The zebrafish as a model for tissue regeneration and bone remodelling

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Chapter 3: Mesoporous silica nanoparticles as a compound delivery system in zebrafish embryos

Abstract

Silica nanoparticles can be efficiently employed as carriers for many drugs *in vitro*. Here, we use zebrafish embryos as a model organism to see whether mesoporous silica nanoparticles (MSNPs) can be used to deliver compounds *in vivo*. We injected 35-40 nl (10mg/ml) of custom-synthesized, fluorescently-tagged 200 nm MSNPs into the left flank, behind the yolk sac extension, of 2 day-old zebrafish embryos. We tracked the distribution and translocation of the MSNPs using confocal laser scanning microscopy. Some of the particles remained localized at the injection site, while others entered the bloodstream and were carried around the body. Embryo development and survival were not significantly affected by MSNP injection. Acridine orange staining revealed that MSNP injections did not induce cell death significantly. We also studied cellular immune responses by means of lysozyme-ds red transgenic embryos. MSNP-injected embryos showed infiltration of the injection site with neutrophils, similar to controls injected with buffer only. In the same embryos, counter-staining with *L-plastin* antibody for leukocytes/macrophages revealed the same amount of cellular infiltration of the injection site in embryos injected with MSNPs or with buffer only. Next, we used MSNPs to deliver two recombinant cytokines (MCSF and RANK-L) to zebrafish embryos. These proteins are known to activate cells involved in bone Remodelling, and this can be detected with the marker tartrate resistant acid phosphatase (TRAcP). Co-injection of these proteins loaded onto MSNPs produced a significant increase in the numbers of TRAcP positive cells after 2-3 days of injection. Our results suggest that MSNPs

can be used to deliver bioactive compounds into zebrafish larvae without producing higher mortality or gross evidence of teratogenicity.

Introduction

Recent years have witnessed an impressive growth of fundamental and applied research in the field of nanoscience and nanotechnology [73]. There is a wide variety of nanomaterials including metal nanoparticles, nanoshells, fullerenes, quantum dots, polymer nanoparticles, dendrimers and liposomes [74-84]. In the future, nanomaterials may be applied in disease diagnostics and for drug delivery targeted at specific sites, for example in the treatment of cancer [85].

The burgeoning field of the nanomaterials brings several advantages to the design of new drug delivery systems (DDSs), as they present very significant properties such as: a high surface area able to incorporate consistent amounts of drug; tunable pore size, shape and diameter; and a chemical surface which can be functionalized to achieve target release [173].

The design of a DDS is crucial for use in medical applications; therefore it has to present some characteristics such as manipulation of biological profiles, such as pharmacokinetics and pharmacodynamics, biodistribution and cellular uptake [86,174] . There are two important challenges in these fields: first, the DDS must protect the cargo from a harsh environment and immune response; second: it has to ensure the delivery to the desired site followed be release of active drug. To meet these challenges, the DDS has to present some prerequisites such as biocompatibility with the biological environment, efficient cellular uptake, and controllable rate of release to achieve an effective local concentration, or concentration gradient [175,176]. Mesoporous silica nanoparticles (MSNPs) can satisfy all these requirements [86].

Previous studies have shown that non-phagocytic eukaryotic cells can endocytose latex beads up to 500 nm in size, and that the efficiency of uptake decreases with increasing particle size [87]. Particles around 200 nm in size or smaller are taken up with highest efficiency, whereas very little uptake is observed for particles larger than 1μm. Therefore MSNPs can be efficiently employed as carriers for intracellular drug delivery [87]. It was found in other

studies that the toxicity of nanoparticles may vary with size, structure and composition [93,94]. Acute toxicity occurs at nanoparticle concentrations in the order of milligrams per litre in the case of the medaka, *Oryzias latipes*, and the largemouth bass, *Micropterus salmoides* [90,177]. Silica nanoparticles were found to be non-toxic to other human and mouse embryonic cells at up to 15 mg/l [178]. In addition silica nanoparticles were not found to have general or overt toxicity between 0.0025 and 200 mg/l [179].

Mice have been used for studying nanoparticle biology [95]. Organs that can take up nanostructures in mice include the spleen, lymph nodes, and bone marrow. All of these are major organs of the immune system and contain large concentrations of phagocytic cells than can ingest the nanostructures. Nanostructures that are coated with polyethylene glycol (PEG), a polymer, have been shown to be more resistant to uptake by phagocytic cells [97].

Zebrafish transgenic lines that express green or red fluorescent proteins (GFP or DsRED2) under a neutrophil-specific promoter, such as the myeloperoxidase (*mpx*) promoter [180,181] or the lysozyme C (*lyz*) promoter [182], provide useful *in vivo* models for real-time imaging and genetic analyses of inflammatory responses. Neutrophils and macrophages in zebrafish larvae can both be visualized by immunolabelling with anti-L-plastin antibody, while the expression of colony stimulating factor 1 receptor *(csf1r*) and the lack of *mpx* and *lyz* expression in macrophages can be used to distinguish them from neutrophils [180,183]. The elongated morphology of the cells expressing these markers further points to a macrophage identity [180]. Both the neutrophil and the macrophage populations are involved in the innate immune response towards infection and injury [180-183]. In order to understand the fate and interaction of nanomaterials with the immune cells, further studies are needed.

Many features of the zebrafish (*Danio rerio*) model make it well-suited for studies of nanomedical applications. It has a short generation time (around three months), and a large clutch size (200-300 eggs), which allows high throughput assays at low cost [4]. Zebrafish embryogenesis is rapid, with most of the internal organs, including the heart, liver, intestine and kidney, developed by 96 h post fertilization (hpf) [3]. The optical transparency of zebrafish embryos, and their fertilization and development outside the mother,

enables an easy and thorough observation of drug effects on internal organs *in vivo*.

