Electronic Supplementary Information
for

Magnetically deliverable calcium phosphate nanoparticles for localized gene expression

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Nanoparticle characterization

Fe_2O_3@TCP nanoparticles were analyzed by nitrogen adsorption according to the Brunauer-Emmett-Teller method (BET; Tristar, Micromeritics), after degassing the nanoparticles at 150°C for 1 h. X-ray diffraction data were acquired using a X'Pert Pro-MPD diffractometer (PANalytical), by exposing the nanoparticle powder to Cu-Kα radiation (λ = 1.54060 Å), over a range of 10 - 70° 2θ with a step size of 0.05°. Iron oxide and TCP phase identification was carried out by means of the X'Pert software PANalytical High Score Plus. Characterization by transmission electron microscopy (TEM) was performed using a Tecnai F30 TEM (300 kV, field emission gun, FEI). Scanning transmission electron microscopy (STEM) investigations were performed on an aberration-corrected HD-2700CS (cold-field emitter; Hitachi), operated at an acceleration potential of 200 kV. An energy-dispersive X-ray spectrometer (EDXS; Gemini system of EDAX) attached to this microscope allowed the recording of EDX spectra and elemental maps. Magnetic properties were investigated by vibrating sample magnetometry (VSM; Princeton Measurements Corporation, model 3900). Particle hydrodynamic size distribution was measured with a X-ray disk centrifuge (XDC; Brookhaven Instruments, United States), using a 1.5 % (wt/vol) powder suspension in ethanol, ultrasonicated at 200 W for 5 min. Nanoparticle behavior in aqueous media was studied by zeta potential measurements (Zetasizer Nano, Malvern).

Particle density

We estimated material density knowing that the produced composite material is 67 wt% TCP and 33 wt% Fe_2O_3:

$$\rho = \frac{1}{\sum_i \frac{m_i}{\rho_i}}$$

where ρ_i is the density of constituent i (TCP: 3.14 g/cm^3, Fe_2O_3: 5.24 g/cm^3), and m_i the mass fraction. Calculated particle density was 3.6 g/cm^3.

Primary particle size

The average primary particle diameter (d_{BET}) was calculated from the measured specific surface area A_s and the density ρ using:

$$d_{BET} = 6/(A_s \cdot \rho)$$

Obtained particle diameter was 25 nm.

Particle size dispersity

Particle size distribution data obtained by XDC were fitted with a lognormal distribution (Fig. S2) to estimate the mean diameter and the geometric standard deviation (GSD). We obtained a mean diameter of 121 nm and a GSD of 1.2, indicating a sufficiently monodisperse distribution.

Number of particles per aggregate

Average number of primary particles per aggregate (n_p) was calculated using the following relationship:

$$n_p = \left(\frac{d_c}{d_p}\right)^D$$

using d_{BET} as primary particle diameter (d_p), mean hydrodynamic size measured by XDC as collision diameter (d_c), and a constant D of 1.8 [1].

1. T. J. Brunner, P. Wick, P. Manser, P. Spohn, R. N. Grass, L. K. Limbach, A. Bruinink and W. J. Stark, *Environ Sci Technol*, 2006, **40**, 4374.

Preparation and transformation of chemically competent *E. coli*

Chemically induced competence followed by transformation was used to introduce plasmids into *E. coli*. 5 ml of LB medium were inoculated with E. coli (DH5α strain*)* and incubated with shaking (250 rpm) overnight at 37°C. 2 mL of the culture were added to 200 mL LB medium and incubated until the absorbance at 600 nm was between 0.4 and 0.6. The culture was chilled for 15 min on ice and then centrifuged (4000 rpm) for 10 min at 4°C. The pellet was resuspended in 100 mL of sterile ice-cold 0.1 M $CaCl_2$. The bacteria suspension was incubated on ice for 15 min before centrifuging again (4000 rpm) for 10 min at 4°C. The pellet was resuspended in 1 mL volume of ice-cold 0.1 M $CaCl_2$/15% glycerol. The bacteria suspension was then aliquoted (50 µL) and stored at -80°.
To transform the competent *E. coli*, an aliquot was mixed with 100 ng of plasmid DNA and incubated on ice for 15 min. The *E. coli* were then heat shocked for 30 s at 42°C in a thermomixer and put back on ice for 2 min. 500 µL of LB media was added and the suspension was incubated for 1 h at 37°C with shaking (1000 rpm). The bacteria were then plated on a LB agar plate supplemented with Kanamycin, and incubated overnight at 37°C.

