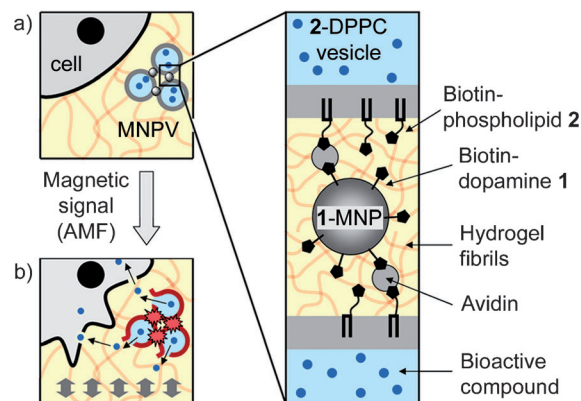


# Conversion of Magnetic Impulses into Cellular Responses by Self-Assembled Nanoparticle–Vesicle Hydrogels\*\*

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Phospholipid vesicles are widely used as nano-sized drug delivery vehicles<sup>[1]</sup> and as biomimetic model systems,<sup>[2]</sup> where the bilayer allows fundamental biomembrane processes like ion transport, signaling, and multivalent recognition to be copied.<sup>[3]</sup> In particular, the remotely triggered transit of stored chemicals across bilayer membranes is a key goal as it will allow vesicles to communicate with cells either in vivo or in vitro during cell culture.<sup>[4]</sup> The latter approach should produce exciting new “smart” biomaterials, although non-invasive and non-chemical control over drug release from vesicles remains challenging.

Most mammalian cells are unaffected by oscillating or permanent magnetic fields. To sensitize cells to magnetic fields, they can be labeled with magnetic nanoparticles (MNPs),<sup>[5]</sup> an approach used to effect gene transfection with static magnetic fields<sup>[6]</sup> or cause hyperthermia with alternating magnetic fields (AMFs).<sup>[7]</sup> Alternatively MNPs can be used to label vesicles, which allows magnetic manipulation and AMF-triggered contents release; magnetic release is attractive as nearby cells would only be affected by the released biochemicals and not the AMF. Recently we used 10 nm Fe<sub>3</sub>O<sub>4</sub> MNPs to crosslink 800 nm diameter phospholipid vesicles and form magnetic nanoparticle–vesicle assemblies (MNPVs).<sup>[8]</sup> Embedding MNPVs within a hydrogel matrix added a further level of assembly, with the hydrogel fibrils acting as an artificial extracellular matrix that reinforced the vesicles, providing robust materials<sup>[9]</sup> that responded to AMFs by releasing stored dyes. Such nanostructured and responsive self-assembled biomaterials have enormous potential in cell culture,<sup>[10]</sup> and replacing these dyes with bioactive species should produce a new type of “smart” cell culture scaffold<sup>[11]</sup> responsive to magnetic impulses. Herein we describe a self-assembled bionanotechnological system able to act as a “smart” biomaterial that translates non-invasive magnetic signals into cellular responses (Figure 1).



**Figure 1.** a) Cells and nanoparticle-vesicle assemblies (MNPVs) are co-immobilized within a calcium alginate hydrogel (yellow). MNPVs are self-assembled nanocarriers composed of magnetic nanoparticles coated with *N*-biotinyl dopamine 1 (1-MNP) and DPPC vesicles containing biotin-DHPE 2 (2-DPPC), which are linked together by avidin. b) Chemical messengers, such as drugs (blue), can be non-invasively released by an alternating magnetic field (AMF), and these released chemicals in turn induce responses from cultured cells.

An important design feature was the self-assembly of Fe<sub>3</sub>O<sub>4</sub> nanoparticles with gel-phase vesicles, an alternative to physical incorporation<sup>[12]</sup> that was designed to allow heat generated in the MNPs by the AMF to be efficiently transferred to the bilayers.<sup>[13]</sup> When heated, gel-phase vesicles “melt” at a triggering temperature ( $T_m$ ), an all-or-nothing event that allows complete and rapid escape of encapsulated compounds. The biotin–avidin interaction was used to link vesicles and MNPs, which improved compatibility across cell types, including myoblasts (Figure 2) and chondrocytes. It also allowed commercially available biotin lipids like *N*-(biotinoyl)-1,2-dihexadecanoyl phosphatidylethanolamine (biotin-DHPE) to be used as vesicle crosslinkers.<sup>[14]</sup>