The zebrafish is also a very attractive model to study the mechanisms underlying bone formation [184,185] because the key regulators of bone formation are highly conserved between mammals and teleosts, and the corresponding orthologs share significant sequence similarities, and an overlap in expression patterns, when compared to mammals [164,186,187]. Molecules essential to promote osteoclastogenesis [188] include: (i) Macrophage colony-stimulating factor (M-CSF); (ii) receptor for activation of tumour necrosis factor kappa B (TNSF-κB or RANK); and (iii) RANK ligand (RANKL, OPGL or TRANCE).

The aims of this study are: first, to test the toxicity of MSNPs on zebrafish embryos; second: to test the effect of MSNPs on immune cells; and third: to see whether MSNPs can be used to deliver bioactive compounds into zebrafish larvae. For this latter objective, we chose M-CSF and RANK-L because they have a clearly defined biological readout, in the activation of osteoclasts [189].

Materials and Methods

Mesoporous Silica Nanoparticle synthesis

The mesoporous silica nanoparticles (MSNPs) were synthesized via sol-gel chemistry using cetyltrimethylammonium bromide (CTAB) and mesitylene as templates. The surfactant, CTAB, is used as template for the mesoporous structure; while mesitylene is used as pore-expanding agent to increase the pore size. The synthetic procedures were done according to Lin and co-workers [174]. The silica nanoparticles were functionalized with 2-[Methoxy (polyethyleneoxy) propyl] trimethoxysilane in order to create a PEGylate surface (Supporting Information 1.1). For laser confocal microscopy, a different batch of MSNPs were synthesized with the fluorescent label fluorescein isothiocyanate (FITC), the synthesis being done according to Zink et al [175] (Supporting Information). Fourier transform infrared spectroscopy (FT-IR) measurement: effective removal of CTAB was confirmed using a FT-IR Biorad Excalibur Series FTS 4000 (RT) and MSNPs were prepared in a KBr tablet.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

MSNPs were suspended and sonicated in methanol in order to avoid aggregation. 10 µl of suspension was deposited on an aluminium stub and coated with pure carbon with a sputter carbon coater. TEM (transmission electron microscope) was performed with a JEOL 1010 instrument at 60 kV. The sample was prepared as stated above and was deposited on a carbon-coated copper grid, and then air dried for 3 h.

X-Ray Diffraction (XRD)

The XRD was measured with a general powder difractometer with Cu radiation at 40 kV and 30 mA.

Release of FITC-BSA from MSNPs

Release of FITC-bovine serum albumin (FITC-BSA) from MSNPs was measured in 1M phosphate buffered saline (PBS) at pH 7.24, by preparing a suspension of 1 mg/ml of loaded MSNPs. MSNPs were washed twice in PBS to remove excess FITC-BSA. The release was determined by taking aliquots at different times. The aliquots were centrifuged and the UV-VIS absorbance of the FITC-BSA was measured to give quantitative information about the release (Fig. 1 E).

Zebrafish embryos

Maintenance of zebrafish (*Danio rerio*) adults took place at 26^{o}C in aerated 5 litre tanks, in a 10 h: 14 h light: dark cycle. In each mating setup two females and one male fish were present. The eggs were collected within the first hour, cleaned, sorted and transferred to Petri dishes filled with egg water (14 gm 'Instant Ocean®' salt in 100 ml of demi water). Embryos were anesthetized with 0.04% MS-222 (tricaine methanesulfonate) at 2 days post fertilization (dpf) for microinjections.

Mesoporous Silica Nanoparticle Injections

The embryos were divided into two groups: **treatment** (injected with MSNPs in phosphate buffered saline) and **control** (injected with PBS only). The embryos

(2 dpf) were anesthetized and transferred to custom-made agarose gel moulds to hold them in place. The moulds were made with 5% agarose (molecular grade, Bioline Cat. BIO-41025) in PBS, heated to dissolve, and then cooled. Borosilicate glass capillaries (1 mm outer diameter x 0.78 mm inner diameter, Cat. GC100TF-10, Harvard Apparatus, Holliston, MA) were pulled using a needle puller. The resulting flexible, thin, closed tip was snapped off to open the capillary for injecting. Each tip was calibrated for the release of fluid in oil, and the diameter of the droplet in oil recorded. About 35-40nl of MSNP suspension was injected into the left flank of each embryo caudal to the yolk sac extension with an air pulse provided by a Parker Picospritzer 3 (Parker Hannifin, Pneutronics Division, NJ) at a pressure of 30 psi and time 10 µsec. The pulse was delivered immediately after the needle had been pushed through the epidermis.

Human recombinant macrophage colony stimulating factor (MCSF) 40 ng/ml and Receptor for necrosis factor ligand (RANK-L) 400ng/ml in combination were loaded into MSNPs by soaking overnight and then centrifuged the MSNPs and protein suspension. The supernatant was removed and the MSNPs were resuspended in 1 x PBS. Resulting MSNP suspension in PBS was injected into 2 days old embryos, and the embryos were fixated at 1, 2, 3, 4 and 5 days post injection for TRAcP enzyme staining. There was no difference between the results from PEGylated and non Pegylated nanoparticles. Therefore we used PEGylated MSNPs for our studies, to avoid immune response. After injection, the embryos were washed twice with fresh egg water and transferred into Petri dishes (30 embryos per dish) and maintained at 28°C until analysis.