DNA binding assay

DNA (2 µg) was mixed with 5 µL 2M $CaCl_2$, particles (8 µg) and water to a final volume of 100 µL. After incubation for 15 min at room temperature and magnetic separation of the particles, the unbound DNA in the supernatant was quantified by Qubit fluorometer (Invitrogen) using Qubit dsDNA HS assay (Invitrogen, cat. no. Q32851). Excess DNA not bound to Fe_2O_3@TCP particles was also visualized on agarose gel (Fig S3). Before loading the DNA solution in the gel, it was dialysed (Millipore 0.025µm VSWP) against water to remove the salt.

Particle long-term storage

Fe_2O_3@TCP nanoparticles can be stored as prepared for long periods. The dry storage does not affect particle size and ability to bind pDNA: both quantities were measured again after storage at ambient temperature for 8 months, attaining the same results previously obtained (*i.e.* a BET particle diameter of 26 nm - BET gives an error ~5% - , and a binding capacity of 0.02 µg DNA/µg particles, as measured by Qubit fluorometer and visualized on the gel in figure S3). An additional proof that particle size is not affected by long-term storage is that a BET particle diameter of 26 nm was measured after sterilization for 30 min at 200°C, a process equivalent to storage at room-temperature for about 10 years according to ASTM F1980 (Accelerated Aging of Sterile Medical Device Packages, Q10=2).

Microscopy

Cells were imaged with a Zeiss Observer microscope (Germany). Cell morphology was observed under bright field (96-well plates) or phase contrast (Petri dishes), while live/dead and transfected cells were identified by fluorescence microscopy using DAPI, FITC and DsRed filter sets. The same exposure times were used for all the conditions and images are shown with minor adjustments (color display, as well as background substraction from a reference image and assembly of tiles for petri dish fluorescence imaging, using Fiji).

Live/dead assay

Simultaneous determination of live and dead cells was performed by labeling live cells with Hoechst stain and dead cells with Ethidium homodimer-1 (EthD-1). Cells were incubated for 1 h in medium supplemented with 10 µg/mL Hoechst and 2 µM EthD-1 (both purchased from Life Technologies).

Cell counting

After staining, cells were counted manually, using Fiji, over an area of 2.4 mm^2 (*i.e.* four times the area illustrated in Fig. S4), with blue nuclei giving the total number of cells, red nuclei giving the number of dead cells, and green cytoplasm/nuclei showing successful transfection. Cell counting data are included in table S1.

Statistical analysis

Data are provided as mean value ± standard deviation. Experiments were performed in triplicates. Statistical analyses were performed with OriginPro 8.6. Data were analyzed by one-way analysis of variance (ANOVA), using Bonferroni test for *post hoc* analysis.

Table S1. Cell counting data.

Cell density:	**312 cells/mm²**			**625 cells/mm²**			**937 cells/mm²**		
	Blue	**Red**	**Green**	**Blue**	**Red**	**Green**	**Blue**	**Red**	**Green**
Fe_2O_3@TCP ($CaCl_2$ +)	3887	110	1294	2088	178	576	2662	237	700
	3913	133	1248	4271	269	1200	5874	217	1582
	3262	92	1255	4567	174	1277	4392	244	1278
Fe_2O_3@TCP ($CaCl_2$ ++)	3914	62	1288	6396	247	1731	7022	203	1759
	3756	93	1305	5623	271	1322	7328	217	1731
	3278	58	1034	5520	243	1348	7659	145	1799
PolyMAG	1075	373	240	2492	1111	586	3468	1180	933
	1007	311	197	2228	1344	660	3378	1322	837
	845	250	137	1624	1126	414	2267	1056	633
NeuroMag	354	-	227	1559	-	932	1670	-	1236
	568	-	378	2485	-	1511	2869	-	2186
	684	-	537	2303	-	1360	2637	-	1995
CaP	1110	74	864	4138	362	1811	6552	291	1530
	1538	84	948	3746	442	1514	6152	423	1754
	1287	122	964	1945	284	753	6565	251	1871
PEI	5169	39	1544	7638	28	1382	7911	28	1628
	5135	57	1798	6763	39	1799	8195	39	1901
	6941	16	2224	6618	22	1724	7388	16	1570
Neg. ctr.	6709	1	0	8159	13	0	8366	8	0
	6620	4	0	8020	5	0	8565	5	0
	6799	1	0	8054	6	0	8794	4	0

Table S2. Results of one-way ANOVA followed by Bonferroni post-test, comparing transfection, proliferation, and viability mean values obtained with Fe_2O_3@TCP particles (100 mM $CaCl_2$) and the other reagents.

