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Title: Mesoporous silica nanoparticles as drug delivery systems
Issue Date: 2012-05-09

Chapter 5

In vivo Evaluation of PEG-coated MSNs as Drug Delivery Systems for Hydrophobic Drugs

Abstract. Herein we investigated the potential use of surface modified mesoporous silica nanoparticles as a drug delivery system for retinoic acid in zebrafish embryos. The release kinetics were studied *in vitro* and *in vivo*, and the bioactivity of retinoic acid was studied by observing embryonic morphology and genetic expression.

5.1 Introduction

Mesoporous silica nanoparticles (MSNs) are nanomaterials which are intensively studied for potential applications in the field of drug delivery.[1] The stable mesoporous structure enables the incorporation of significant amounts of guest molecules.[2] Moreover, the surface chemistry of these materials is readily modified. In this manner, physical properties like *in vitro* release kinetics of encapsulated drugs and dispersibility in aqueous media can be controlled.[3-6] The latter is an important criterion for *in vivo* applications of MSNs as potential drug delivery systems due to the massive clustering of nanoparticles in blood vessel has to be avoided in order to prevent a severe immune response. The biodistribution of silica nanoparticles in adult mice was investigated in several studies by Lu and coworkers. The particles showed to accumulate predominantly in liver and kidneys.[7] However, no signs of degeneration or toxicity were observed. Recently, Suwalsky used silica nanoparticles for the delivery of genes to accelerate the healing of Achilles tendons in injured mice.[8] We recently investigated the potential application of PEGylated MSNs for the delivery of high molecular weight proteins in 2 days old zebrafish embryos. The particles released the proteins in its bioactive conformation and showed to be biological active.[9]

However, the use of MSNs as potential drug delivery systems in living animals is still a pristine area of research. In this contribution we studied the use of silica nanomaterials as a delivery system for hydrophobic compounds in aquatic animals for the first time. Zebrafish are becoming a popular animal system for pharmacological investigations[10] as it enables drug screening studies using morphological and genetic assays within a short time frame.[11] The embryos become larvae in approximately 6-7 days that are optically transparent facilitating microscopic analysis in living and developing animals.[12] High-throughput experiments are also possible due to the high number of eggs obtained after fertilization.[13] Moreover the external development of embryos enables morphogenic investigations during the early stages of life.[14] In addition, 75% of the genome is similar to human genome, thus genetic studies are highly relevant to human development.[12] Another application of zebrafish are toxicity studies of nanomaterials in aquatic environment. Several toxicity studies were performed on nickel, silver or silica nanoparticles.[14-16] These investigations showed that silica nanoparticles are biocompatible and less toxic to the zebrafish as compared to other inorganic nanomaterials.[15]

Until now zebrafish were mainly used for drug screening assays in which the

compounds of interest were dissolved these in the water of the fish tank. However, this approach is less suitable for pharmacological investigation of hydrophobic molecules due to the inherently low solubility. Therefore a system able to deliver these compounds in zebrafish embryos in order to study pharmacological effects *in vivo* is desirable. MSNs can encapsulate hydrophobic guest molecules in the porous structure in order to be released in the targeted site. However, a potential side effect caused by any exogenous material in an *in vivo* environment (i.e. MSNs) is a strong immune response. Therefore we have modified the MSNs surface with polyethylene glycol moieties in order to reduce the immunological effect.[17] To study the *in vivo* release of small hydrophobic drugs from the PEGylated nanoparticles, a model compound was selected which induces an easily detectable biological effect. In this study retinoic acid (RA) was used, as it is known to affect the genetic pathways resulting in malformations due to an excess of RA in the early stages of zebrafish development.[18]

5.2 Results and discussion

5.2.1 PEGylated mesoporous silica nanoparticles synthesis

MSNs were synthesized in the presence of hexadecyltrimethylammonium bromide (CTAB) as a template and tetraethylorthosilicate as the organic precursor. The surfactant was quantitatively removed with an acidic methanol extraction resulting in a mesoporous structure and to ensure non-toxicity for the zebrafish.[19] The absence of the surfactant in the resulting MSNs was confirmed by Fourier-Transform Infra-Red as we have already illustrated in **Chapter 2**. The newly synthesized batch was characterized according to the techniques described in the same chapter. The uniform and spherical shape was investigated using Scanning Electron Microscopy (SEM). X-Ray Diffraction analysis (XRD) revealed the mesoporosity inside the nanoparticles and the obtained average diameter of the pores was 2.8 nm. Transmission Electron Microscopy analysis confirmed the mesoporosity and showed that the internal structure is composed of thousands of parallel aligned channels throughout the particle in which the guest molecules can be loaded and delivered to the targeted site.

