

Functionalized mesoporous silica nanoparticle-based drug delivery system to rescue acrolein-mediated cell death

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Aims: Mesoporous silica nanoparticles (MSNs) were prepared and characterized to develop a drug delivery system by loading them with hydralazine and functionalizing them with polyethylene glycol. These agents restore damaged cell membranes and ameliorate abnormal mitochondria behavior induced by the endogenous toxin acrolein. Such a formulation shows potential as a novel therapeutic agent. **Results & discussion:** MSNs with encapsulated hydralazine and covalently linked with polyethylene glycol were subsequently synthesized and characterized by transmission-electron microscopy, N₂ adsorption/desorption, x-ray diffraction and UV-vis spectroscopy. MSNs exhibited large surface area, pore volume and tunable pore size. The mean particle size was 100 nm and hydralazine encapsulation efficiency was almost 25%. These were tested using PC12 in culture to restore their disrupted cell membrane and to improve mitochondria function associated with oxidative stress after exposure to acrolein. Lactate dehydrogenase, MTT, ATP and glutathione assays were used to examine the physiological functioning of the samples and the loss of lactate dehydrogenase from the cytoplasm assayed the integrity of the membranes. These evaluations are sufficient to initially demonstrate drug delivery (concentrated hydralazine) into the compromised cells cytoplasm using the MSNs as a vehicle. **Conclusion:** MSNs modified with drug/polymer constructs provide significant neuroprotection to cells damaged by a usually lethal exposure to acrolein.

Acrolein is the strongest electrophile of the reactive α , β -unsaturated aldehydes that is formed during lipid peroxidation induced by oxidants and oxidative stress. Acrolein, produced by various and different insults to cells, causes a diverse range of pathological biological cascades in addition to its well-known ability to crosslink biomolecules covalently. It attacks the nucleophile centers in DNA and proteins, which disrupts numerous cellular processes and eventually leads to dysfunction, damage and death by both necrosis and apoptosis. Acrolein production and accumulation is associated with oxidative stress-related diseases, including diabetic kidney disease, Alzheimer's disease, Parkinson's disease, ischemia-reperfusion injury, mechanical trauma, inflammation and atherosclerosis [1–9]. The toxicity of acrolein can be reduced by the activity of nucleophiles containing nitrogen species. Such nucleophiles immediately conjugate with free acrolein in the intracellular environment before forming adducts by alkylation.

Hydralazine, an antihypertension drug, is capable of efficiently binding and thereby decreasing the concentration of acrolein by forming hydrazone. Based on previously published studies, hydralazine is an efficient

trapping agent of acrolein [2,4,10]. Although not a direct antioxidant, polyethylene glycol (PEG) can significantly reduce free-radical-mediated injury through various mechanisms [11,12]. Moreover, particular hydrophilic polymers, such as PEG, poloxamines and poloxamers, have documented abilities to seal membranes [13]. First, they seal the region of damage and subsequently reduce the deleterious exchange of solutes across them. In addition, these strongly hydrophilic polymers interact with the aqueous phase of the damaged region of the bilayer, permitting the lipid core to become continuous once again [11,12,14,15]. Finally, PEG only targets damaged regions of the rat brain and guinea pig spinal cord [16,17]. Targeted drug delivery to specific cells or tissues is a critical issue because localization of drugs or their carriers – in this case, mesoporous silica nanoparticles (MSNs) – can reduce or even eliminate side effects.

Nanoparticle-based drug delivery systems have been developed in order to achieve this aim. Various carriers, such as micelles, liposomes and polymer nanoparticles, have for some time been of interest for this function. More recently, MSNs have become uniquely attractive because [18–22]:

Keywords: acrolein, controlled release, drug delivery, hydralazine, mesoporous silica nanoparticles, PC12 cells, polyethylene glycol

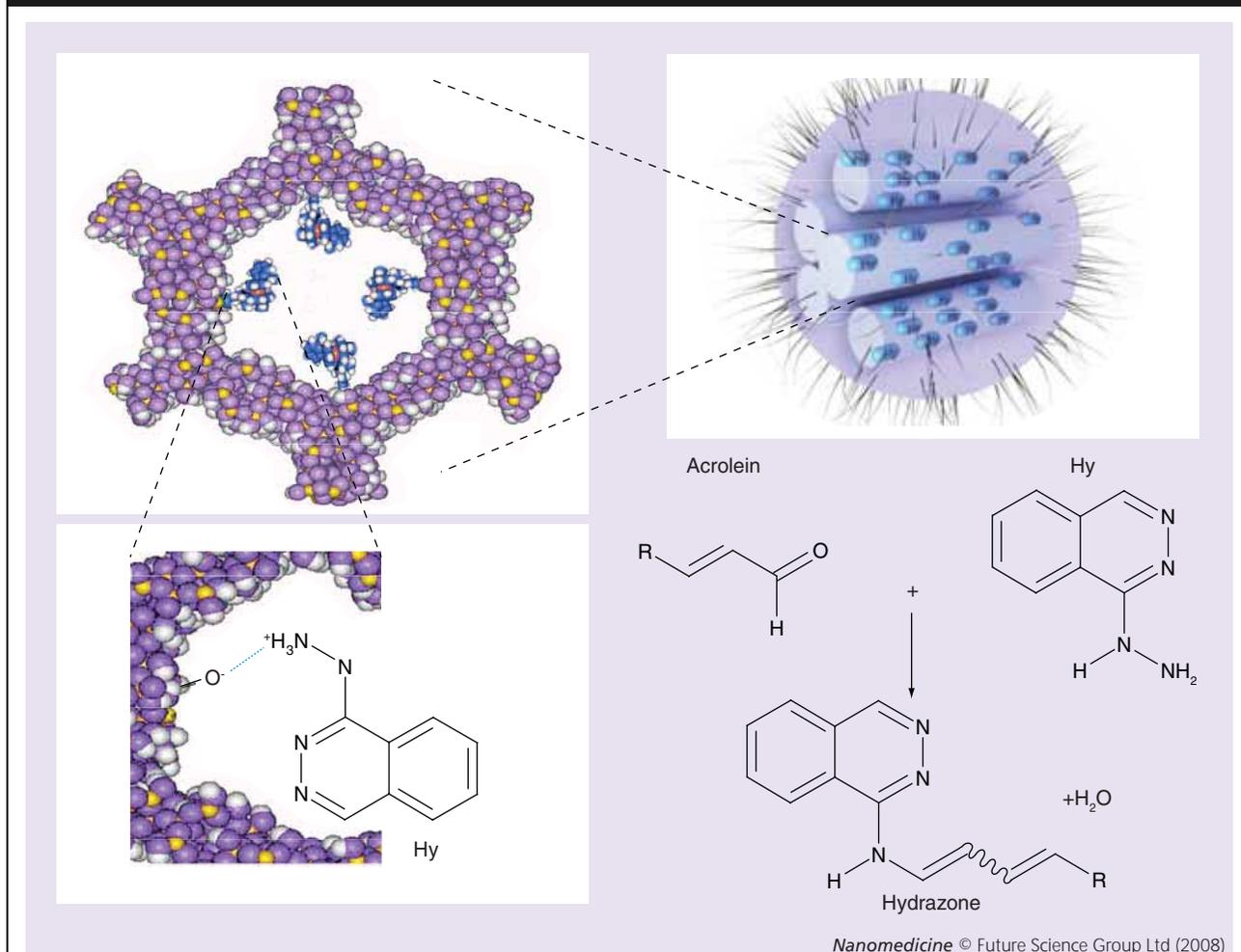
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- MSNs are nontoxic and biocompatible;
- MSNs can be fabricated to include a 'tunable' adjustable pore size, large surface area and a hexagonally ordered and well-defined internal structure;
- MSNs possess thermal and chemical stability and controllable degradation rates.