Cell density:	**312 cells/mm²**		**625 cells/mm²**		**937 cells/mm²**	
Transfection						
	Prob.	**Sig.**	**Prob.**	**Sig.**	**Prob.**	**Sig.**
Fe_2O_3@TCP vs. PolyMAG	1E-1	NS*	1	NS	1	NS
Fe_2O_3@TCP vs. NeuroMag	1E-4	S**	5E-7	S	7E-11	S
Fe_2O_3@TCP vs. CaP	7E-5	S	6E-4	S	8E-1	NS
Fe_2O_3@TCP vs. PEI	1	NS	1	NS	1	NS
Proliferation						
	Prob.	**Sig.**	**Prob.**	**Sig.**	**Prob.**	**Sig.**
Fe_2O_3@TCP vs. PolyMAG	7E-04	S	5E-04	S	7E-06	S
Fe_2O_3@TCP vs. NeuroMag	2E-04	S	6E-04	S	2E-06	S
Fe_2O_3@TCP vs. CaP	2E-03	S	1E-2	S	4E-1	NS
Fe_2O_3@TCP vs. PEI	5E-03	S	6E-1	NS	1	NS
Viability						
	Prob.	**Sig.**	**Prob.**	**Sig.**	**Prob.**	**Sig.**
Fe_2O_3@TCP vs. PolyMAG	1E-7	S	4E-6	S	6E-7	S
Fe_2O_3@TCP vs. NeuroMag	-	-	-	-	-	-
Fe_2O_3@TCP vs. CaP	3E-2	S	2E-1	NS	9E-1	NS
Fe_2O_3@TCP vs. PEI	1	NS	9E-1	NS	1	NS

*NS = the means difference is non significant at the 0.05 level

**S = the means difference is significant at the 0.05 level

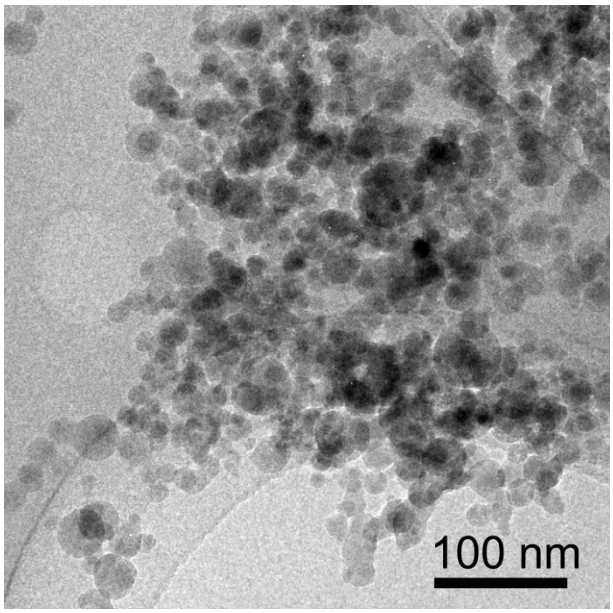

Figure S1. TEM micrograph of Fe_2O_3@TCP nanoparticles.

