Supplementary Information

Biocompatible dextran-coated gadolinium-doped cerium oxide nanoparticles as MRI contrast agents with high T1 relaxivity and selective cytotoxicity to cancer cells

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Methods S1

MTT-assay

The activity of mitochondrial and cytoplasmic dehydrogenases in living cells was analysed using MTT assay, which is based on the reduction of a colourless tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT). A standard MTT assay was performed 24 h after the incubation of cells with $Ce_{0.9}Gd_{0.1}O_{1.95}$ nanoparticles.

Live/dead assay

Live/Dead Viability Kit (Invitrogen, Life Technologies) was used to evaluate the cytotoxic effects of $Ce_{0.9}Gd_{0.1}O_{1.95}$ nanoparticles. The kit contained two fluorescent dyes for selective cell labelling. The green signal (SYTO 9 dye, λ = 485/498 nm) is characteristic of live cells and the red signal (propidium iodide dye, λ = 535/617 nm) is characteristic of dead cells. Cells were seeded in 96-well plates and a kit was used according to the manufacturer's protocol. Cells were visualised and photographed 25 minutes after adding the dyes, using an Axiovert 200 fluorescence-light microscope (Carl Zeiss, Germany) and a Canon A620 digital camera (Canon, USA). For each cell group, four fields in each well were examined.

Intracellular localisation of nanoparticles

The intracellular localisation of nanoparticles was studied by fluorescence microscopy with an Axiovert 200 Cell Observer Microscope (Carl Zeiss, Germany) equipped with a 63× oil immersion objective. Cells were seeded in a 35 mm imaging dish with a polymer coverslip bottom (Ibidi, Germany) at a density of 3×10^4 per cm^2, and then left overnight. After that, FITC-labelled $Ce_{0.9}Gd_{0.1}O_{1.95}$ nanoparticles (100 μg/ml) were added. After 24 h incubation of the cells with nanoparticles, the cells were washed twice with PBS and stained using Hoechst 33342 (Hoechst, Germany) and LysoTracker Red DND-99 (Invitrogen), to visualise nuclei and lysosomes, respectively.

Comet-assay

The genotoxic property of $Ce_{0.9}Gd_{0.1}O_{1.95}$ nanoparticles was studied using the DNA-comet assay. This method is based on the registration of DNA fragments' mobility in a constant electric field, using agarose gel. The analysis of DNA damage in cells was carried out using the alkaline version of the comet assay [1]. The microscopic slides were made of two layers of 1% agarose gel (Sigma, type II, USA). The cell suspension (cell concentration was determined in the Goryaev chamber and was equal to 10^6 cell/cm^3) was introduced into low-melting agarose gel (Sigma, type VIIa) prepared in phosphate-buffered saline (PBS) solution at 37°C. After gel hardening, the lysis was performed for 60 minutes at 4°C in a special buffer (10 mM Tris-HCl (pH 10), 2.5 M NaCl, 100 mM EDTA-Na, 1% Triton-X-100). Then, the samples were subjected to alkaline denaturation (20 minutes, 4°C; 0.3 M NaOH, 1мM EDTA-Na, pH 13). The horizontal electrophoresis was performed under a thin layer (2-3 mm) of the same buffer at an electric field density of 1 V/cm for 20 minutes. The slides that were prepared were washed twice with distilled water, stained with ethidium bromide for 1 h at 4°C, in the dark, and finally washed in distilled water (2-3 times). The samples were studied using an Axiovert-200 fluorescent microscope, DNA-comet parameters being analysed using recorded digital images and Open Comet software [2].

Analysis of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was determined by fluorescence microscopy using JC-1 dye. JC-1 accumulates in the mitochondrial membrane in a potential-dependent manner. The high potential of the inner mitochondrial membrane facilitates formation of dye aggregates (J-aggregates) which are characterised by red-shifted excitation and emission (530 nm/590 nm) when compared with that for JC-1 monomers (485 nm/538 nm). Cells were seeded into 96-well tissue culture plates (Greiner) at a density of 5×10^4 cells/well and cultured in a CO_2 incubator at 37°C for 24, 48 and 72 h, with different concentrations of $Ce_{0.9}Gd_{0.1}O_{1.95}$ nanoparticles. The cells were preincubated with 5 μM JC-1 in the PBS, in a CO_2 incubator at 37°C for 30 minutes. At the end of the incubation, cells were stained with 10 μM JC-1 in PBS and, again, incubated at 37°C for 30 minutes. Next, the cells were washed twice by PBS and analysed using an Axiovert 200M inverted fluorescence microscope (Carl Zeiss, Germany) at 200× magnification. MMP were calculated as a ratio of fluorescence measured at 530 nm/590 nm (JC-1 aggregates) to that measured at 485 nm/538 nm (JC-1 monomers) and represented as a percentage of the untreated control.