Embryo imaging and analysis

Imaging (including time-lapse recording) was done with confocal microscopy (Zeiss observer LSM 500 inverted microscope), immediately after injection, and 24 h post injection, to assess the uptake and distribution of the fluorescent MSNPs in the body of the living embryo. We recorded mortality, malformations and cell death as described in Supplementary information ‘Analysis of zebrafish embryos’.

Transgenic lysC::DsRED2 embryos for Neutrophils

The transgenic lysC::DsRED2 embryos used in this study have been described previously [182]. The neutrophil-specificity of this line is supported by other studies [190]. Two groups, each consisting of 35 eggs, were injected with or without MSNPs. Embryos were imaged within 2-5 h post injection, at 24 h, 2 d and 3 d post-injection with confocal microscopy.

L-plastin immunostaining

For L-plastin immunostaining, we used the procedure adapted after Cui C, [191]. Incubation was done overnight at 4°C with rabbit anti-*L-plastin* (Mathias *et al.*, 2007) in blocking buffer (1:500 dilution). Embryos were incubated for 2 h at room temperature in Alexa Fluor 405 Goat-anti-Rabbit antibody (Invitrogen, 1:200). They were stored at 4°C and imaged using confocal microscopy.

TRAcP staining

Tartrate resistant acid phosphatase (TRAcP) staining was done with TRAP kit 387A-1KT according to the manufacturer's instructions (Sigma Aldrich Chemie GmbH, Steinheim, Germany).

Quantitative analysis

For *lysC::DsRED2* transgenic embryos and L-plastin immunostaining the quantitative analysis was done by counting the total number of cells present in the area around the injection site (10 embryos/ group) from flattened *z* stacks of confocal images. The total number of TRAcP positive cells was counted manually under a compound microscope from the whole body on both sides (10 embryos/ group). Statistical analysis was done with one way Anova using Bonferroni post hoc test.

Results

Characterisation of MSNPs

We synthesized mesoporous silica nanoparticles via a sol-gel technique with a pore-expanding agent (mesitylene) to a CTAB-templated emulsion system. The template was removed with a methanol acidic wash because CTAB has been demonstrated as a very toxic compound for cells [192] due to its capability to damage biological membranes and cause the release of intracellular enzymes. The effective removal of CTAB was checked via FT-IR (Fig. 1 A and B). The nanoparticle surface was modified with a PEGylated methoxysilane in order to reduce cluster formations in physiological fluids. The nanomaterials thus synthesised present an inner structure comparable to the MCM-41, as previously described [173,193] with a honeycomb arrangement of the channels, as shown by the electron micrographs (Fig. 1 C and D).

The particle size of MSNPs was measured with dynamic light scattering (DLS) in PBS solution (pH 7.2) before and after the PEGylation step. The hydrodynamic diameter of the particles, before the surface functionalisation is 985 nm (PDI $\pm$0.21) indicating an elevated tendency toward aggregation. Nevertheless, after surface modification the diameter measures 255 nm (PDI $\pm$0.198) showing capability of particles to avoid clustering. A sharp peak was detected at, 2.8 theta, showing the presence of a mesoporous structure with a pore size of ca. 8 nm. We used bovine serum albumin (BSA) to study the properties of release and loading capacity of MSNPs. BSA was modified with fluorescein isothiocyanate (FITC) and then used to measure the release profiles of MSNPs. Loading capacity of MSNP-BSA in the supernatant was 65 μg/ml of loaded compound. The release curve shows the concentration of released BSA via UV-VIS in the supernatant (Fig. 1 E). It shows a delay of ca. 1 hour, due mainly to two factors: (i) the steric hindrance of the BSA; and (ii) some ionic interaction between the silica scaffold of the nanoparticles and the positive charges of the amino acids of the chain. Variation of the ζ-potential of the MSNPs and protein loaded MSNPS was measured as a function of pH Fig. 1 F.

Distribution of nanoparticles after injection into embryos

MSNPs conjugated with FITC-dextran were injected into the left flank of zebrafish embryos at 2 dpf, caudal to the yolk sac extension (Fig. 2 A). We found that some nanoparticles were seen circulating in the bloodstream (Fig. 2 A). while others were visible in the tissue at the site of injection, and remained there for the duration of experiment i.e. up to 5 dpf (Fig. 2 B). The earliest time examined was with CLSM was 10 minutes post injection, at which time the particles were already in the circulation.

Toxicity Testing

To see whether nanoparticles had an adverse effect on the development of the embryo we first recorded the cumulative mortality at 5 dpf (Fig. 3 A). Percentage mortality, pericardial oedema and morphological abnormalities are shown in Fig. 3A. Mean mortality in the MSNP-injected group was 5.8% and 4.1% in the control group (PBS only; Fig. 3 A). In the MSNP-injected group, 4.68% of embryos had pericardial oedema and skeletal abnormalities compared with 1.5% of controls. The skeletal abnormalities were of Meckel's cartilage, the pharyngeal arches and the ethmoid plate, as revealed by Alcian blue staining (Figs. 3 B, C). Cell death, as indicated by acridine orange staining (Fig. 3 E), was quantified for the whole embryo at 24 h post injection, and was similar in MSNP-injected and control groups (Fig. 3 D). Statistical analysis showed that there was no significant difference between the MSNP-injected and control (PBS-injected) groups in terms of cell death, mortality or malformations.