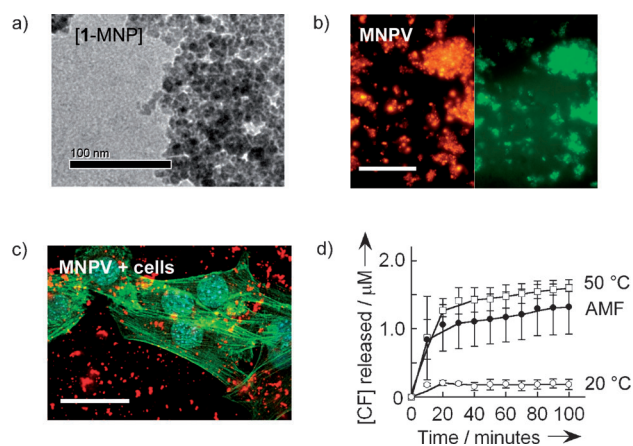
*N*-Biotinylated dopamine (1)<sup>[15]</sup> was used to give Fe<sub>3</sub>O<sub>4</sub> MNPs an adhesive coating. MNPs were formed by coprecipitation<sup>[16]</sup> then sonicated with 1 in deoxygenated methanol (0.7 mM) to give 1-coated Fe<sub>3</sub>O<sub>4</sub> nanoparticles ([1-MNP]), with a coating efficiency of 50 ± 20 % (Figure 2a). Dipalmitoyl phosphatidylcholine (DPPC) vesicles (800 nm diameter) were chosen as the nanocontainers as these bilayers have  $T_m \approx 42^\circ\text{C}$ ,<sup>[17]</sup> a triggering temperature above cell culture conditions (37 °C). Vesicles with stored chemical payloads were created by extrusion of 0.2 % mol/mol biotin-DHPE 2 in DPPC in a solution of the compound to be encapsulated. The thermal release of encapsulated 5/6-carboxyfluorescein (5/6-CF) showed these [2-DPPC] vesicles had  $T_m \approx 40^\circ\text{C}$ . Addition of the avidin “glue” to a mixture of [1-MNP] and [2-DPPC] vesicles produced large magnetic

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**Figure 2.** a) TEM image of [1-MNP] (scale bar 100 nm). b) Fluorescence microscopy images of MNPVs formed from [1-MNP], rhodamine-labeled [2-DPPC] (0.1% rhodamine-dipalmitoyl phosphatidylethanolamine) and fluorescein isothiocyanate (FITC)-avidin (left: rhodamine channel, right: fluorescein channel, scale bar 20  $\mu\text{m}$ ). c) Confocal microscopy image of myoblasts and rhodamine-labeled MNPVs co-immobilized in alginate (scale bar 25  $\mu\text{m}$ ). Cells stained with 4',6-diamidino-2-phenylindole (DAPI, nuclei) and FITC-conjugated phalloidin (F-actin). d) 5/6-CF release at (○) 20  $^{\circ}\text{C}$  (□) at 20  $^{\circ}\text{C}$  after incubation at 50  $^{\circ}\text{C}$  (4 min) (●) at 20  $^{\circ}\text{C}$  after exposure to an AMF (4 min).

nanoparticle-vesicle assemblies (MNPVs); using fluorescein-labeled avidin with rhodamine-labeled vesicles revealed these components were co-localized in the MNPVs (Figure 2b).<sup>[18]</sup> These MNPVs were susceptible to external magnetic fields, a useful feature that allowed magnetic sedimentation and separation from non-encapsulated material. Six-fold repetition of magnetic sedimentation, supernatant removal (60–80% of the total volume), and re-suspension in buffer could remove 99.5% of non-encapsulated material.

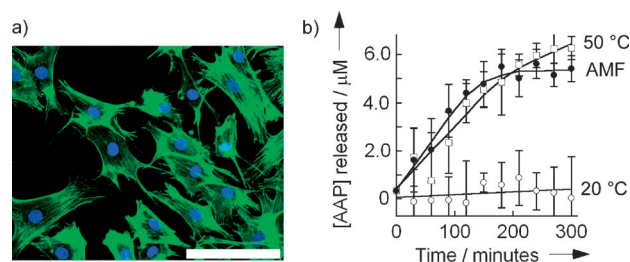
To obtain robust biomaterials, these MNPVs were immobilized in a calcium alginate matrix. This hydrogel allows these smart biomaterials to be compatible with many cell types, particularly chondrocytes,<sup>[19]</sup> while being easy to form and manipulate at physiological temperatures. Purified MNPVs were magnetically sedimented then re-suspended in 2% wt/vol sodium alginate in phosphate buffered saline (PBS). The mixture was then gelled by infusion of  $\text{CaCl}_2$  (0.1M) through a polycarbonate membrane; adding cells prior to gelation produced materials with cells and MNPVs co-immobilized. Confocal microscopy showed cells surrounded by the smaller MNPVs (Figure 2c); adhesive interactions between cells and MNPVs occur if they are co-incubated for > 4 h before gelation.<sup>[20]</sup>