Detection of intracellular reactive oxygen species (ROS)

The level of intracellular ROS was determined using dichlorodihydrofluorescein (DCF). DCF is a dye that diffuses through the lipid membrane into the cell and is subsequently oxidised by intracellular ROS (preferably by hydrogen peroxide), with the formation of highly fluorescent dichlorofluorescein. Upon treatment of hMSc and MCF-7 cells with $Ce_{0.9}Gd_{0.1}O_{1.95}$ NPs, the medium was aspirated and cells were washed twice with HBSS and incubated in 1 ml of medium without serum. DCF was added at a final concentration of 40 μM. After 20 minutes' incubation at 37°C, the cells were washed once with HBSS and kept in a fresh HBSS. The quantitative analysis of fluorescence was performed using a Tecan 200 PRO plate reader (485 nm/520 nm). The average fluorescence intensity was measured in five random wells, n = 4.

Detection of apoptosis using Yo-Pro-1 dye

To evaluate the number of apoptotic cells formed upon incubation with $Ce_{0.9}Gd_{0.1}O_{1.95}$ nanoparticles, a YO-PRO-1 dye (1 μM) was used. YO-PRO-1 dye has found a niche in identifying apoptotic cells because apoptotic cells are permeable to this dye but not permeable to propidium iodide, which is used for staining dead cells. Cells attached to a 96-well plate were stained according to the manufacturer's protocol and visualised, 25 minutes after adding the dye, using a Zeiss Axiovert 200 fluorescence-light microscope coupled to a Canon A620 digital camera. For each cell group, three fields in each well were examined.

RT-PCR

Reverse transcription was performed with a Sileks kit (Russia), using an oligodT primer according to the manufacturer's protocol. The cDNAs produced served as a real-time PCR matrix. For PCR reaction, a mixture was used with SYBR Green dye (Syntol, Russia). To conduct a PCR-RT analysis, a BioRad CFX-96 amplifier or an Applied Biosystems ABI 7500 Fast Real-Time PCR System was used. The expression of 96 genes responsible for key cell processes under oxidative stress was thus determined (**Table S1**). For PCR profiling, the genes to be analysed were selected from the database http://www.sabiosciences.com/. The level of gene transcription was normalised by the levels of expression of housekeeping genes β-*actin, rplp0* (ribosomal protein, large, P0) and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase). The gene-specific primers were selected using Primer Express software (Applied Biosystems, USA). Each measurement was made twice (internal repetition) and averaged for two independent samples. A sample without a reverse transcription stage was used as the control. The expression data obtained were analysed using online services http://www.sabiosciences.com/, mayday-2.14 software (Center for Bioinformatics, Tübingen, Germany) and Genesis software.

Statistical analysis

Statistical analysis was performed using variation statistics, with GraphPad Prism 6.0 and Microsoft Excel 2007 software. The mean values and the standard deviation of the mean were determined. The significance of differences was assessed using the Mann-Whitney U criterion.

References

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Table S1. Selected gene groups for PCR-RT analysis.