This versatility makes them ideal for adsorption and release of a variety of compounds by organically modifying them with the desired functional group that is attached to and within the walls of the pores. In addition, their controllable pore size and volume can contribute to the protection of the 'passenger' drug from unwanted

leakage and optimization of drug loading capacity. To date, research using MSNs has been focused on various biomedical applications, such as MRI permitted by the encapsulation of magnetic particles, labeling, diagnostics and drug delivery [19,23–28]. This report is focused on the application of nanotechnology to novel biomedical/bioengineering therapies in the treatment of trauma. In particular, we show that MSNs incorporated with hydralazine and functionalized with PEG restore disrupted cell-membrane function – leading to cellular rescue – after challenge by an endogenous toxin, the most potent of those causing secondary injury in the nervous system subsequent to the initial mechanical insult.

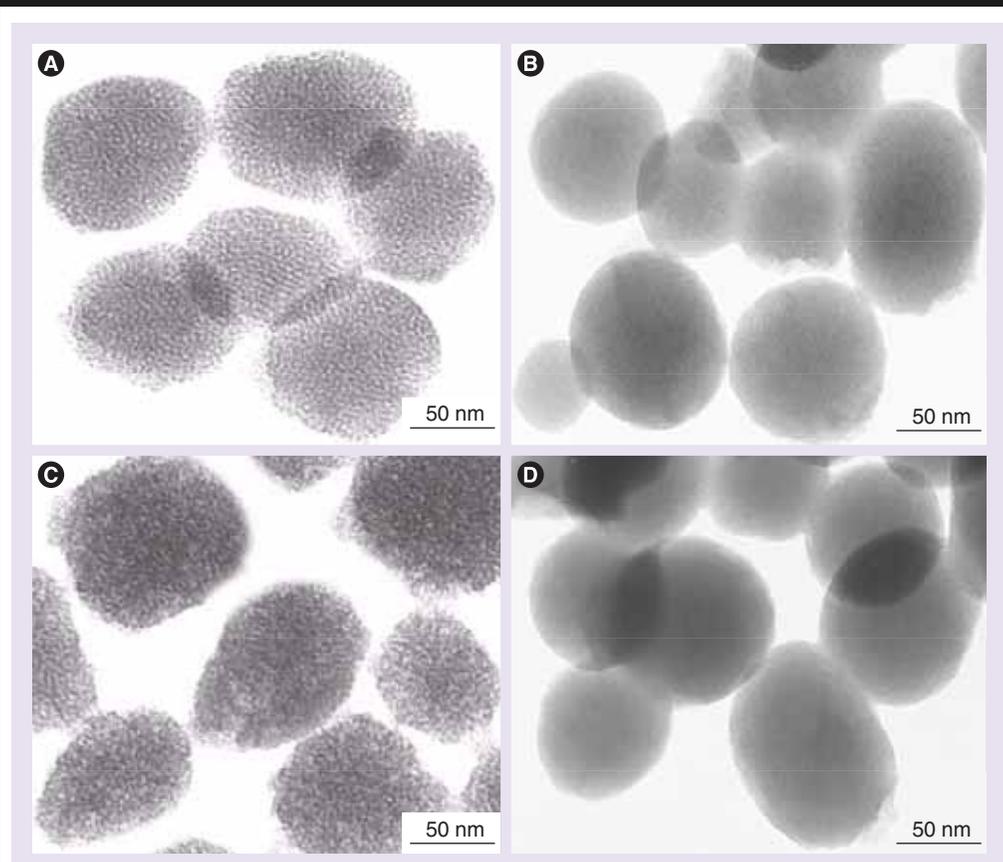
Figure 1. Illustration of mesoporous silica nanoparticles loaded with hydralazine and functionalized with PEG.



Hy was incorporated on the ordered silica framework by electrostatic interaction. PEG was coated on the nanoparticle by a two-step modification using an aldehyde moiety and subsequently functional precursor PEG-NH₂ (MW: 3000). The PEG coating was confirmed using Fourier transform infrared spectroscopy and showed peaks at approximately 2920, 1450 and 1030 cm⁻¹, which are characteristic of PEG (C-H stretching, C-H bending and C-O stretching) (data not shown). The inset shows the interaction of the nucleophile drug hydralazine with acrolein to form hydrazone, the nontoxic end product.

Hy: Hydralazine; MW: Molecular weight; PEG: Polyethylene glycol.

Figure 2. Transmission-electron microscopy images of (A) as-synthesized MSNs, (B) MSNs surface functionalized with PEG (MSN-PEG), (C) MSNs encapsulating hydralazine (MSN-Hy) and (D) MSNs loaded with hydralazine and surface modified with PEG (MSN-Hy-PEG).



Well-ordered hexagonally shaped MSNs with a mean diameter of ~100 nm were synthesized by sol-gel chemistry. After coating with PEG (B), the average particle size increased and the pore volume was decreased. The loading with hydralazine (C) caused the surface to become darker owing to the presence of drug inside the pores of MSNs, although the pores were still visible. By contrast, in (D), the pores were barely visible as a result of the addition of drug/polymer.

Hy: Hydralazine; MSN: Mesoporous silica nanoparticle; PEG: Polyethylene glycol.