The efficiency of magnetic release was quantified using encapsulated 5/6-CF. Fluorescence spectroscopy indicated little release of 5/6-CF (0.05M inside the vesicles) occurred during gelation and the MNPVs largely remained intact. However 5/6-CF rapidly escaped from a gel block (25 mm<sup>3</sup>) after exposure to a 392 kHz AMF pulse (240 s) (Figure 2d). The amount of 5/6-CF that escaped from the gel was quantified after 2 h, which gave 1.3  $\mu\text{M}$  5/6-CF in the solution covering the gel block (2 mL); without an AMF only 35% escaped after 3 days. The corresponding concentration within

the hydrogel after magnetic release but prior to diffusion out of the gel block was calculated as ca. 100  $\mu\text{M}$ , which should allow biochemicals with micromolar  $K_d$  values to induce cellular responses.

Biomaterials composed of chondrocytes encased within calcium alginate have shown promise as engineered scaffolds to replace articular cartilage,<sup>[21]</sup> with the cells producing cartilage markers such as glycosaminoglycans and collagen II.<sup>[22]</sup> For the production of extracellular collagen by chondrocytes and osteoblasts, which normally occurs over several days, ascorbic acid is an essential additive.<sup>[23]</sup> Therefore either ascorbate (150–300  $\mu\text{M}$ )<sup>[24]</sup> or ascorbic acid-2-phosphate (AAP, a polar and air-insensitive precursor that is readily converted to ascorbate by cells) are typically added to chondrocyte cell culture media.

The response of chondrocytes to AAP was the ideal model system to illustrate magnetically-induced changes in cellular behavior. Chondrocytes show good tolerance for calcium alginate matrices, with the inert 3D alginate matrix maintaining their rounded morphology and therefore their phenotype.<sup>[22]</sup> Furthermore, adding MNPVs ( $\approx$  20 mM DPPC) did not significantly affect the growth of chondrocytes in the gel (Figure 3a).<sup>[19b]</sup>

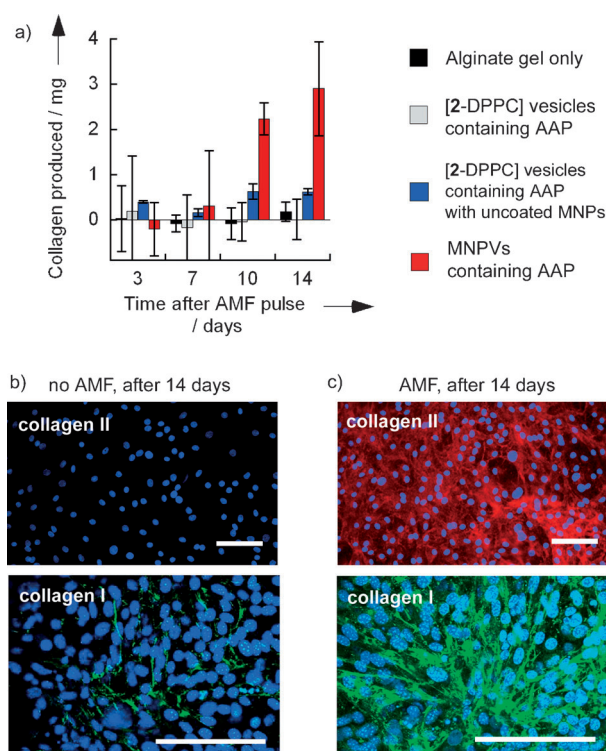


**Figure 3.** a) Confocal microscopy image of chondrocytes and unlabelled MNPVs co-immobilized in alginate (scale bar 100  $\mu\text{m}$ ). b) Ascorbic acid-2-phosphate (AAP) release from MNPVs immobilized in alginate at (○) 20  $^{\circ}\text{C}$  (□) at 20  $^{\circ}\text{C}$  after incubation at 50  $^{\circ}\text{C}$  (4 min) (●) at 20  $^{\circ}\text{C}$  after exposure to an AMF (4 min).