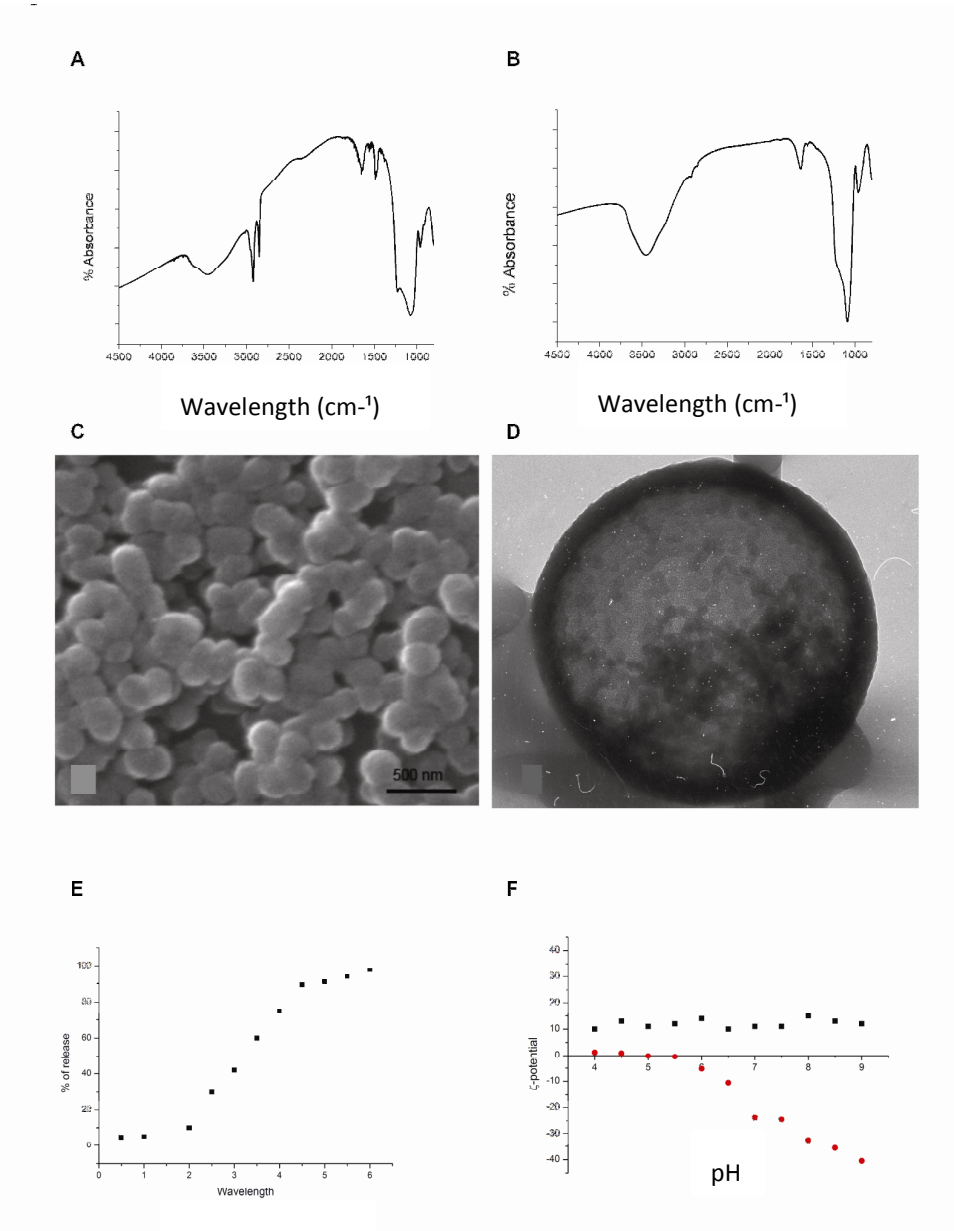


Fig. 1 Characterization of silica nanoparticles. **A** FT-IR of MSNPs before removal of CTAB template, double peak of quaternarium ammonium present at 2750 cm^{-1}. **B** FT-IR of MSNPs after the removal of CTAB template; the double peak at 2750 cm^{-1} is removed with acidic methanol washing. (**C**) SEM image of MSNPs showing the homogeneous

shape and diameter. **D** TEM image of MSNP showing homogeneous displacement of parallel channels. **E** Release of FITC-BSA from MSNPs, a delay of ca. 1.5 h is observed due to the interaction of the BSA with the silica scaffold of the nanoparticle, (y axis shows % of release). **F** Variation of the ζ-potential of the MSNPs (dots) and protein loaded MSNPS (squares) as a function of pH, (Y axis ζ-potential).

Immune cell response to injection

We examined the neutrophil response to the injection of MSNPs or PBS (control) in 2 dpf *lyz::DsRED2* embryos from 2 h until 3 days after injection. At 2 h post injection, the site of injection was already infiltrated by neutrophils (Fig. 4 A, B). Neutrophils were still in the vicinity of the injection site at 24 h and 2 days post injection (Figs. 4 C, D and E), but very few neutrophils remained at the injection site at 3 days post injection (Fig. 4 H).

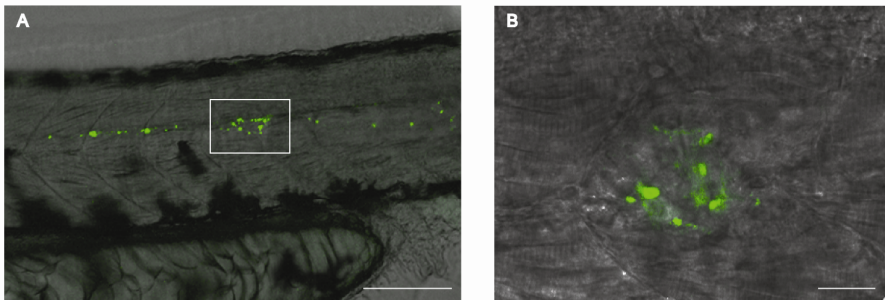


Fig. 2 Fluorescent MSNP injected zebrafish embryos. **A** MSNPs (green) distributed anteriorly and caudally from site of injection (boxed area); the black cells are pigment cells (melanocytes); scale bar, 100 µm. **B** MSNPs aggregated at the site of injection; scale bar 20 µm.