Materials & methods

Synthesis & functionalization of MSNs

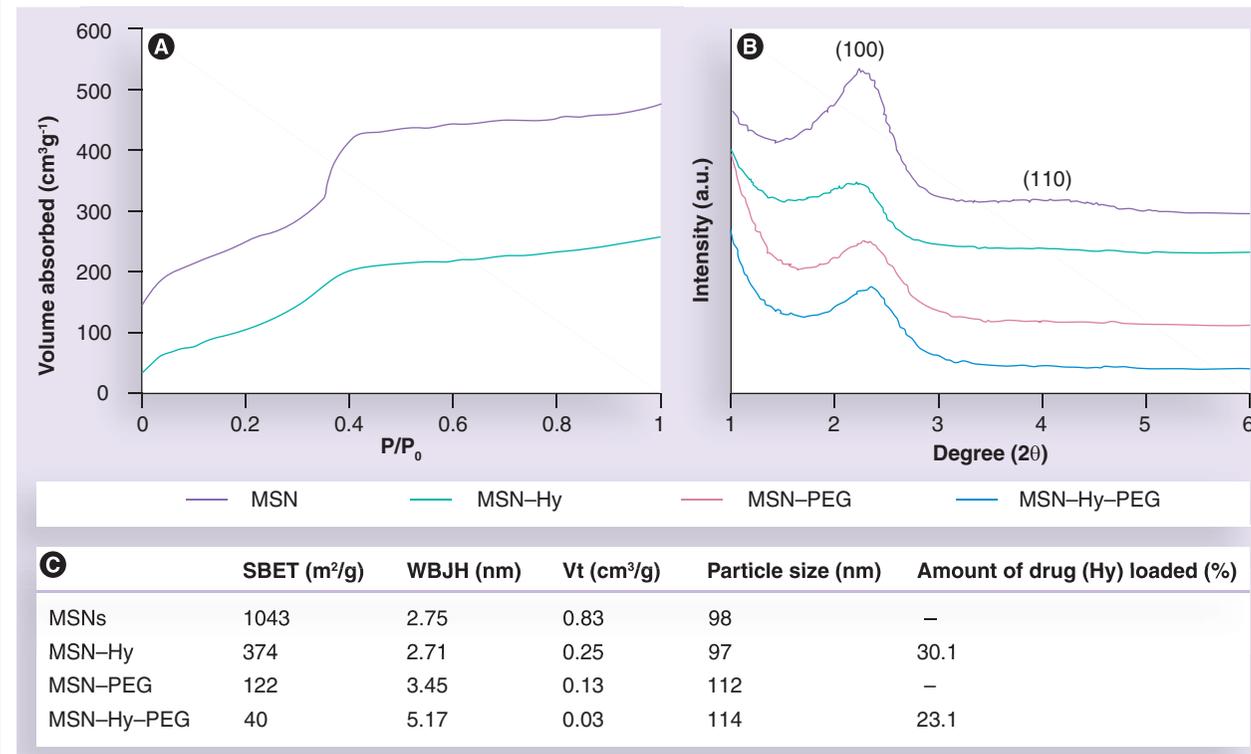
All chemicals were purchased from Sigma-Aldrich unless otherwise specified. MCM 41-type MSNs were synthesized according to the procedure described by Slowing *et al.* [29]. First, cetyltrimethylammonium bromide (CTAB), used as a template, was dissolved in a solution of deionized water and ammonia. After stirring at 80°C for 2 h, tetraethyl orthosilicate was added slowly to the mixture. The solutions were stirred at elevated temperature for another 3 h and then the white precipitate was collected by filtration, rinsed with water and dried at 100°C for 12 h. Finally, an acidic-extraction method (0.75 ml concentrated HCl/100 ml methanol solution)

was performed overnight to remove the CTAB template. MSN incorporating hydralazine (MSN-Hy) was prepared by adding 20 mg of as-synthesized MSNs to 10 ml of a 50 mM hydralazine solution. The mixture was shaken at room temperature for 24 h. The product was separated by centrifugation and dried in an oven overnight. The particles were further modified to link PEG covalently to the MSN surface using 3-(trimethoxysilyl) propyl aldehyde followed by coupling with PEG-NH₂ (3000 molecular weight).

Characterization of nanoparticles

The structural properties of MSNs were analyzed by nitrogen adsorption/desorption measurements at -196°C using an ASAP 2010 sorptometer

Figure 3. Characterization of MSNs.



(A) BET N₂ adsorption-desorption isotherms of as-synthesized MSNs (purple line) and MSN-Hy (green line), (B) x-ray diffraction patterns of as-synthesized MSNs and functionalized colloids, MSN-Hy, MSN-PEG and MSN-Hy-PEG. (C) Characterization of differently functionalized particles. The SBET, WBJH and V_t were measured using BET and BJH methods. The particle size was estimated by dynamic light scattering in deionized water.

BET: Brunauer–Emmett–Teller; BJH: Barrett–Joyner–Halenda; Hy: Hydralazine; MSN: Mesoporous silica nanoparticle; PEG: Polyethylene glycol; SBET: Brunauer–Emmett–Teller surface area; V_t: Pore volume; WBJH: Pore size.

(Micromeritics, USA). Before each measurement, as-synthesized particles and functionalized MSNs were outgassed at 300°C and 80°C, respectively. The morphology of MSNs was observed by transmission-electron microscopy (TEM; JEOL 2000FX). Particle size was measured by dynamic-light scattering. Powder x-ray diffraction was performed to collect the structural information using Siemens D500 diffractometer with Cu K α radiation.

Hydralazine release from particles

The fully dried hydralazine-loaded particles were suspended in modified Krebs' solution that contained 124 mM NaCl, 2 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 2 mM CaCl₂ and 26 mM NaHCO₃. Krebs' solution is appropriate for *in vitro* drug release tests owing to its similar ionic composition to human-body plasma. Hydralazine solutions with different concentrations were then prepared and measured by UV spectroscopy to obtain standard

curves. At different time intervals, the released drug was extracted and centrifuged to analyze the supernatant spectroscopically. The concentration was calculated by linear equation to determine the hydralazine-release curve.

Cell cultures

PC12 cells (density = 1 × 10⁶ cells/ml) were cultured in Dulbecco's modified eagle's medium (DMEM; Invitrogen) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 U/ml penicillin and 5 mg/ml streptomycin, at an incubator setting of 5% CO₂ and 37°C. After trypsinization and centrifugation, cell pellets were resuspended in Hank's balanced salt solution (HBSS) for acrolein and MSN testing. Acrolein was prepared fresh daily in phosphate-buffered saline solution. Differently functionalized MSN suspensions were applied at a concentration of 20 μ l/ml in medium, with a delay for 15 min after the application of acrolein (100 μ M).

Lactate dehydrogenase assay for cell membrane integrity

The lactate dehydrogenase (LDH) assay is used to evaluate cell-membrane integrity because the release of this large (9–160 KD) enzyme from the cytoplasmic compartment to the supernatant of cells is indicative of membrane damage. Based on the reduction of NAD by the action of LDH to form a tetrazolium dye, the amount of LDH was measured spectrophotometrically at 492 nm. The background absorbance measured at 660 nm was subtracted from the reading at 492 nm. Cells were grown in a 12-well plate at a density of 1×10^6 cells/well in HBSS and were used for LDH release into the medium and determination of total LDH, respectively.

$$\text{LDH(\%)} = \frac{A_{\text{medium}}(492 - 660\text{nm})}{A_{\text{total}}(492 - 660\text{nm})} \times 100$$

where A = absorbance of the resulting compound.

