Magnetic nanoparticles for "smart liposomes"

Yoshitaka Nakayama[1], Mislav Mustapić[2], Haleh Ebrahimian[1], Pawel Wagner[2], Jung Ho Kim[2], Md Shahriar Al Hossain[2], Joseph Horvat[2], Boris Martinac[1,3,#]

[1]Molecular Cardiology and Biophysics Division, Victor Chang Cardiac Research Institute, NSW 2010, Australia, [2]Institute For Superconducting and Electronic Material (ISEM), University of Wollongong, Squires Way, North Wollongong, NSW 2500, [3]St Vincent's Clinical School, University of New South Wales, Darlinghurst, NSW 2010

[#]Corresponding author: Dr Boris Martinac
Victor Chang Cardiac Research Institute
Lowy Packer Building
405 Liverpool St
Darlinghurst, NSW 2010
Australia

Abstract: (173/200 words)

Liposomal drug delivery systems (LDDS) are promising tools used for the treatment of diseases where highly toxic pharmacological agents are administered. Currently, a major challenge remains to destabilise LDDS by a specific stimulus at a target site. The bacterial mechanosensitive channel of large conductance (MscL) presents an excellent candidate biomolecule that could be employed as a remotely controlled pore-forming nanovalve for triggered drug release from LDDS. In this study, we have developed superparamagnetic nanoparticles for activation of the MscL nanovalves by magnetic field. Synthesised $CoFe_2O_4$ nanoparticles with the radius less than 10 nm were labelled by SH-groups for attachment to MscL. Activation of MscL by magnetic field with the nanoparticles attached was examined by the patch clamp technique showing that the number of activated channels under ramp pressure increased upon application of magnetic field. In addition, we have not observed any cytotoxicity of the nanoparticles in human cultured cells. Our study suggests the possibility of using magnetic nanoparticles as a specific trigger for activation of MscL nanovalves for drug release in LDDS.

Keywords

Magnetic nanoparticles, $CoFe_2O_4$, mechanosensitive channels, MscL, liposomes, drug delivery system, patch clamp

Introduction:

Liposomes are one of the mainstream particulate drug carriers used in modern medicine (Janoff et al. 1999, Svenson et al. 2004). Liposomal drug delivery systems (LDDSs) provide a widely applicable method of drug encapsulation and delivery. In their basic form liposomes consist of naturally occurring biocompatible phospholipid vesicles (Suntres et al. 2011) that act as physical barriers to protect the drug cargo from degradative enzymes. Due to their stability during blood circulation liposomes are useful as sustained-action delivery vehicles for drugs for which low bioavailability or toxicity is a problem. Ideally, a LDDS should be stable, have a long half-life during circulation, concentrate at a target site and release the cargo it contains in a controlled manner upon a specific stimulus (Koçer, 2007). Currently, the major shortcoming of LDDS is that they lack an effective drug release mechanism, which would help to increase their efficacy.

In recent years, a rapidly growing interest for superparamagnetic nanoparticles has been motivated by numerous existing and expected applications in biomedicine such as separation of magnetically tagged cells, targeted drug or radionuclide transport into the cells or tissues and contrast improvement in diagnostic MRI and magnetic hyperthermia, amongst many others (Pankhurst et al. 2003, Pankhurst et al. 2009). The use of magnetic nanoparticles as a drug delivering system is still defined by its biocompatibility and selective targeting to the desired target tissue under the guidance of external magnetic field. Advances in current technologies and the development of magnetic nanoparticles as drug delivery systems to deliver drugs to tumor hypoxic zones have fast-tracked in the past decade and led to the development of various magnetic nano-formulations including liposomes. This novel drug delivery system has increased the ability to deliver drugs for which conventional therapy has shown limited efficacy (Sun et al. 2008). The field of magnetic drug delivery is still in its infancy, and synthesis of better magnetic drug delivery systems and integration of

multifunctional ligands are being continuously investigated so as to carry it from the bench-top to the clinic (Wahajuddin et al. 2012).