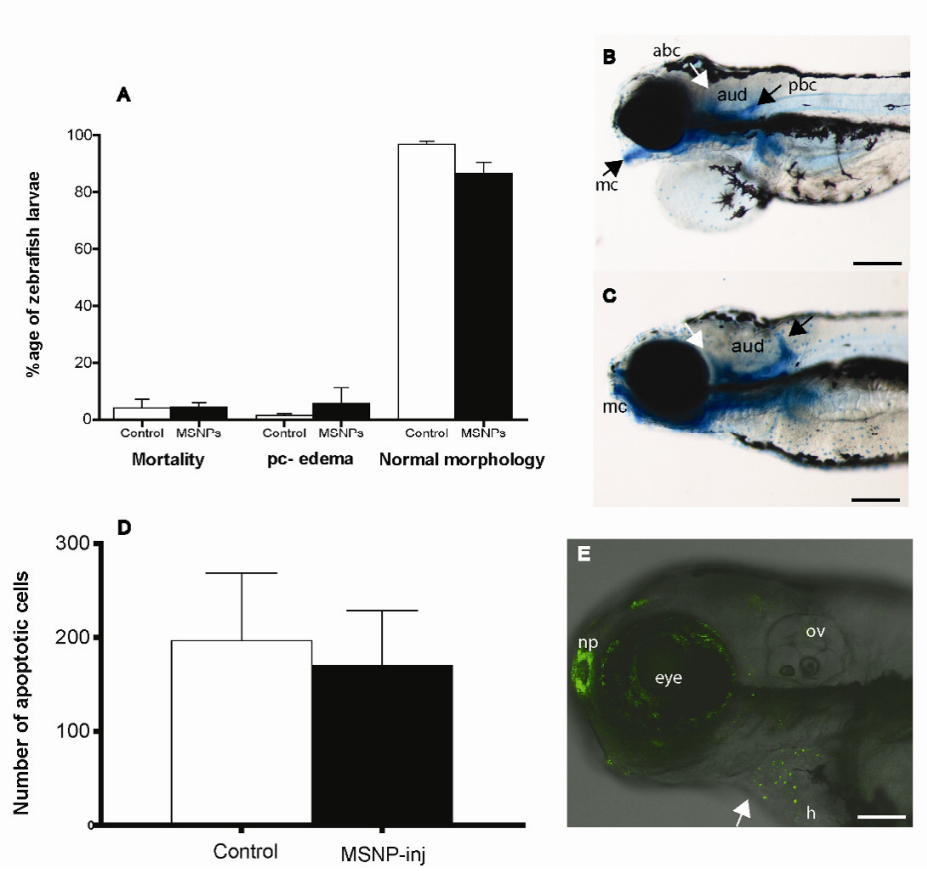
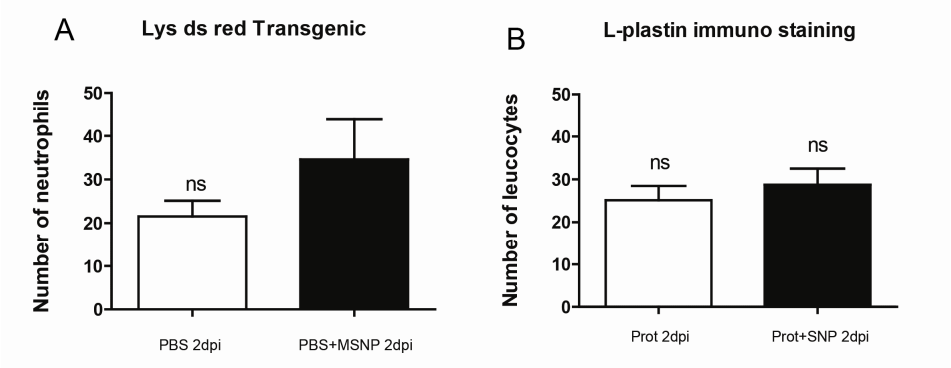


Fig. 3 Toxicity of MSNPs in zebrafish larvae. **A** Graph showing percentage of 1) mortality in larvae injected nanoparticles, 2) larvae affected by pericardial edema 3) normal morphology. The differences between buffer-injected (Control) and MSNP-