MTT assay for cellular viability

The MTT assay is based on a colorimetric assay system, in which tetrazolium rings of the pale yellow MTT are cleaved to form dark blue

formazan crystals by the activity of a mitochondrial dehydrogenase enzyme from viable cells. The number of healthy cells can be quantified by spectrophotometric measurements. The cells were seeded in a 12-well plate at 1×10^6 cells/well in HBSS. MTT reconstituted in phosphate-buffered saline was added to each well. After incubation, formazan crystals were pelleted by centrifugation and dissolved in a MTT solubilization solution. The absorbance was read at 550 nm minus the background at 660 nm.

ATP assay

The ATP assay provides useful information regarding the intracellular ATP concentrations that are one of the indicators for the metabolic state and integrity of the cell. In the presence of ATP, luciferase induces the oxidation of luciferin, which produces yellow-green light and can be measured conveniently by luminometers. The amount of light produced is directly proportional to the concentration of ATP. The ATP kit was purchased from Molecular Probes (Eugene, OR, USA). The cell pellets at 1×10^6 cells/well were lysed in lysis buffer and sonicated at two bursts of 10 sec each. After centrifugation, supernatant was boiled at 100°C for 4 min to denature proteins. The solution containing supernatant and standard-reaction buffer was evaluated by a FLx800 multidetection microplate reader.

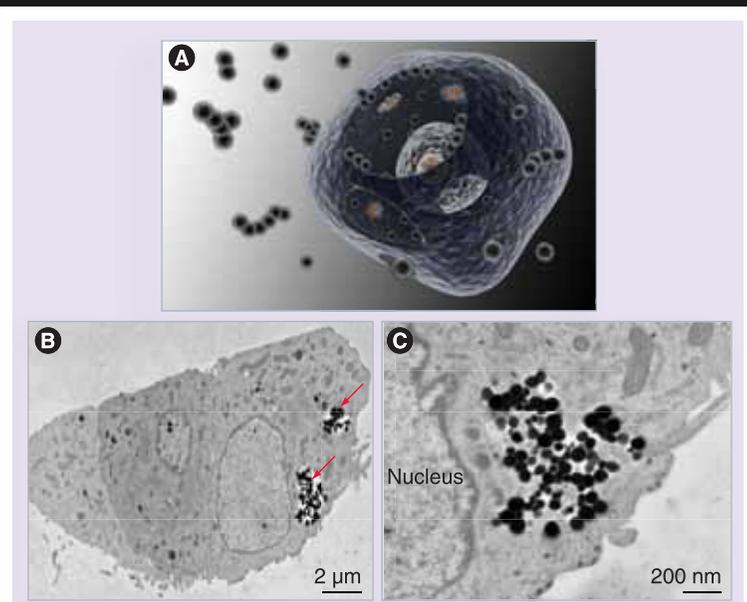
Glutathione assay

The glutathione (GSH) assay kit was purchased from Chemicon (Temecular, CA, USA). This assay is based on the fact that reduced GSH diminishes an endogenously induced oxidative stress by glutathione peroxidase and xenobiotics by glutathione transferase. After inducing apoptosis, cell lysates were incubated with monochlorobimane (MCB), a glutathione-specific dye. MCB has a high affinity for GSH, resulting in a blue fluorescence (excitation: 380 nm; emission: 461 nm). Fluorescence was detected by an FLx800 multidetection microplate reader.

Statistical analysis

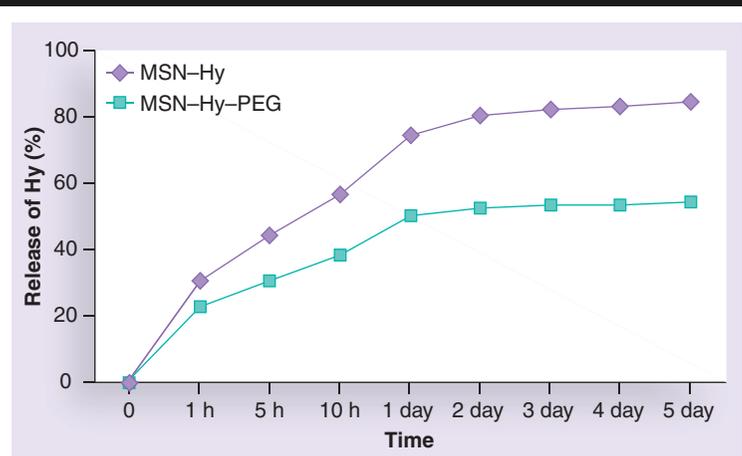
One-way ANOVA was used to determine the statistical significance between control and experimental groups using InStat software. Results are expressed as mean \pm standard deviation. All experiments were conducted with 3–5 repetitions; $p < 0.05$ was considered statistically significant.

Figure 4. Functionalized mesoporous particles in association with the cell.



(A) Representation of the endocytosis of PEG-functionalized MSNs containing Hy (MSN–Hy–PEG) by PC12 neuronal cells. **(B)** Transmission-electron microscopy images showing cellular uptake of MSN–Hy–PEG by PC12 neuronal cells. The arrows indicate the presence of MSN–Hy–PEG in PC12 cells after incubation for 2 h. **(C)** Magnified transmission-electron microscopy image of **(B)**. Hy: Hydralazine; MSN: Mesoporous silica nanoparticle; PEG: Polyethylene glycol.

Figure 5. Release profile of Hy from MSN-Hy and MSN-Hy-PEG materials.



At 1 day, 80% of Hy was released from pores inside MSN-Hy, whereas only 40% of Hy was dissolved from MSN-Hy-PEG. The coating with high molecular weight substance, PEG, decreases the release rate of drug substance compared with that of MSN-Hy.

Hy: Hydralazine; MSN: Mesoporous silica nanoparticle; PEG: Polyethylene glycol.