The encapsulation and release of AAP (8.6 mM) from alginate-embedded MNPVs was demonstrated in a similar manner to 5/6-CF. A pulse of AMF (240 s) released encapsulated AAP from a 25 mm<sup>2</sup> gel block, and the concentration of the AAP in the surrounding PBS solution (2 mL) was determined by phosphatase hydrolysis followed by titration with triiodide (Figure 3b).<sup>[25]</sup> This showed that the concentration in the gel block after release was ca. 400  $\mu\text{M}$ , which should be high enough to induce a strong cellular response. The release rate of anionic AAP<sup>[26]</sup> at pH 7.4 was akin to that observed for 5/6-CF, with 40% release after 0.5 h and 80% release after 1.5 h (compared to incubation at 50  $^{\circ}\text{C}$ ). This suggests that the passage of bioactive anions through calcium alginate is not significantly impeded by the hydrogel matrix.

To measure the response of chondrocytes cultured in these AAP-loaded MNPV hydrogels to an AMF, alginate gels containing chondrocytes (25000 cells per 500 mm<sup>3</sup>) and MNPVs ( $\approx$  20 mM DPPC) were fabricated in glass vials, while control hydrogels containing [2-DPPC] vesicles with

stored AAP but no 1-MNP were also formed. Media (1 mL) was added to cover the gel and the samples incubated at 37 °C. Collagen production was measured at regular intervals over 14 days using the Sircol collagen assay. After an initial lag period following application of the AMF, the chondrocytes in the gel responded to the magnetic release of AAP by producing collagen at levels comparable to literature studies (up to 6 mg mL<sup>-1</sup>, Figure 4a).<sup>[27]</sup> Little cell response was observed with uncoated MNP and [2-DPPC], showing an adhesive link between both components of the MNPVs is needed to produce a cellular response to the AMF.



**Figure 4.** a) AMF-induced production of collagen by chondrocytes: (■) calcium alginate only; (■) AAP-containing [2-DPPC] vesicles in calcium alginate; (■) AAP-containing [2-DPPC] vesicles with uncoated MNP in calcium alginate; (■) AAP-containing MNPVs in calcium alginate. b,c) Confocal microscopy images of alginate gels containing chondrocytes and MNPVs, with fluorescent staining of cell nuclei (blue). Top: stained for collagen II (stained red with tetramethyl rhodamine isothiocyanate (TRITC)–secondary antibody). Bottom: stained for collagen I (stained green with FITC–secondary antibody).

The production of collagen by chondrocytes in response to the magnetic signal was visualized after 14 days using fluorescence microscopy on gels containing AAP-loaded MNPVs. Little collagen was produced in samples that had not been exposed to an AMF (Figure 4b). However, large amounts of extracellular collagen I and collagen II were produced in samples exposed to an AMF, which formed a mesh around the chondrocytes (Figure 4c). This response shows the potential of these 3D cell culture scaffolds for non-invasive production of cartilage-like extracellular matrices.<sup>[28]</sup>

In summary, we have developed a self-assembled and biomimetic biomaterial that responds to a magnetic signal by

releasing the contents of phospholipid vesicles. Crosslinking thermally-sensitive DPPC vesicles with magnetically-responsive Fe<sub>3</sub>O<sub>4</sub> nanoparticles allowed remote magnetic release of the vesicle contents, which occurred at low loadings of Fe<sub>3</sub>O<sub>4</sub> and did not otherwise affect cells within the material. An alginate gel was the synthetic extracellular matrix around the MNPVs, producing robust materials suitable for cell culture. We found that encapsulating polar compounds at ca. 10 mM within the vesicles gave sufficient concentrations after magnetic release to generate cellular responses. The exciting potential of these materials was exemplified using magnetic release of AAP, which switched on collagen production by chondrocytes and added this extracellular matrix protein to the alginate scaffold. The transparency of tissue to magnetic fields means that cellular responses could be initiated non-invasively in vivo, as recently illustrated with in vivo magnetic release of cells from ferrogel-based scaffolds.<sup>[29]</sup> Given external magnetic fields can pattern MNPVs within these materials, we hope in future to initiate cellular responses that are both spatially and temporally controlled.

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