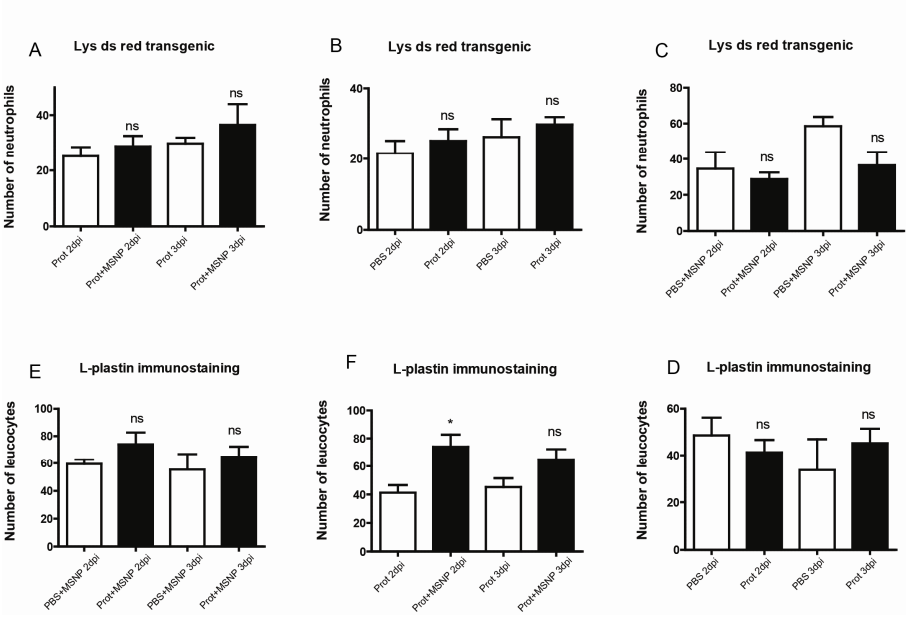
injected (MSNP) embryos are not statistically significant. Bars indicate standard error of mean. **B** Embryo injected at 2 dpf with MSNPs and fixed and stained at 5 dpf with Alcian blue; it has a combination of abnormalities as follows: pericardial oedema, a malformed Meckel's cartilage (Mc), anterior brachial cartilage (white arrow), posterior branchial cartilage (black arrow), and auditory capsule (aud). Scale bar, 200 µm. **C** Embryo injected at 2 dpf with buffer only, then fixed at 5 dpf and stained with Alcian blue. This embryo shows a normal pharyngeal skeleton. Scale bar, 200 µm. Although we selected this malformed embryo from the MSNP group, and the normal from the controls, there was no significant difference in the incidence of malformed embryos between buffer-injected and MSNP-injected groups. **D** Quantification of Acridine orange stained cells in control (buffer-injected) and MSNP groups. There was no significant difference after 24 h of injection between the two groups (10 embryos per group). **E** Acridine orange stained embryo with dead cells (green) in the eye, heart (h) and nasal placode (np). Other symbols: ov, otic vesicle. Scale bar, 100 µm.

Large aggregations of neutrophils were seen at the site of injection regardless of whether MSNPs or buffer alone was injected (Fig. 4 A, B). There was no difference between MSNP-injected and PBS-injected embryos in terms of neutrophil infiltration in the larvae 2 days after injection (See supplementary Fig. 1). Additionally, we performed L-plastin immunostaining to image and quantify the accumulation of leukocytes. Like lyz-DsRED2 cells, *L-plastin* positive cells accumulated around the injection sites in both MSNP injected and control larvae (Fig. 4 G, H). Quantitative analysis revealed no significant difference between MSNP and control injections (Supplementary Fig 2).



Supplementary Figure 1 Morphometric analysis of immune cells in *lysC::DsRED2* transgenic embryos, (**A)** Number of neutrophils 2 days post injection of PBS (veh) and MSNPs. (**B**) L-plastin positive leucocytes 2 days post injection of PBS (veh) and

MSNPs.



Supplementary Figure 2(A) Number of neutrophils 2 and 3 days post injection with Proteins or [Prot-MSNP]. **(B)** Number of neutrophils 2 and 3 days post injection of [PBS] and [Protein]. **(C)** Number of neutrophils 2 and 3 days post injection of MSNPs with or without proteins. **(D)** Number of total leucocytes 2 or 3 days post injection of [PBS] or [protein]. **(E**) Number of leucocytes 2 or 3 days post injection of [MSNP + PBS] or [Proteins+MSNP]. **(F)** Number of leucocytes 2 or 3 days post injection of protein or prot-MSNPs.

Compound delivery

In embryos injected with MSNPs loaded with a combination of 40 ng/ml of MCSF and 400 ng/ml of RANK-L, and then suspended in buffer, TRAcP positive cells were observed around the injection site (Fig. 5 A) and at many other locations distributed all over the body of the larvae (Fig. 5 A-F). We made a quantitative analysis of the total number of TRAcP positive cells throughout the body of the larvae 1-5 days after the injection of protein-loaded MSNPs. We found a significant increase in the number of TRAcP positive cells from 2 days post injection up to 4 days post injection compared to the controls. This increase in number seems to be transient as it returns to normal after 5 days. There was no significant difference in the number of TRAcP positive cells in the cytokine and cytokine-MSNP injected embryos 2 days and 3 days post injection (supplementary Fig. 2).

