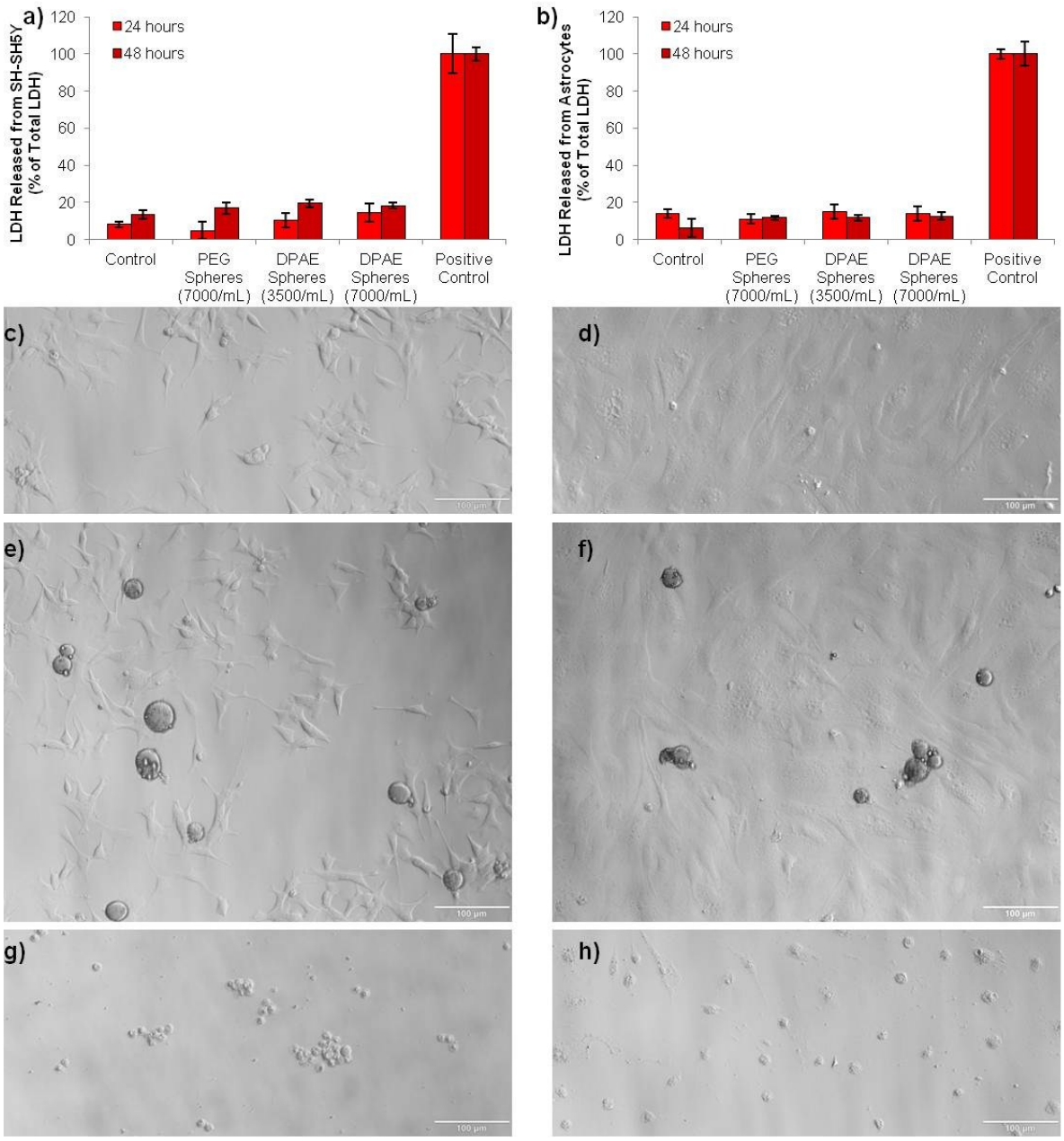

*Supplementary Information Figure S2 - DPAE microsphere size distribution after filtration through a 100 µm mesh with and without the use of Pluronic surfactant during the synthesis process.*



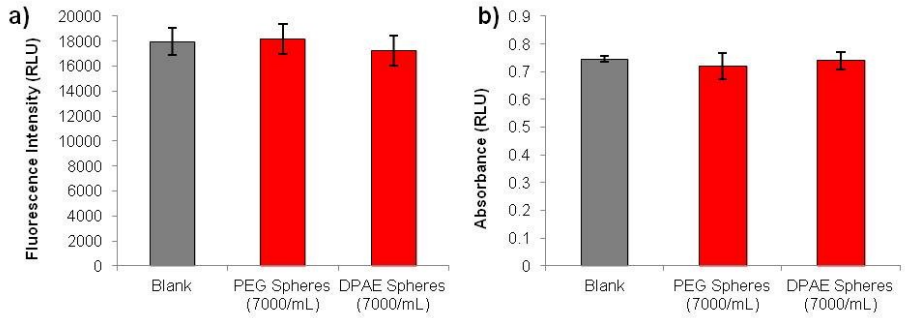

*Supplementary Information Figure S3 - PEG microsphere size distribution after filtration through a 100 µm mesh with and without the use of Pluronic surfactant during the synthesis process.*




*Supplementary Information Figure S4 - No significant mass loss was observed for the photo-crosslinked DPAE polymer in PBS at 37°C for up to three weeks (longest time analysed).*




*Supplementary Information Figure S5 - Analysis of the effect of PEG and DPAE microspheres on LDH release from SH-SY5Y cells (n=4) (a) and primary astrocytes (b) showing all groups to be under 20% of the total LDH present in the cells. The control group received no microspheres, and the positive control group received media containing 10% DMSO. Light microscopy analysis of SH-SY5Y cell morphology (c, e, g) showed no effect of DPAE spheres (7000/mL) (e), compared to the cell alone control group (c), however the effect of DMSO treatment can be observed in (g). The same trend was true for the primary astrocytes (d, f, h) with both cell alone (d) and DPAE (7000/mL)(f) show the spread morphology of the astrocyte extracellular matrix before confluency (when the well known projection like morphology can be observed), but the damage that occurs by treatment with DMSO (h). Scale bars represent 100 µm.*




*Supplementary Information Figure S6 - Control groups for the PrestoBlue analysis (a) and LDH analysis (b) which both showed no interaction of the microspheres with the assay components.*

## References


1. DenizáYilmaz, M. and J. FraseráStoddart, *Esterase-and pH-responsive poly (β-amino ester)-capped mesoporous silica nanoparticles for drug delivery.* Nanoscale, 2015. **7**(16): p. 7178-7183.
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