Bacterial mechanosensitive (MS) channel MscL, when activated, protects the microbial cell from sudden increase in membrane tension due to a hypo-osmotic shock by allowing rapid efflux of intracellular solutes (Levina et al. 1999). The salient feature of MscL is its very large non-selective channel pore of 28 Å in diameter (Cruickshank et al 1997, Corry et al. 2010, Wang et al. 2014), which is the largest of all known MS ion channels (approx. 10-100 times larger than typical eukaryotic MS channels). It is permeable to molecules as large as most peptides and even small proteins of up to 6.5 kDa (van den Bogaart et al. 2007). Together with its ability to be activated by membrane tension in liposome membranes (Martinac et al. 2011, Teng et al. 2015), its large pore makes MscL an ideal candidate for a triggered nanovalve able to release a cargo of small molecules from LDDS. For this purpose MscL has already been engineered as a remote-controlled nanovalve switchable by light (Koçer, 2007).

In this study, we have developed superparamagnetic $CoFe_2O_4$ nanoparticles for binding to MscL. We show their lack of toxicity on a human cell culture. In addition, we demonstrate the activation of MscL by magnetic field in the presence of the superparamagnetic nanoparticles using patch fluorometry.

Materials and Methods:

Synthesis of $CoFe_2O_4$

The synthesis of $CoFe_2O_4$ was performed by wet chemical process. $FeCl_3 \cdot 6H_2O$ and $Co(aceta)_2 \cdot 4H_2O$ (molar ratio 2:1) were dissolved in DI water previously bubbled with pure argon. Whole experiment was maintained under inert atmosphere, with temperature of reaction kept at 0°C. Hydrazine was used as a reducing agent in alkaline environment. Following the hydrazine, 1,6 hexanedithiol was added as a surfactant. Black precipitates started to appear after few minutes. Shortly after that, reaction was stopped and nanoparticles removed using external magnet, washed with DI water and acetone, and finally dried. Conducting the experiment at low temperature, high concentration of starting precursors in presence of surfactant and in limited time enables the preparation of very small and uniform nanoparticles.

Transmission electron microscopy

TEM imaging was performed in a probe-corrected JEOL ARM200F equipped with cold field emission gun and an UltraScan CCD. The microscope is also equipped with a Centurio EDS detector with collection angle of ~1 sr. The sample was diluted in emulsion of DI water and cyclohexane, centrifuged, and drop-casted in a Holey carbon Cu grid for TEM observation.

Protein purification

The MscL protein contains no cysteine residues, so we were able to generate a site that could be uniquely labeled with superparamagnetic nanoparticles coated with SH-groups using site-directed mutagenesis to introduce the substitution M42C. This site is located on the outer edge of the extracellular side of the protein. According to the crystal structure of the MscL protein in a closed state (Chang et al. 1998) it is comprised of five identical subunits

surrounding a central pore. Thus, the M42C mutation introduced five identical cysteine sites. The mutant was cloned into a pQE-32 expression vector as BamHI-SalI fragments, expressed in *E. coli* and purified using Co-NTA affinity chromatography for reconstitution into liposomes, as described previously (Perozo et al. 2002). Consequently, each channel protein could theoretically have had five nanoparticles attached in our patch flurometry experiments.

Liposome preparation

Liposomes made of azolectin (Sigma) were produced using the Dehydration/Rehydration method as reported previously (Häse et al. 1995). Briefly, the lipids were dissolved in chloroform and dried with a N_2 gas stream until a thin film of lipid was formed on the wall of a glass tube. 0.2 ml of D/R buffer (200 mM KCl, 5 mM Hepes-KOH) was added to the tube. After sonication for 5 min, purified M42C MscL protein was added in a 1/1,000 [w:w] ratio in the liposome suspension. 2.8 ml of D/R buffer was added and incubated for 1 h on a rotatory shaker when biobeads (Bio-rad) were added and the suspension was incubated for further 3 h to remove detergent. . The suspension was centrifuged at 250,000 × *g*. The liposome pellet was resuspended in 60 µl of D/R buffer and spotted onto a glass slide. The sample was dehydrated overnight under vacuum, and rehydrated next morning by adding 20 µl of D/R buffer of each dehydrated lipid spot on the slide. The rehydrated liposomes were used for experiments after minimum three hours and up to three days after rehydration.