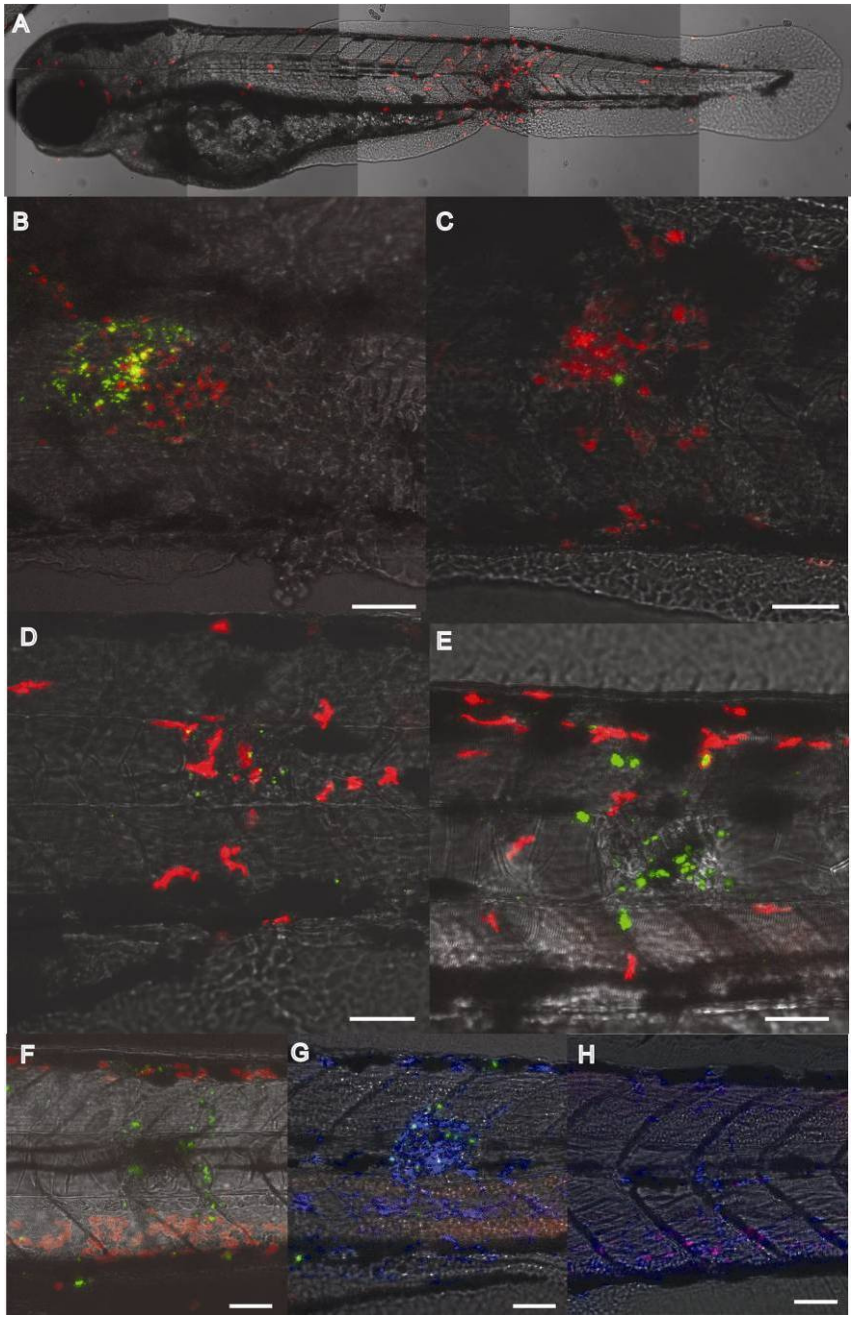


Fig. 4 Confocal images showing injection site of *lysC::DsRED2* transgenic zebrafish embryos/larvae. **A** Buffer-injected 2 dpf embryo 2 h post injection (overlay of tiles with z stacks). Note the neutrophils (red) at the injection site (arrow) and at more remote

sites. **B** An overlay of transmitted and confocal image of a different embryo showing MSNPs (green) Neutrophils (red) at the injection site 2 h post injection. **C** Representative overlay image of a different embryo injected at 2 dpf with MSNPs and analysed at 2 h post injection. This case shows a much smaller accumulation of MSNPs (gree**n**) neutrophils (red) at the injection site than B. **D** MSNP-injected embryo 24 h post injection. MSNPs (green), Neutrophils (red). **E** MSNP-injected embryo, 2 days post injection showing MSNPs (green), Neutrophils (red). **F** MSNP injected 3 days post injection (green), neutrophils (red), leucocytes (blue). **G** MSNPs (green) in 2 days post injection larva, Leucocytes (blue). **H** MSNP injected 2 dpf larva B-H are all to the same scale, bar= 50µm.

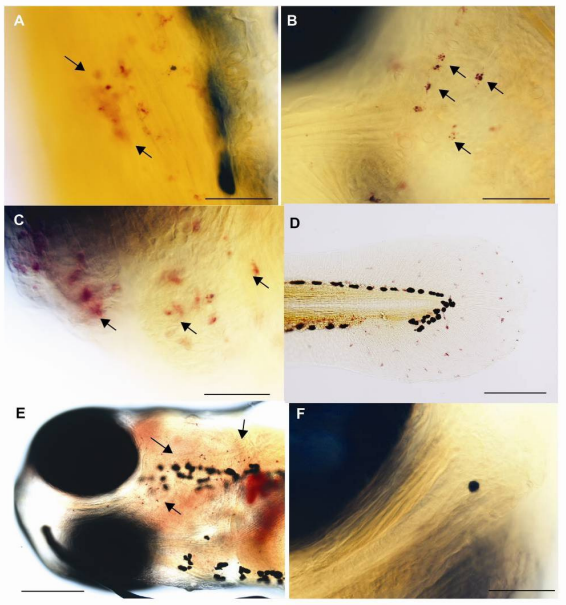


Fig. 5 TRAcP enzyme staining in MSNP injected larvae, **A** TRAcP positive cells at the site of injection in 3 days post injection larva (arrows). **B** TRAcP positive cells in the head region under the eye (arrows) 3days post injection) **C** TRAcP positive cells in the heart region in 3 days post injection embryo (arrows). **D** TRAcP positive cells in caudal fin of a 4 days post injection larva. **E** TRAcP positive cells in ventral side of head of a 4days post injection larva (arrows). **F** PBS injected larva 3 days post injection with no expression below the eye. Scale bar A, B, C, F, H = 50 µm, D 100 µm and E, 200 µm).

There was also the rare presence of apparently multinucleated TRAcP positive cells in cytokine-loaded MSNP injected embryos (Fig. 6 A-D). Hematoxylin and TRAcP double-staining showed 3-5 nuclei in a cell (Fig. 6 B). One of these apparently multinucleated TRAcP positive cells in the somite area of a 3 days

post injection larva in Fig. 6C was counter-stained with (DAPI) to visualise the nuclei (Fig. 6 D boxed area). Quantitative analysis of PEGylated cytokine-loaded MSNPs or buffer-loaded MSNPs in 2 days post injection and 3 days post injection groups showed that the number of TRAcP positive cells significantly increased compared to the buffer-loaded MSNP injected controls (Fig. 6 E).