Results & discussion

Functionalization of MSNs with drug-polymer conjugation

The polymer-drug conjugation on MSNs has significant advantages, as follows:

- Hydralazine incorporated inside the channels of the silica framework can be delivered safely in the cytoplasm to scavenge reactive oxygen species associated with acrolein
- Application of PEG after injury inhibits the process of necrosis occurred by acute membrane disruption and facilitates the integrity of the cell membrane, thus eventually maintaining the intracellular level of ions

Particles of approximately 100 nm diameter are able to be internalized efficiently into cytoplasm by endocytosis to interact with cell compartments directly. The well-ordered internal structure of MSNs is a template for hydralazine adsorption by favorable electrostatic interaction between free silanol groups on the wall of pore and positively charged amine groups of drug (Figure 1). The successful encapsulation of hydralazine into the surface of the pores was confirmed by TEM, N₂ adsorption, x-ray diffraction, UV spectroscopy and Fourier transform infrared spectroscopy (data not shown). Figure 2 shows the TEM images that observe the morphology and interior structure of as-synthesized MSNs, MSN-Hy, MSNs functionalized with PEG (MSN-PEG) and MSNs with

hydrilazine encapsulation and PEG coating (MSN-Hy-PEG), respectively. CTAB-removed MSNs exhibit uniformity in size with regular spheres and well-defined hexagonal array. The TEM image of MSN-Hy displays the characteristic pore filling represented by dots and indicates the distribution of hydrilazine both on and in the silica framework (Figure 2B). The as-synthesized MSNs were further modified to covalently link PEG to silica surfaces (Figure 2C), resulting in an interruption of the porous structure of MSNs by bulky polymer.

In addition, MSN-Hy has further undergone functionalization with PEG to the surfaces of silica (Figure 2D). The physical properties of as-synthesized MSNs and modified MSNs were investigated by N₂ adsorption/desorption isotherm (Figure 3). The curves from MSNs and MSN-Hy exhibit no hydrolysis loop, which represents stable mesoporous features. As-synthesized MSNs show a 1043 m²/g of Brunauer, Emmett and Teller surface area, 0.83 cm³/g of total pore volume and 2.75 nm of pore diameter, respectively. However, the uptake of hydrilazine causes the decrease in surface area, total pore volume and pore diameter significantly, indicating the pore filling with drug. The covalent cross-linking with PEG results in low surface area and pore volume and high pore diameter, which becomes an obstacle for further coupling with drug owing to the steric hindrance of PEG with large molecular weight (3000 molecular weight). In the case of MSN-Hy-PEG, it is anticipated that the particles will possess a hydrilazine core and a PEG-modified silica structure, which can be confirmed by an obvious decrease of Brunauer, Emmett and Teller surface area and pore volume compared with those of MSNs. The loading degree of hydrilazine corresponded to 30.1% of MSN-Hy and 23.1% of MSN-Hy-PEG, respectively. The further modification of MSN-Hy with PEG would attribute to the lower hydrilazine incorporation as a consequence of some loss of hydrilazine entrapped inside the pore through two-step PEG modifications. The powder x-ray diffraction presents specific information regarding the change of internal structure before and after loading with hydrilazine and/or coating with PEG. As-synthesized MSNs showed a strong reflection at (100 and 110 2θ). After functionalization, mesopores still displayed their inherent hexagonal array but the intensity of scattering was decreased in an obvious fashion. This different behavior is attributed to the

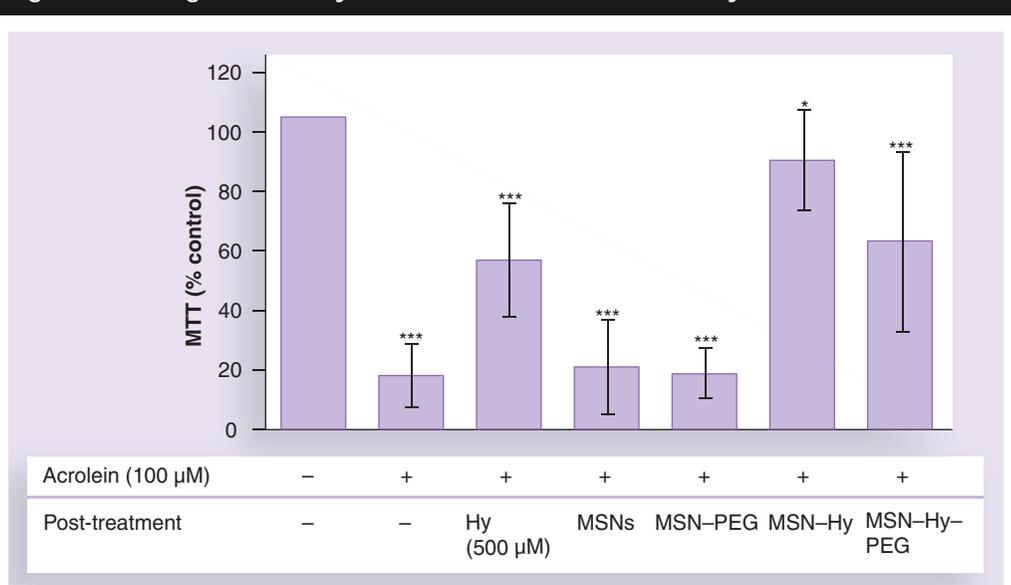
pore-filling effect, which is consistent with other studies [18,27]. As an ideal delivery system, a drug has to be localized specifically and directly to its intended target. Compared with intravenous administration of free drug/polymer, the attraction of a MSN-based system is the capability of nanoparticles to cross membrane barriers, especially with specificity. The cellular uptake of MSNs was observed by TEM as shown in Figure 4, indicating that the particles entered into the cell by endocytosis and accumulated in the cytoplasm. Nanoparticle-based drug delivery not only protects drugs from denaturation and degradation but also maintains the activity of the drug and enhances the bioavailability through uptake. Figure 5 shows the release behavior of hydralazine from MSNs in Krebs' solution over 5 days. A total of 80% of the adsorbed hydralazine was released from MSNs within 1 day whereas the MSNs coated with PEG delay hydralazine release in an obvious way. The slower release rate could be explained by the presence of PEG that covers around the external surface of silica particles. When PEG is conjugated to the MSN, the bulkiness of the PEG polymer would enhance the stability of the encapsulated drug and prevent release [30]. This

suggests that the release behavior of a drug would be controllable by varying the type and concentration of the polymer agent.

Understanding the injury & cytotoxicity of acrolein

Mechanical damage to cell membranes, referred to as the 'primary injury', produces a break in the semipermeable membrane, causing a loss in ionic sealing. This results in the poor regulation of ionic species crossing or being transported across the membrane. Eventually, ions, especially Ca^{2+} , are able to move freely between intracellular and extracellular compartments, causing significant cell pathology. Such ionic derangement causes the progressive destruction of cytoarchitecture (through climbing concentrations of free Ca^{2+}) and, eventually, of the cell body through a cascade of pathophysiological processes. The membrane endures the collapse of mitochondrial anatomy and physiology. Aberrant oxidative metabolism by then-compromised mitochondria accelerates the production of free radicals, including super oxide, hydroxyl ions and hydrogen peroxide. The overproduction of such 'antioxidants' leads to further deterioration of the integrity of cell membranes through the

Figure 6. Testing the viability of PC12 cells with the MTT assay.