5.2.2 Influence of PEGylation on hydrodynamic radius

The surface of MSNs was modified with poly(ethylene glycol) chains as it is known to be non-toxic and is commonly used in drug and food formulations.[20] This surface coating results in particles that have a minimal interaction with serum proteins, thereby avoiding opsonisation.[21] In addition this hydrophilic shield also increases the colloidal

stability in solution.[22] The average hydrodynamic diameter of PEG-modified MSNs showed to be 215 nm, as measured with dynamic light scattering (DLS) analysis in phosphate buffered saline (PBS pH=7.24 **Figure 1**) In contrast unmodified MSNs showed to cluster and are therefore unsuitable for *in vivo* applications.[23]

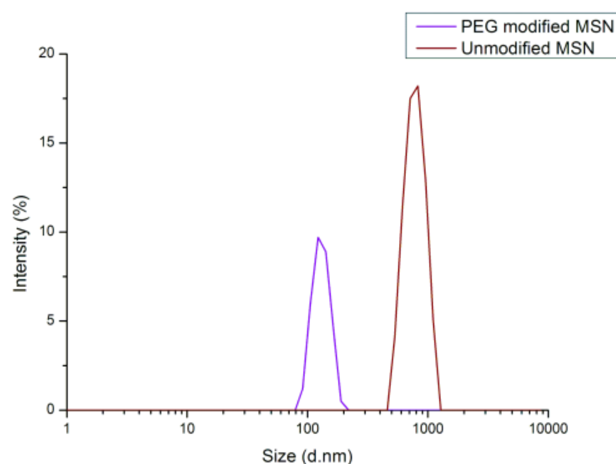

Figure 1. Dynamic Light Scattering (DLS) analysis of unmodified MSNs and PEG modified MSNs. The presence of surface modification prevents from clustering in PBS solution as the average diameter is 150 nm which is comparable with the observed dimension in SEM image. Unmodified MSNs observed diameter was 950 nm.

5.2.3 Release studies *in vitro* and *in vivo*

For the *in vivo* studies PEG-modified MSNs were loaded with retinoic acid (RA) as the hydrophobic model drug. The nanoparticles were incubated with a 1 µM solution of RA in DMSO and the PEGylated MSNs typically contained 0.4 mmol of RA per gram of silica, while the loading capacity of unmodified MSNs was slightly higher (0.63 mmol/g). This shows that the PEG layer influences the loading capability of the nanoparticles to some extent. The nanoparticles were rinsed with PBS (3x1 mL) before use, in order to remove excess of molecules loosely bound to the surface. Next, PEG-modified nanoparticles were dispersed in PBS and the release of retinoic acid (RA) in the supernatant was measured using UV/VIS spectroscopy as a function of time (**Figure 2**).

The RA release from unmodified MSNs was also determined for comparison to understand the influence of the surface pegylation on the drug delivery characteristics. Approximately 50% of the retinoic acid was released in 1 hour from unmodified MSNs. Interestingly, the release from PEG-modified MSNs was considerably slower as the same amount was in approximately 3 hours showing that the hydrophilic PEG layer has a significant effect on the RA-release.