Patch clamp experiments

Glass pipettes [Drummond Scientific, Broomall, PA] were pulled fresh before every experiment using a Narishige gravity puller [PP-83; Narishige, Tokyo, Japan]. Negative ramp pressure was applied by a high-pressure clamp apparatus (HSPC-1 ALA Scientific Instruments, USA). Currents were amplified with an Axopatch 200B amplifier (Axon

Instruments), and data were acquired at a sampling rate of 5 kHz with 2 kHz filtration. The channel currents were recorded at +30 mV and data were analysed using the pClamp 10 analysis software (Molecular Devices, Sunnyvale, CA). Magnetic particles were put inside a pipette at a concentration of 100 µg/ml. Magnetic field was applied to the liposome membrane patch by putting ring shaped magnets on the patch clamp chamber. Pressure threshold was adjusted before applying magnetic field.

Patch fluorometry

Creeping of fluorescently labelled liposome patches in the patch pipettes was observed as previously described (Nomura et al. 2012). To visualize liposome patches the pipette tip was bent ~30° with a microforge (Narishige; MF-900) to become parallel to bottom face of the chamber. Liposomes consisted of azolectin (99.9%) and rhodamine-PE (0.1%) containing M42C MscL. The fluorescence was detected using a Zeiss LSM 700 confocal microscope. The pipette solution and magnetic particles were the same as in the patch-clamp experiments. A 555-nm laser line was used to excite the fluorophore labelled patches with emission detected using a long pass 560-nm filter.

Testing cytotoxicity of the nanoparticles

The cultures of the human A549 lung cancer cells were started with approximately 360,500 cells/mL to 584,375 cells/mL in 10 % FBS supplemented DMEM media in 24 well microtiter plate. The cultures with or without the nanoparticles were then incubated for 24 hrs in an incubator with 5% CO_2 and 95% humidified atmosphere. The cells were removed from the culture by trypsinization after 24 hrs and washed 2 times with Dulbecco's PBS (pH 7.4) to remove any presence of serum, which interferes with the staining. The cells were then re-suspended in PBS and aliquots of 20 µL were made from all the different cultures. Equal

amount (*v/v*) of 0.4% trypan blue stain, which was filtered previously, was added to the aliquots, and were allowed to settle for 1 min. The live cells are observed as non-colored, as the cell surface is intact and in turn will repel the stain, whereas the cells with damaged cytoplasm are observed as blue colored cells. In all cases the cells were counted as dead (coloured) or alive ones (non-coloured). Their ratio was used to calculate the percentage of viability of a culture treated with a particular nanoparticles. The percentage of cell viability was calculated using the following expression,

$$\%\ of\ viability = \frac{Total\ number\ of\ viable\ cells/mL}{Total\ number\ of\ cells/mL} \times 100$$

Results:

The Characterization of the superparamagnetic nanoparticles

X-ray diffraction (XRD) patterns of the synthesised $CoFe_2O_4$ nanoparticles are shown in Figure 1*A*. The XRD pattern consists of fairly broad but still resolved peaks superimposed on smoothly varying background intensity. Such broad XRD peaks are characteristic for nanoparticles with relatively large thickness of the disordered surface layer in comparison to their diameter. By comparing our results to the XRD pattern of crystalline $CoFe_2O_4$ (Traces 00-002-1054, space group Fd3m) a good correspondence is obtained (Fig. 1A). Therefore, the obtained XRD pattern is consistent with the presence of $CoFe_2O_4$ nanoparticles and there is no indication for existence of any other crystalline phases in the obtained powders. TEM observations show that the diameter of our nanoparticles is ~5nm (Fig. 1B). For this diameter, the thickness of the disordered surface layer is about 0.8 nm (Sun et al., 2012). Therefore, the volume of the disordered surface layer is estimated as 40% of the total nanoparticle volume. This is the reason for obtaining the broad XRD peaks, which still resemble the crystalline XRD pattern.