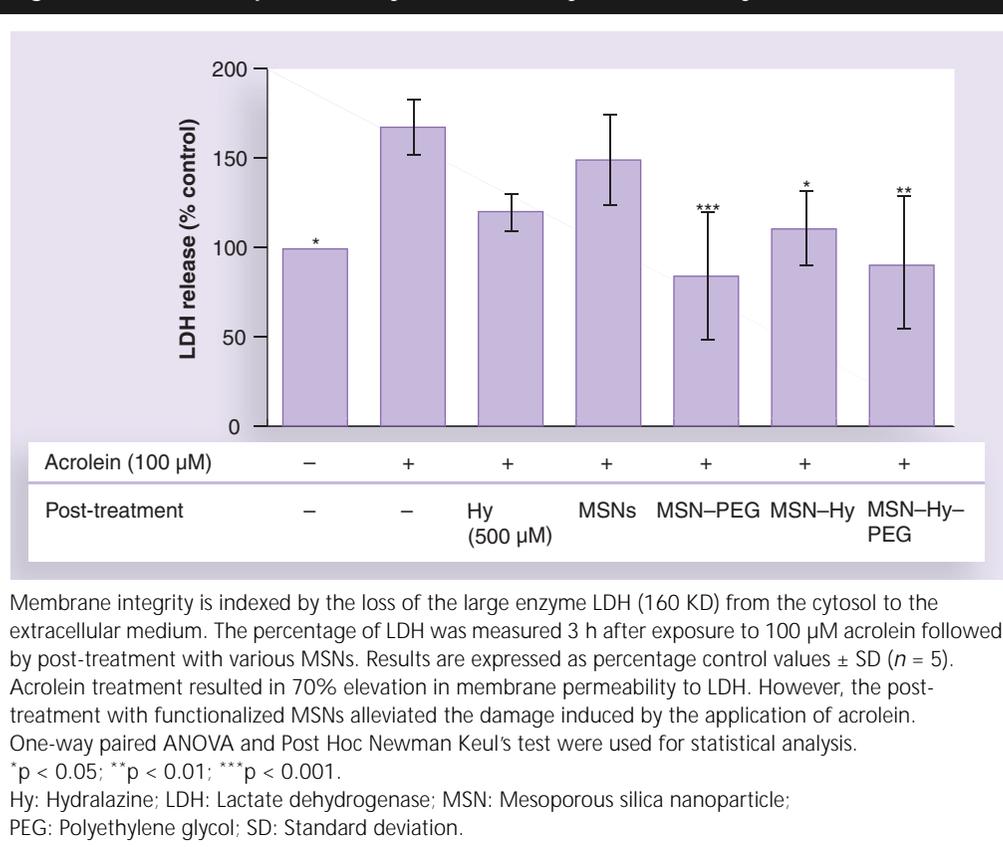


The MTT assay is correlated positively with mitochondrial function. Approximately 15 min after acrolein challenge (100 μM), cells were exposed to 500 μM Hy and various MSNs. Results are expressed as percentage control values ± SD (n = 5). The addition of 100 μM acrolein significantly reduced MTT level, whereas immediate applications of 500 μM hydralazine or functionalized MSNs obviously alleviated the acrolein-mediated toxicity.

*p < 0.05; ***p < 0.001.

Hy: Hydralazine; MSN: Mesoporous silica nanoparticle; PEG: Polyethylene glycol; SD: Standard deviation.

Figure 7. Membrane permeability as revealed by the LDH assay.



release of free fatty acids. Continuing peroxidation of free membrane lipids results in the production of endogenous aldehydes into the cytosol – all of which are toxic – principally acrolein. Such endogenous toxins can pass the intact membrane freely, thus the extracellular concentration of these cellular poisons increases and, as cells die, even more acrolein is released, which induces the progressive destruction of nearby 'healthy' cells. These independent and overlapping chemistries, as well as the progressive destruction of tissue they cause, are referred to as secondary injury.

Effect of PEG as a fusogen & hydralazine as a scavenger of acrolein

PEG, a widely used biocompatible polymer, exhibits nontoxic, nonimmunogenic and excellent solubility. Our group has reported numerous studies documenting the effectiveness of PEG in repairing damaged cell membranes [11,14,31], interfering in mitochondrial dysfunction and restoring the anatomy, physiology and functioning of traumatized spinal cord and brain in adult mammals [12,14,16]. The actions of PEG on injured cell membranes after mechanical

damage is largely twofold: first, the application of PEG improves the 'fence' property of the membrane through its 'surfactant-like' seal. Second, the rearrangement of water at the membrane through its strongly hydrophilic character permits the lipidic core of membranes in the vicinity of the damage to resolve into each other – largely erasing the defect. PEG participates in part to reduce secondary injury induced by acrolein and other aldehyde-mediated cell death, probably by such membrane fusion. Although the mechanisms of membrane fusion/self-assembly by PEG are not understood clearly, studies *in vivo*, *in vitro* and with various model membranes document the sealing, properties and rearrangement of damaged plasmalemma [14,32–34]. In the end, sealing and spontaneous assembly of the damaged membrane leads to recovery of its functionality and, in white matter, restores the conduction of action potentials within minutes to hours [35].

Another important property of PEG in combating soft-tissue trauma is that PEG preferentially seeks out the membranes of damaged cells – it is believed by attraction to the relative

hydrophobic character of this region. Thus, PEG can be used as a targeting molecule. Fluorescently labeled PEG administered intravenously marks the damaged tissue in both rat brain [17] and guinea spinal cord [36].

Some of the obstacles to PEG’s effectiveness in combating neurotrauma include:

- Inability of PEG to cross intact or lightly damaged membranes [12,31];
- A restriction of direct internalization limits its efficiency, for example, in interfering with mitochondrial dysfunction (unpublished observation);
- Relative restriction on the molecular weight to induce repair;
- Low molecular weight (<1000 D) can be toxic;
- Because the polymer is metabolized, production of monomers induces ethylene glycol poisoning and kidney failure;
- Higher molecular weights (>3000 D) produce solutions too viscous to be used easily in the clinic [37].