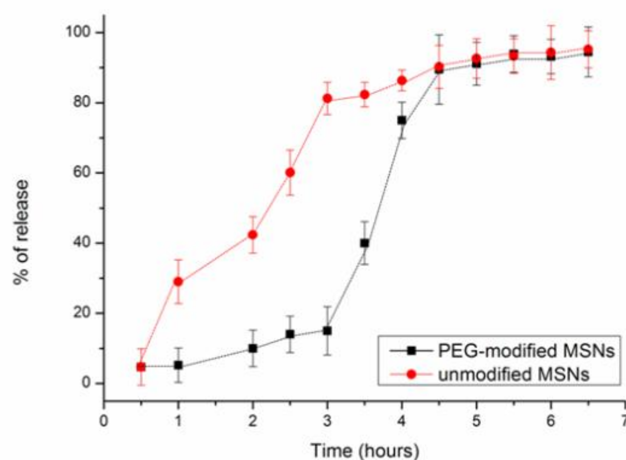

Figure 2. Release of retinoic acid in presence and absence of PEG surface modification on the nanoparticles. The retention of guest compound is observed when PEG-modified MSNs are analyzed.

For the *in vivo* release studies of RA from PEGylated MSNs, 5 nl of a stock solution containing 1 mg/mL nanoparticles was injected in the zebrafish yolk. We investigated the toxicity of the nanoparticles and analyzed the RA release from PEG-modified MSNs by analyzing the phenotype with a particular focus on the expression of some genes responsible for the cranio-facial structures of the embryo. Three different groups of embryos were studied: control embryos soaked in PBS, embryos injected with PEG-modified MSNs, and embryos injected with RA-loaded PEG-modified MSNs. In this study RA was not injected directly in the zebrafish embryo to prevent photoisomerization due to the powerful light sources of the microscope setup. The biological effects analyzed in this report were compared with previous experiments in which *all-trans*-RA was the active compound causing malformation in zebrafish embryos. Thus the photoisomerization of *all-trans*-RA would have caused a reduction in acitivity of this molecule with subsequent modification of the biological results. The morphological development and RA-induced malformations were studied in 2 days old embryos using optical microscopy as shown in **Figure 3**.

The majority of injected embryos with PEG-modified MSNs were found to be healthy (n=89), while only few zebrafish showed severe malformation (n=2) or were found to be dead (n=9 **Figure 4**). This showed that the injected PEG-modified MSNs are compatible with zebrafish embryos and no deteriorating effect on the development was observed. Control siblings soaked in growing medium and PBS were performed and a similar behaviour was observed (**Figure 3** and **Figure 4**).

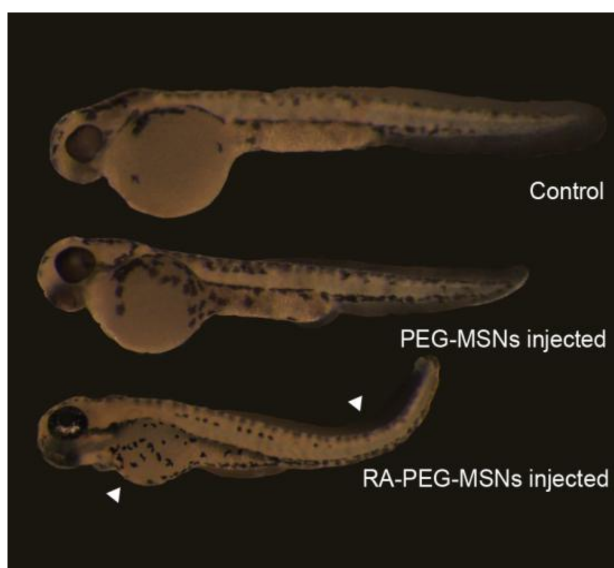

Figure 3. Bright field image of Zebrafish embryos. In the picture are reported control embryos (top) treated with PBS solution and PEG-MSNs injected embryos. The modified mesoporous silica nanoparticles have biocompatible properties as they do not affect the embryological development (centre). The release of RA is observed when PEG/modified MSNs are injected in the embryos. The curved tail and the not developed yolk are evident signals of the release (bottom).