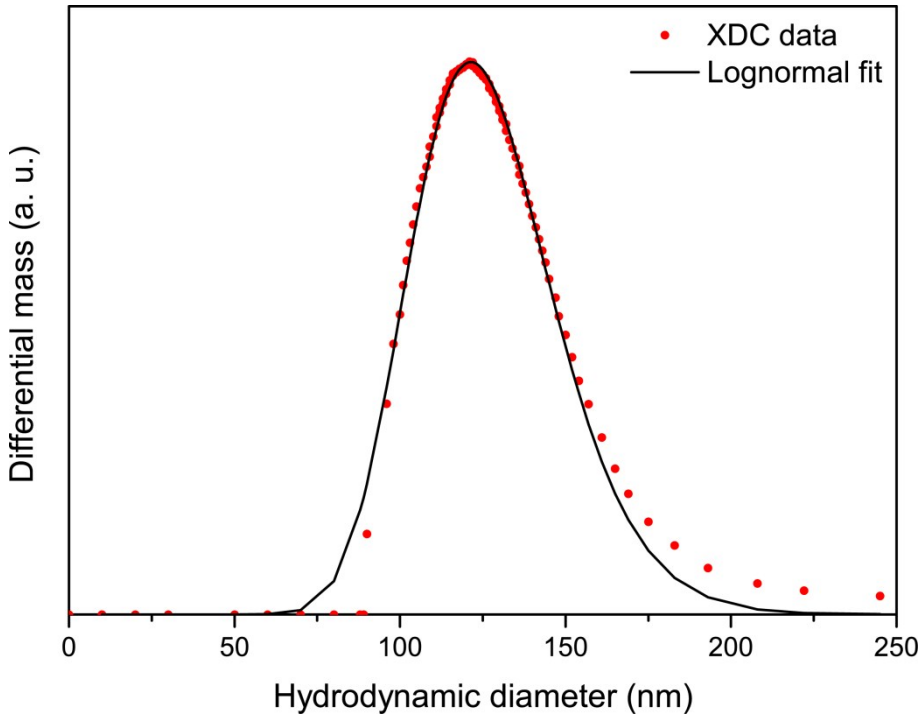

Figure S2. Hydrodynamic size distribution of Fe_2O_3@TCP particles.

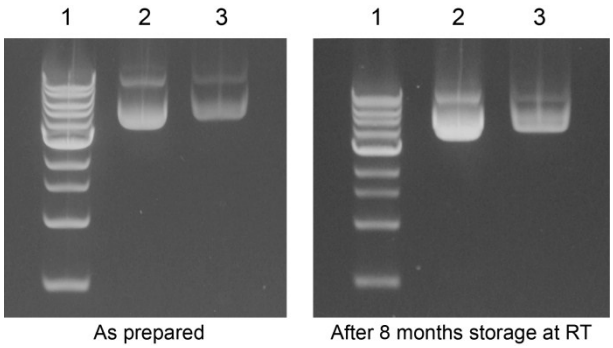

Figure S3 Gel electrophoresis showing DNA Ladder (1), control DNA solution (2) not treated with nanoparticles, and residual DNA in the supernatant treated with nanoparticles (3) as prepared (left) and after storage for 8 months at room temperature (right).

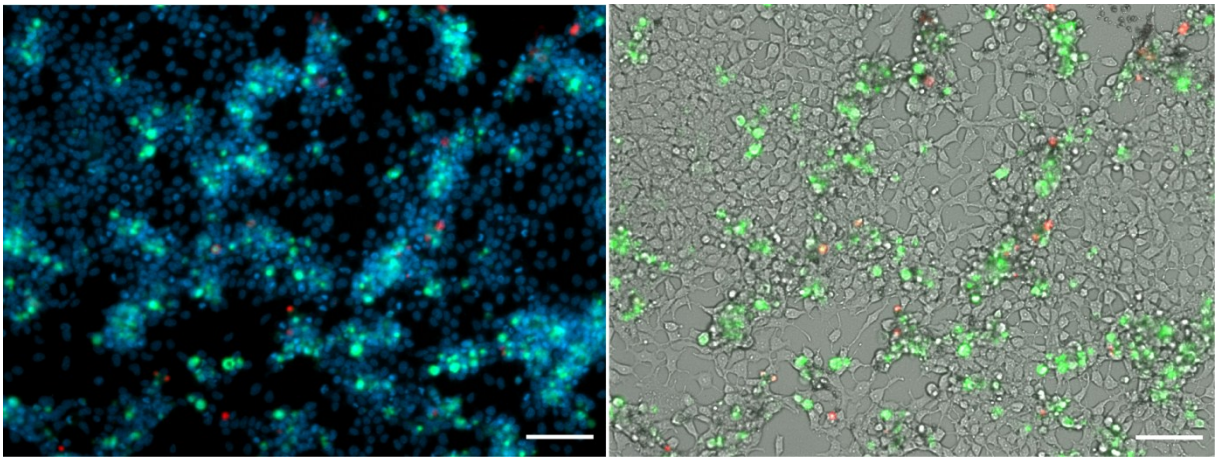

Figure S4 Fluorescence microscopy picture (left) showing Hoechst stained cells (blue), EthD-1 stained cells (red), and cells expressing GFP (green); merged image (right) of green, red, and bright field, showing cell morphology. Scale bar: 100 µm.