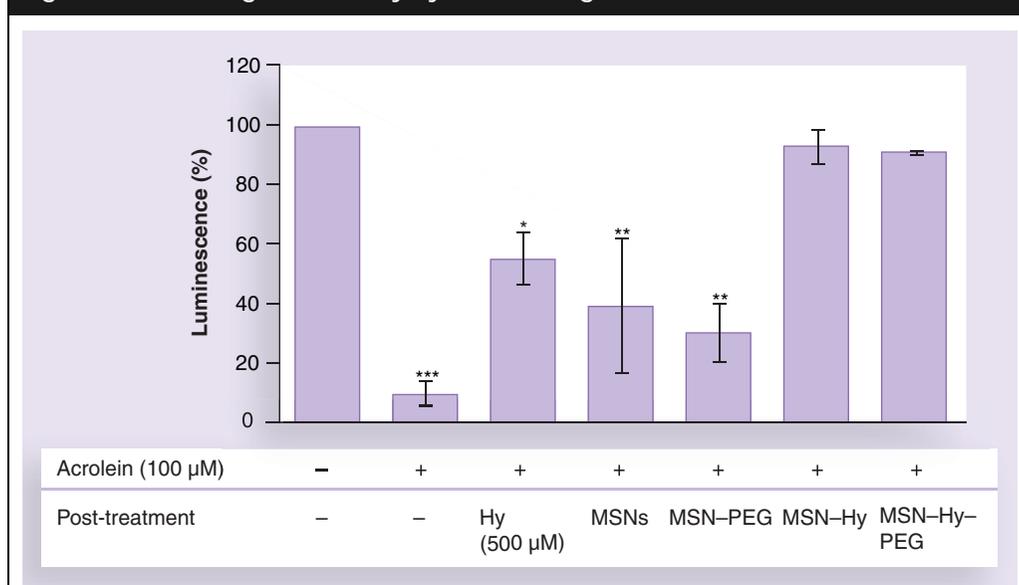
It is well-documented that the exposure of acrolein at various concentrations to PC12 cells induces oxidative stress and consequently

results in anatomical and functional damage [3,6–8]. However, on immediate treatment with hydralazine, the cells suffering from acrolein-mediated attack can be rescued. Catastrophic consequences, such as membrane damage, oxidative stress and mitochondrial injury, can be alleviated and, in many cases, cell death can be prevented. Previous reports reveal that cell viability is directly proportional to the concentration of hydralazine present in the medium [2,10]. If one can develop a system in which highly concentrated hydralazine and PEG can be administered simultaneously, initial and secondary injury after mechanical attack could perhaps be relieved.

Cell viability test by MTT & LDH assay

Exposure to 100 μM acrolein can induce the death of over 80% of a population of PC12 cells in a few hours and 100% by 8–12 h of observation [3]. In this study, we compared the effectiveness of hydralazine and MSNs functionalized with hydralazine and PEG to rescue injured cells from acrolein toxicity using the MTT and LDH assays. First, we observed the responses of the entire cell population in seven different groups:

Figure 8. Evaluating cell viability by determining intracellular ATP levels.

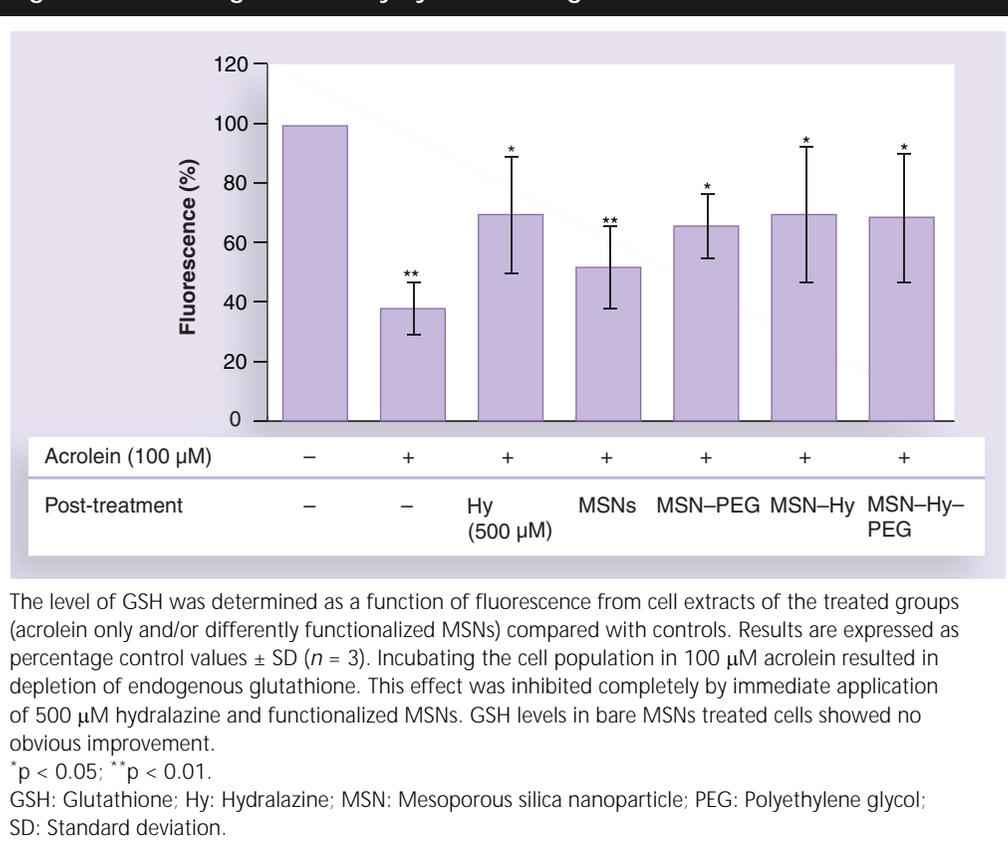


The level of ATP was expressed as the percentage of luminescence intensities in the control group versus experimental groups treated with various MSNs after the incubation with 100 μM acrolein. Results are expressed as percentage control values ± SD (n = 3). The application of acrolein reduced the intracellular ATP level of PC12 cells. Meanwhile, the toxic effect of acrolein-induced damage was significantly inhibited by treatment with functionalized MSNs.

p < 0.01; *p < 0.001.

Hy: Hydralazine; MSN: Mesoporous silica nanoparticle; PEG: Polyethylene glycol; SD: Standard deviation.

Figure 9. Evaluating cell viability by determining intracellular GSH levels.



- A control group, in which cells were cultured in HBSS and 'treated' with HBSS as the vehicle instead of acrolein;
- A 100 μ M acrolein-exposed group;
- A 100 μ M acrolein-exposed and 500 μ M hydralazine-treated group, in which hydralazine was added in the cell medium within 15 min after the exposure to acrolein;
- An acrolein-exposed and post-treated with MSNs group with 15 min delay after the exposure to acrolein;
- An acrolein-exposed and post-treated with MSN-PEG group with 15 min delay after the exposure of acrolein;
- An acrolein-exposed and post-treated with MSN-Hy group with 15 min delay after the exposure to acrolein;
- An acrolein-exposed and post-treated with MSN-Hy-PEG group with 15 min delay after the exposure to acrolein.