When RA was dispersed in the medium the majority of the embryos were found dead (n=64) while others presented mild or severe malformations (n=10, n=30 respectively). In **Figure 4** the statistical results are summarized and are in agreement with previous reports.[24] Injection of RA-loaded-PEGylated MSNs resulted in a majority of the embryos to be severly malformed (n=74). This shows that it is possible to study embryos malformations as a consequence of minute amounts of RA released by injecting PEG-modified MSNs. In fact, the injected suspension contained 5 ng of PEG-modified MSNs which released only 0.5 nmol of RA in the embryo. This implies that 6 orders of magnitude less of RA are required to observe the effects on zebrafish development as compared to the traditional way of drug testing in which 10 μmol of RA were dissolved in the fish tank.[25]

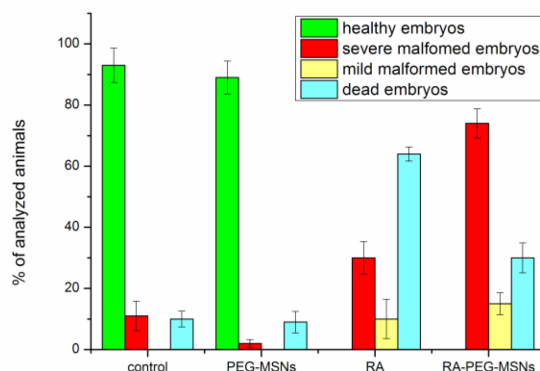

Figure 4. Toxicity study of zebrafish after injection with unloaded and loaded PEG-modified MSNs versus controls and embryos treated with RA only. The biocompatibility of PEG-modified

MSNs is confirmed by the high number of healthy embryos found after the injection, while RA loaded PEG-modified MSNs cause significant mortality due to the release of RA in the living system.

5.2.4 *In Situ* Hybridization

The genetic changes during the development were also studied after the injection of PEG-modified MSNs to evaluate the toxicity of this material for developing embryos. Using an *in situ* hybridization technique the expression of *msx*B gene in the zebrafish embryo 1 day old was investigated. This gene is crucial for the development of the neural tissue and it is expressed mainly in the encefalus area.[26] Nanoparticles could possibily interact with embryonic dividing cells ultimately resulting in cellular death. Thus the genetic expression may be affected by these materials. When zebrafish embryos were injected with PEGylated MSNs the *msx*B gene presented a normal expression pattern.[27, 28] In **Figure 5** the head region of 20 hours old zebrafish embryos is highlighted with number 1. This indicates that these particles do not affect or interfere with brain development. In contrast, the detrimal effect at the gene level of released retinoic acid from injected PEGylated MSNs was observed (**Figure 5**). An abnormal development of the brain area was also revealed and the expression of *msx*B was shown to be deeply disturbed by the action of released RA in brain dividing cells.[29] Thus, this MSN delivery system enables the investigation of the positive or negative effects of drugs on the development of zebrafish embryos using minute amounts of a drug**.**

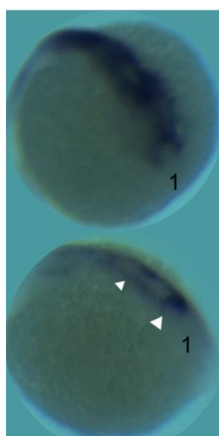

Figure 5. *In situ* hybridization bright field image of gene msxB. The expression of msxB gene was studied in PEG-MSNs injected embryos (above) and in RA loaded PEG-MSNs treated embryos (below). The head region, highlighted with number1, is the most interested area and a different genotype is observed when RA loaded nanoparticles are injected in zebrafish embryos (white arrows).

5.3 Conclusions

In this study we demonstrated the potential application of mesoporous silica nanoparticles as drug delivery system in zebrafish embryos as these nanoparticles showed to be biocompatibile. Moreover, we have observed that the surface PEGylation does not lead to any toxicity. Retinoic Acid loaded nanoparticles were injected in the yolk of 4 hours old zebrafish embryos and the effect of the released drug on the embryonic development was studied. Through morphological studies we showed the biocompatibility of PEG-modified MSNs with the zebrafish. Moreover, the effective release of RA in significant a concentration to induce morphological abnormalities was shown. The biological effects of RA were also observed by developmental changes in gene expression of *msx*B.

This delivery system has potential application as versatile tool for drug screen of hydrophobic drug. PEGylated MSNs enables to perform high-throughput drug screenings using minute amounts of compounds as compared to current methods. Finally these PEGylated MSNs enable investigations of the pharmacological effects caused by poorly water soluble drugs on aquatic animals. However, we envision that these particles can also be used in other animal model systems.