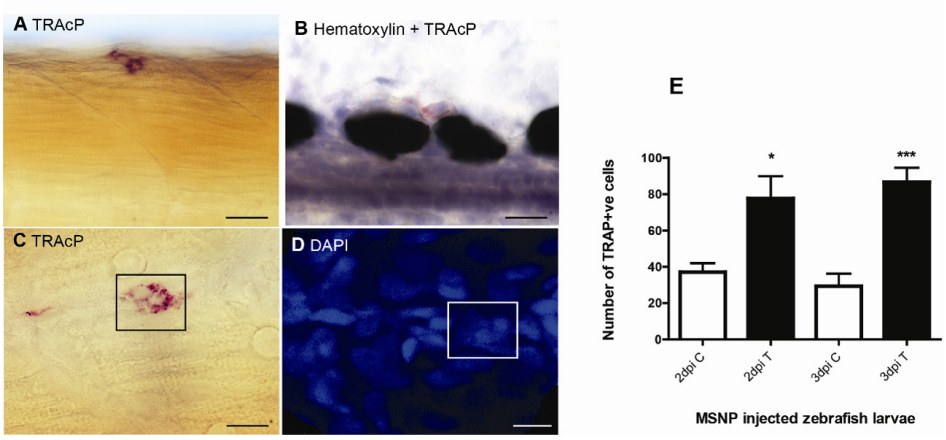


Fig. 6 (**A**) TRAcP expression in the somites of a 3 days post injection larva with apparently multinuclear cell. (**B**) TRAcP positive cells counter stained with Hematoxylin **C** TRAcP positive zebrafish larva showing apparently multinuclear cells (box) **D** TRAcP enzyme stained larva counter stained with DAPI showing 5-6 nuclei in the same area as C (box) Scale bar ABC=12.5 µm and D=12 µm **E** Quantitative analysis of TRAcP positive cells in 2 days or 3 days post injection, 10 larvae/group were studied for the number of TRAcP positive cells, analysis was done with One way analysis of variance * = p< to 0.1,**= $p < 0.01$, *** $p < 0.001$.

Discussion

We have tested the toxicity, and the capacity for controlled drug delivery, of MSNPs injected into living zebrafish embryo. We find that there is no significant difference between the toxicity of MSNP injections, and buffer-only injections, as measured by several parameters (mortality, cell death, gross malformations). Injection of MSNPs led to an influx of immune cells at the site of injection that persisted for 2-3 days. However, the same type of influx was also seen in embryos injected with buffer alone, suggesting that it is not the MSNPs that are

responsible for the immune response, but some aspect of the injection procedure itself (tissue damage, for example). Indeed, it has previously been shown that trauma to zebrafish embryos (e.g. a fin clip or mechanical wounding with an injection needle) causes an influx of macrophages and neutrophils to the wound site [180-183].

In all the studies reported here, we used PEGylated MSNPs because PEGylation is thought to avoid triggering an immune response against the particles [97,194]. With time-lapse imaging it is clear that some MSNPs remain at the site of injury, while others travel in the blood stream from the site of injection. The MSNPs localized at the site of injection were found to be there even after 3 days.

Even though the MSNPs are very small in size with an average diameter of 200nm these particles have a very high surface area that can be used to carry drugs in our case cytokines. This small size helps in fast endocytosis by non-phagocytic cells [96]. In our studies we found no significant toxic effects of injected MSNPs in the living embryos. This means that at low concentrations, MSNPs could be a very good delivery system in the whole organism. Nanomaterials such as fullerenes have been found to be excreted following oral administration or injections in mice [195] while others were shown to be taken up by immune cells. We observed very little co-expression of lyz: DsRED2-labeled cells and MSNPs. This suggests that only a few of the particles are actively phagocytosed by neutrophils. With L-plastin immunolabeling, which stains leucocytes, more overlap with the fluorescent signal of the particles was observed, suggesting that part of the MSNPs may be phagocytosed by immune cells.

Other studies have shown that, in the presence of artificially provided cytokines like MCSF and RANK-L, immune cells are activated into TRAcP+ osteoclasts [196]. We were not able to combine the double staining of fluorescent immune cells with the histochemical staining of TRAcP enzyme activity because the two protocols were not compatible. Using single-labeling, we found a significant increase of TRAcP positive cells in zebrafish larvae injected with MSNP-cytokines, while there was no significant increase in the total number of immune cells in these embryos. Some of these TRAcP positive cells had multiple nuclei, as shown by DAPI staining. By 4-5 days post injection, TRAcP positive

cells appeared to become small and darkly-stained. One possible explanation for these observations is that the TRAcP positive cells were transiently induced by the injected cytokines in the early period (2-4 days) after injection, and returned to a normal level later on.

Further work is required to clarify the differentiation status of the TRAcP positive cells. If it can be confirmed that young larvae have inducible osteoclast-like cells, this would be interesting because zebrafish larvae have not previously been reported to have osteoclasts at the stages studied here. Furthermore, the presence of osteoclast-like cells in zebrafish larvae could lead to a disease model for bone disorders and for the study of effects, for example, of anti osteoporotic drugs. What is clear from our findings is that MSNPs can be used for drug or compound delivery in the zebrafish embryo with no excess of gross toxic effects or immune responses attributable to the nanoparticles themselves.