Typically, populations of cells with acrolein-mediated damage show a significant reduction in MTT activity owing to the collapse of mitochondria function. However, hydralazine or MSNs functionalized with hydralazine recover

from mitochondrial injury and abnormal oxidative metabolism. In PC12 cells, the exposure of 100 μ M acrolein decreased the absorbance to $11.0 \pm 10.2\%$ of control values (100%, $p < 0.001$, $n=5$) after 5 h (Figure 6). In cells treated with acrolein and hydralazine, this reduction was only $53.8 \pm 18.6\%$ of controls. However, the cells treated with MSN-Hy and MSN-Hy-PEG displayed significant enhancement in cell viability recovering to $85.5 \pm 16.6\%$ and $59.8 \pm 29.6\%$ after 5 h, respectively. The significant performance of MSN-Hy-PEG in the MTT assay may be owing to the presence of PEG on exterior surfaces of MSNs, where PEG could seal against the back-diffusion of hydralazine from the cytosol. According to the hydralazine-release profile, the amount of hydralazine escaping from incubation during 5 h was approximately 42% from MSN-Hy and 26% MSN-Hy-PEG, respectively. This result suggests that increasing the incubation time would enhance the adsorption of hydralazine into cell, thus increasing its effectiveness. However, cultures treated with MSNs and MSN-PEG did not exhibit a significant increase of MTT activity. This result is consistent with a previous report, in which secondary injury associated with

acrolein toxicity cannot be protected with the immediate application of PEG because PEG must gain access to the cytoplasm to reduce the concentration of reactive oxygen species – and early after injury the membrane suffering from acrolein exposure is not compromised sufficiently to enable the entry of PEG [31].

LDH results were also consistent with the MTT assays (Figure 7). After exposure to 100 μ M acrolein, LDH release increased to $168 \pm 15.4\%$ of control values. After exposure to acrolein, treatment with hydralazine reduced LDH release to $120 \pm 10\%$ of control values ($p > 0.05$), whereas MSN-Hy reduced this amount to $107 \pm 20\%$ of control values ($p < 0.05$). Significantly, MSNs coated with PEG, such as MSN-PEG and MSN-Hy-PEG, reduced LDH release to a level even below that of controls (untreated cells; $100 \pm 2\%$ of control values), to $84 \pm 31\%$ ($p < 0.001$) and $90 \pm 39\%$ of control values ($p < 0.001$), respectively. These data indicate a more complete membrane seal by PEG concentrated on the surface of particles. Finally, 'as-synthesized MSNs' as a formal control treatment were completely unable to protect cells from acrolein treatment, showing values similar to that of simple acrolein challenge ($17 \pm 10\%$; $p < 0.001$).

Effect of acrolein & functionalized MSNs on GSH depletion & intracellular ATP

Intracellular ATP levels are an excellent indicator of impairment of mitochondria function because continuous depletion of ATP results directly in a decrease in energy generation and ATP-mediated cell signal transduction, and may consequently induce cell death [10,38]. The mechanism by which acrolein inhibits mitochondria function is still not understood completely but there are two likely scenarios:

- Acrolein can form Michael adducts with mitochondrial proteins
- Acrolein prevents the coupling of oxidative phosphorylation and ATP production

Here, ATP levels are expressed as the emitted luminescence in the control group versus experimental groups, as shown in Figure 8. As expected, acrolein treatment of PC12 cells caused a dramatic decline in the intracellular ATP level (to $9 \pm 4\%$ of control values [$p < 0.001$]). However, once cells were exposed to hydralazine (15 min delay), after application of acrolein, ATP levels were enhanced significantly ($55 \pm 8\%$ of control values [$p < 0.05$]). The post-treatment with MSN-Hy and MSN-Hy-PEG enhanced ATP

levels more significantly ($93 \pm 5\%$ and $91 \pm 1.6\%$ [$p > 0.05$], respectively). By contrast, MSNs that were not loaded with hydralazine, such as MSNs and MSN-PEG, did not show significant acrolein-scavenging capability; correspondingly, the concentration of ATP after these attempts was only $39 \pm 10.6\%$ and $30 \pm 9.9\%$ of control values, respectively ($p < 0.01$). It is noteworthy that MSNs themselves are not an efficient scavenger of acrolein and intracellular ATP level is related linearly to the concentration of hydralazine loaded in the MSNs.

Inactivation of GSH function induces oxidative stress and facilitates the action of acrolein directly by increasing free radicals and lipid peroxidation unabated, whereas GSH levels normally restrict these biochemistries. Therefore, cells can be protected partially from apoptosis and necrosis by maintaining intracellular levels of GSH. The measurement of the intracellular level of GSH was dependent on the degree of affinity between GSH and MCB, which is expressed as the percentage of fluorescence intensity of the thiol-bound MCB dye. Intracellular GSH levels are decreased significantly after exposure to acrolein, even at low concentration (10 μ M). This sensitivity is owing to acrolein interacting rapidly with GSH by forming a glutathion-acrolein adduct [10]. As shown in Figure 9, exposure of acrolein decreased the intracellular GSH level significantly ($38 \pm 9.9\%$; $p < 0.01$) as expected in PC12 cells. After the application of hydralazine, GSH levels were increased significantly (as a function of MCB fluorescence ($70 \pm 20\%$; $p < 0.05$)). MSNs without any modification show extraordinarily similar values of intracellular GSH as the acrolein group ($52 \pm 14\%$ vs $38.9 \pm 9.9\%$, respectively; $p < 0.01$). MSNs functionalized with different species, MSN-PEG, MSN-Hy and MSN-Hy-PEG, all improved the support of GSH levels after exposure, to $66 \pm 11\%$ ($p < 0.05$), $70 \pm 13\%$ ($p < 0.05$) and $69 \pm 12\%$ ($p < 0.05$), respectively.

Conclusions

From this study, we conclude that a nanoparticle-based strategy to rescue cells from secondary injury (particularly acrolein-mediated injury) is supported by the results of all tests we designed to explore this possibility *in vitro*. Incorporation of hydralazine and PEG inside or on the surface of MSNs not only increases the efficacy of the 'experimental therapy' treatment through controlled and concentrated release of drug/polymer but also produces an enhanced cellular internalization with a prolonged duration.

Future perspectives

We are extending the MSN-based drug/polymer delivery system to animal models of spinal cord [36] and head [17] injury. Although we are relatively unconcerned regarding the possibility of toxicological problems from the use of silica-based nanocarriers in the mammal, the use of a natural polymer (e.g., chitosan) as a base is also an attractive perspective.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Mesoporous silica nanoparticles were synthesized, characterized and further functionalized to be used as a drug/polymer delivery vehicle.
- Acrolein-mediated toxicity resulted in significant pathology in all populations of PC12 neuronal cells studied in culture.
- Mesoporous silica nanoparticle-based vehicles exhibited constant and concentrated drug release in a controlled manner to the poisoned cells.
- Treatment of acrolein-challenged PC12 cells with functionalized mesoporous silica nanoparticles repaired and sealed compromised cell membranes through the polymer component and restored biochemical equilibria, reducing biochemical pathology, after acrolein-mediated attack, through hydralazine scavenging.

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