5.4 Experimental part

Materials and Methods. Acetic anhydride, alkaline phosphatase (AP), alcian blue, bovine serum albumin (BSA), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), ethylenediaminetetracetic acid (EDTA), ficol 400, formamide, heparin, hexadecyltrimethylammonium bromide (CTAB), yeast RNA, magnesium chloride ($MgCl_2$), methyl salicylate, paraformaldehyde (pFA), potassium chloride (KCl), retinoic acid (RA), 2-propanol, sodium chloride (NaCl), sodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide, triethanolamine (TEA), tetraethyl orthosilicate (TEOS), tri-chloroacetic acid (TCA), tris(hydroxymethyl)aminomethane HCl (TRIS-HCl), Tween 20, hydrochloric acid (HCl) and retinoic acid (RA) were purchased from Sigma Aldrich and used as received.

Milli-Q water with a resistance of more than 18.2 MΩ/cm was provided by a Millipore Milli-Q filtering system with filtration through a 0.22 µm Millipak filter. 2-[Methoxy(polyethyleneoxy)propyl] trimethoxysilane was purchased by ABCR GmbH & Co. KG, while all the reagents for *in situ* hybridization (ISH) have been purchased by Roche Diagnostics.

Phosphate buffer saline (PBS) was composed of 2.74 M of NaCl, 54.0 mM of KCl, 210 mM of $Na_2HPO_4*2H_2O$ and 30.0 mM of KH_2PO_4 and this solution was diluted 20 times to reach the final concentration. The solution of PBS-Tween (PBS-T) was composed of PBS containing 0.1 % of Tween 20.

All the buffers used during the ISH protocol have been prepared according to Bardine et al. [30, 31].

Animal Handling. *Danio rerio* adults (AB wild type) were bought at a local pet shop (Selecta, Leiden) and kept for at least 2 months before used for breedings. The aquarium room had a light-dark cycle of 14 hours of light and 10 hours of dark. The temperature of the water was 24° C and the air temperature 23° C. Fish were kept in 7,5 liter containers (Tecnilab-BMI, Someren, The Netherlands) at a density of 12 fish/container. The fish were fed daily with dry food (DuplaRin M, Gelsdorf, Germany) and frozen artemias (Select Food, Aquadistri BV, The Netherlands).
The afternoon before egg collection, a mesh-net was introduced in the container to allow the eggs to fall through (and thus preventing the eggs from being eaten). Then the morning of the day after the eggs were collected and placed in petri dish. Eggs were allowed to develop in a climatized room with a temperature of 28° C with the same dark-light cycle of adult fish.

The water used in tank was tap water; however the water used for zebrafish eggs was composed of 0.21 g of instant ocean sea salt (purchased by the local pet shop mentioned above) in 1L water and add 0,0005% (v/v) of methylene blue.

Needle preparation for injection in *Xenopus laevis*. Needles were pulled with a Flaming/Brown Micropipette Puller, Model P-97 purchased from Sutter Instrument & Co. and the microcapillaries used were thin wall glass capillaries TW100F-3 with filament purchased by World Precision Instrument. The parameters used for preparation of the needle were heat=515, pull=60, velocity=60 and time=40. Subsequently, the needles were opened by breaking the point with Dumont forceps. Injections were done with a picospritzer purchased from Narishige USA IM-200.

PEG-modified MSNs synthesis, characterization and loading. The synthesis of nanoparticles was performed accordingly with the procedure explained in **Chapter 4**. The characterization of the nanomaterial was execute as described in the same chapter.

FT-IR measurements of PEG-modified MSNs. FT-IR spectra were recorded with a BIORAD Excalibur series FTS 4000 instrument. For this, 2 mg of synthesized MSNs were dispersed in 200 mg of KBr. The powders were mixed in a mortar and compacted into a tablet with a Graseby Specac powder presser, at a pressure of 13 tons. In general, all spectra were collected with 254 scans and 4 cm^{-1} resolution.