Figure 1C shows the magnetic hysteresis loops of $CoFe_2O_4$ nanoparticles at different temperatures. The obtained S-shaped hysteresis loops with negligible coercive field (H_c) are a typical feature of superparamagnetic nanoparticles. Due to small nanoparticle size, the magnetic moment of individual nanoparticles is small enough to be misaligned by thermal excitations. Application of magnetic field aligns the magnetic moments of nanoparticles and the magnetization of the sample is almost fully saturated at low fields (300 mT). The saturation moment (M_s) observed in this study was 43.5 emu g^{-1} at 10 K and 37.3 emu g^{-1} at 310 K for $CoFe_2O_4$ with average nanoparticle size of less than 6 nm. While H_c for the nanoparticles is negligible at room temperature, it becomes 180 Oe at 10K. For comparison, the size, Ms and H_c of the nanoparticles in previous studies are listed (Table 1). Magnetic

saturation of our nanoparticles is consistent with the values reported for nanoparticles of various sizes, prepared by sol-gel method and annealed at different temperatures (Lee et al. 1998). Strong magneto-elastic coupling in $CoFe_2O_4$ allowed for studying the evolution of the surface spin disorder using infrared spectroscopy methods (Sun et al. 2012). Ferrimagnetic order of $CoFe_2O_4$ is disrupted in the disordered surface layer, resulting in a smaller magnetic moment. The decrease of the magnetic saturation of the nanoparticles occurs as the ratio of the disordered surface volume to the total nanoparticle volume increases. This effect is accelerated as the nanoparticle size goes below 10 nm (Sun et al. 2012).

FTIR spectroscopic analysis provided a clear signature of 1,6-haxanedithiol (Ozturk et al. 2005) in the nanoparticles (Figure 1D). The absorption bands at 2927 and 2853 cm^{-1} are attributed to stretching modes of -CH in 1,6-haxanedithiol. The bands at 1459 and 1432 cm^{-1} are due to bending modes of $-CH_2$. The bands at 1351, 1269 and 1234 cm^{-1} are from the wagging modes of $-CH_2$. The band at 1124 cm-1 is from the CH_2 twisting mode and the band at 1071 cm^{-1} is from the C-C stretching mode (Ozturk et al. 2005). The strong absorption band just at the edge of the sensitivity window of our spectrometer at ~570 cm^{-1} is the ν_1 vibrational mode of $CoFe_2O_4$ (Pauline et al. 2012, Guilherme et al. 2009).

The effect of magnetic nanoparticles on the gating of MscL in patch clamp experiments

To bind the $CoFe_2O_4$ magnetic particles coated with SH-groups to MscL, we used the M42C mutant MscL, which was labelled with nitroxide spin labels or Alexa-dyes having the SH-group, as shown previously (Perozo et al. 2001, Perozo et al 2002). M42C mutation positions at the end of the TM1 transmembrane domain facing the outside of the membrane (Fig. 2*A*). Since MscL is incorporated into the liposome membrane in a right-side out orientation (Ajouz et al., 2000), magnetic nanoparticles can attach to MscL from the pipette in the inside-out excised patch configuration. Magnetic field was applied to the membrane

with ring shaped magnets providing 300 mT field (Fig. 2*B*). When 4 s pressure ramp (-25 mmHg/s) was applied to the patch membrane before applying the magnetic field with the nanoparticles attached, the M42C MscL channel started to open at approximately -70 mmHg and up to four channels were activated at the maximum ramp pressure. In contrast, 5 min after holding the same patch under the influence of the magnetic field the MscL activation threshold did not change, however, the number of the activated MscL channels increased to 11 or more channels (Fig. 2*C*). We examined also the patch creeping using patch fluorometry to find out if magnetic particles had any effect on it. The creeping under the magnetic field and pressure ramp was very similar to that without magnetic field (Figure 2*D* and supplementary video S1). These observations indicate that magnetic particles in the presence of the magnetic field are the sole contributor to the increase in number of the activated MscL channels.

In order to examine whether the channels would stop gating if the pressure was not changing due to the ramp profile of the applied pressure, we also applied constant pressure to the patch pipette in the presence of the magnetic field. At constant pressure and the magnetic field present, the number of activated channel increased after application of the pressure ramp and then remained constant (Figure 2*E*). Moreover, we applied and removed the magnetic field sequentially to observe the effect of the magnetic field and superparamagnetic nanoparticles on the gating of M42C channels under constant pressure. The pressure was continuously applied by HSPC until a few channels opened and it was further kept at a constant value. When in addition the magnetic field was applied, the open probability of the channels increased to NP_o = 0.19. However, the open probability decreased to NP_o = 0.05 after the magnet was removed. When the magnetic field was applied again, the channels started to open again frequently after several tens of seconds (Figure 2*F*). These results

further indicate that the increase in the channel number and open probability were caused by the magnetic field.