Electron Microscope analysis. Transmission electron microscopy (TEM) was conducted on a JEOL 1010 instrument with an accelerating voltage of 60 kV. Samples for TEM were prepared by placing a drop of each MSNs solution on carbon-coated copper grids. After approximately 10 minutes the droplet was removed from the edge of the grid. In order to characterize the exterior and the shape of the particles a scanning electron microscopy (SEM) analysis was performed, with a NovaSem microscope. Samples were dispersed in 2-propanol and sonicated for 30 seconds, then 10 µL of this suspension was placed on an aluminum stub and dried in at 37 °C under vacuum conditions for 30 minutes. The samples were coated twice with a layer of carbon and analyzed with an acceleration voltage of 15 kV.

UV-VIS measurements of RA release form PEG-modified MSNs. UV-VIS absorbance spectra were measured with a PerkinElmer Lambda 25 UV-VIS spectrometer. The loading capability of MSNs and PEGylated MSNs was first measured. Two suspensions were prepared cotaining1 mg of MSNs and 1 mg of PEGylated MSNs in 1 mL of a 1 µM RA solution of DMSO overnight. The suspensions were kept in a dark chamber to prevent any photoisomerization of the drug. The particles were then centrifuged and washed once with 1 mL of DMSO to remove unloaded RA. The loading capability was measured as the difference in

absorbance of the starting solution and the absorbance of the solution after overnight nanoparticles loading. Next, the particles were suspended in 1 mL of PBS and the RA release was measured in the supernatant as a function of time. The results were expressed as the average of three measurements. The amount of drug in the supernatant was measured at λ=350 nm, ε=45,000 $M^{-1}cm^{-1}$.

Dynamic light scattering measurements. Dynamic light scattering (DLS) analysis was measured with a NanoSeries DLS zetasizer (Malvern). The hydrodynamic radius was measured in PBS pH=7.24 at room temperature. A suspension of 1 µg/mL MSNs particles was sonicated for 2 hours before the measurement. The mean diameter was calculated assuming a spherical shape of the particles. The analysis was performed in triplo and for each time the sample has been measured 24 times.

Zebrafish injection of PEG-modified MSNs. A stock solution containing 1 mg/mL of PEGylated MSNs loaded with 1 µM solution of retinoic acid was prepared. Then, 5 nL of the stock solution were injected using a picospritzer in the zebrafish yolk. In control experiments embryos were exposed to PBS, and to 1 µM RA solution with 5% of DMSO in PBS. In all these experiments the embryos have been exposed to the buffer solution and to the drug for one week and then fixated with 4% pFA in PBS.

In situ hybridization (ISH). For ISH the gene *msxB* was selected. The antisense RNA probe labelled with digoxigenin (DIG) were synthesized according to Bardine and collaborators [30]. The ISH was performed on a whole embryo. After rehydration the embryos were treated with proteinase K (0.5 µg/mL in PBS-Tween) for 8 minutes at room temperature, followed by enzyme inactivation with a solution of TEA 0.1 M and acetic anhydride 0.5 µL/mL in PBS-T for 10 minutes. The sample were washed 3 times with PBS-T and fixated in pFA 4% in PBS. The basic solution used for prehybridization and hybridization contained 50% deionized formamide, saline citrate, Denhart's, 1% of Tween-20, 0.1% of CHAPS (Sigma-Aldrich), 50 mg/mL of yeast tRNA (Sigma Aldrich) and EDTA. The Denhart's solution was prepared according to the procedure published by Jansen and collaborators [31]. The embryos were prehybridized in this solution for 5 hours at 65 °C, followed by an overnight hybridization at 65 °C with the hybridization buffer containing the DIG labelled probe. Then the samples were washed with sodium chloride sodium citrate (SSC) buffer for six times and then with maleic acid sodium chloride buffer solutions with 10% of Tween-20 (MNT). Next, they were incubated for 1 hour with the blocking buffer followed by soaking for 4 hours with anti-DIG antibody (Roche Diagnostics) with a dilution of 1:2000, at room temperature. Several MNT washes were performed after and then the samples have been incubated with alkaline phosphatase (AP) buffer and stained with BM purple.

5.5 References and notes

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