We measured further the changes in time course of the channel activation threshold and the number of channels with and without the magnetic nanoparticles being present during application of the magnetic field. Opening threshold in the absence of magnetic nanoparticles was slightly decreased 5min after applying magnetic field due to patch creeping known to occur in patch clamp experiments (Nomura et al. 2012). This suggested that patch creeping may also occur in the pipette under the influence of the magnetic field meaning that the number of activated channels could also increase due to the reduced activation threshold caused by the patch creeping (Fig. 2*G*). Interestingly, with nanomagnetic particles present we did not observe the reduction of the activation threshold indicating that patch creeping did not occur in this situation (Fig. 2D; Video S1). Nevertheless, the number of activated channels increased with time after application of the magnetic field (Fig. 2*G*). This strongly suggests that the increase in the number of channels was affected by the movement of magnetic particles in the patch membrane under the influence of the magnetic field.

Cytotoxicity of the superparamagnetic nanoparticles

To examine the cytotoxicity of the nanoparticles we used cultures of A549 human cancer cells to which the nanoparticles were added at different concentration of nano-conjugates ranging from 10 to 100 µg/mL. One culture was kept blank for reference. After incubation, the microscopic observation revealed a dense covering of the cells by nano-conjugate suspension and the density of the suspension increased with the increase in the concentration of the nano-conjugates. However, the blank culture showed no such coverage by the suspension of nanoparticles. Instead, the culture showed clear surface of the attached cells, as shown in Fig. 3*A*. Even though the nanoparticle suspension covered the cells, they showed no change in their shape, size, growth pattern or number. The cell culture was also observed

under an inverted microscope in the ‘Fuchs-Rosenthal’ hemocytometer to determine the cell viability, as shown in Fig 3*B*. The cells from all the cultures with different nano-conjugates exhibited little or no cytotoxicity with cell viability ranging close to the blank culture, as shown in Fig. 3*C*.

Discussion:

We developed superparamagnetic $CoFe_2O_4$ nanoparticles for the activation of the MscL channels reconstituted into azolectin liposomes. Magnetic hysteresis loops (Fig. 1C) are typical for superparamagnetic particles, comparable to those described in the previous studies (Pauline et al. 2012, Sun et al, 2012, Ayyappan et al, 2009, Lee et al. 1998). Our magnetic measurements are consistent with XRD and TEM observations of nanoparticles small enough to be superparamagnetic (Sun et al. 2012). Magnetically ordered nanoparticle core has large magneto-crystalline anisotropy and large value of Hc (Pauline et al. 2012, Sun et al. 2012). However, H_c of our nanoparticles is negligible (Fig. 1C). Consequently, the change of magnetic moment of our nanoparticles with application of H occurs through rotation of nanoparticles themselves and not by rotation of the magnetic moment within the nanoparticles. No magnetic irreversibility was observed, indicative of negligible inter-particle interaction. Magnetic field of 300 mT is considered strong enough to move the nanoparticles on the liposome membrane (Supplementary Video S2).

By recording the channel activity in patch clamp experiments we found that the number of activated channels increased upon application of the magnetic field in the presence of the nanoparticles, which was not the case in the absence of the magnetic field. The nanoparticles coated with SH-groups on their surface can bind to the cysteine residue of each subunit of the M42C MscL channel mutant by forming disulphide bond, as previously shown in several EPR and FRET spectroscopic studies (Corry et al. 2010, Wang et al. 2014, Perozo et al. 2002). Alternatively, it is also possible that the nanoparticles could attach to the liposome membrane due to the viscosity of the lipid bilayer. This is because there are two possibilities to explain the increased number of activated channels in the presence of nanoparticles and magnetic field. The nanoparticles bound to the channels would pull on the MscL channel proteins in the presence of an inhomogeneous magnetic field, and thus open

the channels. In contrast, the nanoparticles attached to the liposome membrane would pull on the membrane itself. Local nanoparticle density on the membrane is likely to be inhomogeneous and therefore, different sections of the membrane would experience different pull by the nanoparticles in an inhomogeneous field. The membrane is then locally stretched, which would have a similar effect to the externally applied pressure opening the channels. The strength of the magnetic field of 300 mT in our experiments was sufficient to move the nanoparticles (Supplementary Video S2). Consequently, irrespectively of the way nanoparticles were attached in our patch fluorometry experiments the increased number of activated channels resulted from the movement of the nanoparticles induced by the magnetic field.

Conclusions:

In summary, our study indicates that superparamagnetic nanoparticles of average size of less than 6 nm coated with SH-groups are highly suitable as components of a trigger mechanism for opening MscL nanovalves. Due to their very large open pore and property of being modulated by mechanical stimuli MscL channels present an ideal candidate for use as nanovalves in liposomal drug delivery. Consequently, a combination of MscL channels and the magnetic nanoparticles generated for this study holds promise for use in development of "smart liposomes", a new generation of LDDS.

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Author contributions:

Y. N., M. M., J.H. and B. M. wrote the main manuscript text. M.M. prepared the magnetic particles. Y. N., M. M. and H. E. conducted the experiments and analysed data. All authors reviewed and edited the manuscript.

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Figure legends:

Figure 1. Magnetic properties of the superparamagnetic nanoparticles. *A.* X-ray diffraction pattern of $CoFe_2O_4$ nanoparticles. ***B.*** TEM image of $CoFe_2O_4$ nanoparticles, with EDS results in inset. ***C.*** The magnetic hysteresis loops of $CoFe_2O_4$ nanoparticles at various temperatures. **D.** FTIR spectra of the $CoFe_2O_4$ nanoparticle coated with 1,6 hexanedithiol.

Figure 2. Activity of the M42C MscL channels with nanoparticles in the patch pipette with magnetic field. *A.* The crystal structure of MscL on the view from side (left) and top

(right). Red spheres show methionine 42 of each subunit, which is replaced with cysteine in this study. ***B.*** Patch clamp experimental chamber with a ring-shaped magnet (left) and a protocol of applying magnetic field (right). ***C.*** Patch clamp recording from M42C MscL upon application of a pressure ramp in the absence of the magnetic field (left) and 5 min after application of the magnetic field (right; same patch). ***D.*** Patch creeping before (left) and after (right) applying the magnetic field. Double arrows show the distance from the tip of the pipette and the patch membrane. Scale bars show 5 μm. ***E.*** Recording from M42C MscL under constant pressure. Left trace was recorded before applying magnetic field (no magnet) and right trace shows a recording 5 min after applying the field (with magnet). Both traces were recorded from the same patch. ***F.*** The effect of the magnetic field on the gating of M42C MscL under constant pressure. The magnet was placed on the patch chamber (ON) and taken off (OFF) during the recording. Open probability (NP_o) was calculated with Clampfit software over periods indicated by horizontal bars. ***G.*** Time course of opening threshold in relative units (left) and number of activated channels in the patch given in relative units (right) in the presence of the magnetic field. The threshold and number of activated channels are normalized by the value of control, which is measured before applying magnetic field (n = 5). Black and red traces show without and with $CoFe_2O_4$ nanoparticles in the pipette, respectively. All experiments were performed independently more than three times (Bars show standard error, Student t-test, *; $P = 0.036$).

Figure 3. Cytotoxicity of the nanoparticles. ***A.*** Images of cells in culture with the increasing concentration of nanoparticles. ***B.*** Cells after staining with trypan blue showing uncolored alive cells and blue dead cells. ***C.*** Cell viability in the different concentrations of different nanoparticles.

Table 1. Comparison of magnetic $CoFe_2O_4$ particles

Reference	Particle size (nm)	M_s (emu/g)	H_c (Oe)
This study	≈ 5	37.3	180
Pauline et al., 2012	17-22	58-65	233-253
Ayyappan et al., 2009	≈ 15	60	250-300
Lee et al., 1998	bulk	80	5400

Supplementary video legends

Video S1

Patch creeping of liposome membrane in the presence of nanoparticles during application of the magnetic field. 4-s pressure ramps were applied to the membrane without the ring-shaped magnet. Then, the magnet was placed on the chamber in the middle of this video and the same ramp pressure was applied.

Video S2

The movement of $CoFe_2O_4$ nanoparticles by ring-shaped magnet.

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