Function	Description	GeneBank	Symbol	Forward 5'-3'	Rewerse 5'-3'
Glutathione Peroxidases (GPx)	Glutathione peroxidase 1	NM_000581	GPX1	CCTCCCCTTACAGTGCTTGTTC	GCACACATGGCGCAATTG
	Glutathione peroxidase 2 (gastrointestinal)	NM_002083	GPX2	CCGATCCCAAGCTCATCATT	TCTCAAAGTTCCAGGCCACAT
	Glutathione peroxidase 3 (plasma)	NM_002084	GPX3	CATCCCCTTCAAGCAGTATGCT	GCCCGTCAGGCCTCAGTAG
	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	NM_002085	GPX4	CCGATACGCTGAGTGTGGTTT	GCTCCTGCTTCCCGAACTG
	Glutathione peroxidase 5 (epididymal androgen-related protein)	NM_001509	GPX5	TCACCACACTCTCTTCCTGCAT	AGAGTGGGAATTCTGGCAGTATG
	Glutathione S-transferase pi 1	NM_000852	GSTP1	CAGGAGGGCTCACTCAAAGC	GTGAGGTCTCCGTCCTGGAA
	Glutathione transferase zeta 1	NM_001513	GSTZ1	CCCAGAACGCCATCACTTG	TGCCCGCTGTGCTCTGT
Peroxiredoxins (TPx)	Peroxiredoxin 1	NM_002574	PRDX1	CTGGGACCCATGAACATTCC	AAGACCCCATAATCCTGAGCAA
	Peroxiredoxin 2	NM_005809	PRDX2	TCCTTCGCCAGATCACTGTTAA	CAGCCGCAGAGCCTCATC
	Peroxiredoxin 3	NM_006793	PRDX3	GCATTTGAGCGTCAACGATCT	TCACCAAGCGGAGGGTTTC
	Peroxiredoxin 4	NM_006406	PRDX4	GAGGCATCCCGGGTATCG	GGCTTGGAAATCTTCGCTTTG
	Peroxiredoxin 5	NM_181652	PRDX5	AGATGATTCGCTGGTGTCCAT	ACTATGCCATCCTGTACCACCAT
	Peroxiredoxin 6	NM_004905	PRDX6	GGCCGCATCCGTTTCC	CCCGAGGGTGGGAGAAGA
Other Peroxidases	Catalase	NM_001752	CAT	CAGGGCATCAAAAACCTTTCTG	CGGATGCCATAGTCAGGATCTT
	Cytochrome b-245, beta polypeptide	NM_000397	CYBB	CCTTTGAGTGGTTTGCAGATCTG	AGCCGGCATTGTTCCTTTC
	Cytoglobin	NM_134268	CYGB	GCAGCACCTCGAGCAGAAG	CCTTGGCACCCAGAAATGG
	Dual oxidase 1	NM_175940	DUOX1	TGAGCGGCACTTCCAGAAG	GACGGCCAAAGTGGGTGAT
	Dual oxidase 2	NM_014080	DUOX2	CCTTCGAGCCCTTCTTCAACT	CAGCTGAACACCCCGATCTT
	Lactoperoxidase	NM_006151	LPO	CAAGCTTTTCCAGCCAACTCA	CCGGCAACGCTGTGTGT
	Myeloperoxidase	NM_000250	MPO	CCTGAAATTGGCGAGGAAACT	GCCGCCCATCCAGATGT
	Prostaglandin-endoperoxide synthase 1	NM_000962	PTGS1	TGTTCGGTGTCCAGTTCCAATA	TGCCAGTGGTAGAGATGGTTGA
	Prostaglandin-endoperoxide synthase 2	NM_000963	PTGS2	AATTGCTGGCAGGGTTGCT	GGTCAATGGAAGCCTGTGATACTT
Other Antioxidants	Albumin	NM_000477	ALB	TGAGAAAACGCCAGTAAGTGACA	GAAAAGCATGGTCGCCTGTT
	Apolipoprotein E	NM_000041	APOE	CTGCGTTGCTGGTCACATTC	CTCTGTCTCCACCGCTTGCT
	Glutathione reductase	NM_000637	GSR	TGCAGGGACTTGGGTGTGA	GCCTTCGTTGCTCCCATCT
	Metallothionein 3	NM_005954	MT3	AGTGCGAGGGATGCAAATG	GCCTTTGCACACACAGTCCTT
	Sulfiredoxin 1	NM_080725	SRXN1	TGCTGTATCCCCAAGAATCATG	GCTAGTTTGGCCCTTCCTCTTC
	Superoxide dismutase 1, soluble	NM_000454	SOD1	TGGTGTGGCCGATGTGTCT	GTGCGGCCAATGATGCA
	Superoxide dismutase 2, mitochondrial	NM_000636	SOD2	TCCGCAGAAAGGAACATTAAGG	TGACCTCCATTCTTTGCTCTCA
	Superoxide dismutase 3, extracellular	NM_003102	SOD3	GCGGAGCCCAACTCTGACT	TGCCAGATCTCCGTGACCTT
Genes Involved in Reactive Oxygen Species (ROS) Metabolism	Arachidonate 12-lipoxygenase	NM_000697	ALOX12	CCACCCACCACCAAGGAA	TGCCGGACATCAGGTAGTGA
	Nitric oxide synthase 2, inducible	NM_000625	NOS2	CCGCATGACCTTGGTGTTT	TCCAGCATCTCCTCCTGGTAGA
	NADPH oxidase 4	NM_016931	NOX4	AAGAGCCCAGATTCCAAGCTAATT	CGGCACAGTACAGGCACAAA
	NADPH oxidase, EF-hand calcium binding domain 5	NM_024505	NOX5	AGGCACCAGAAAAGAAAGCATACT	ATGTTGTCTTGGACACCTTCGAT
	Uncoupling protein 2 (mitochondrial, proton	NM_003355	UCP2	CAGTTCTACACCAAGGGCTCTGA	CCTGTGGTGCTGCCTGCTA

Function	Description	GeneBank	Symbol	Forward 5'-3'	Rewerse 5'-3'
	carrier)				
	Aldehyde oxidase 1	NM_001159	AOX1	GGTGTTCCGTGTTTTTCGCTAT	GGTCCATGCAGGCCTCTCT
	BCL2/adenovirus E1B 19kDa interacting protein 3	NM_004052	BNIP3	TCCATCTCTGCTGCTCTCTCATT	AGGTTGTCAGACGCCTTCCA
	Epoxide hydrolase 2, cytoplasmic	NM_001979	EPHX2	AACTGGGCCTCTCTCAAGCA	AGCCATGTACCACACCAGCAT
	MpV17 mitochondrial inner membrane protein	NM_002437	MPV17	TCTATGGCCTGCTGTGCAGTT	GGACAACGGCCAACCTGTA
	ATX1 antioxidant protein 1 homolog (yeast)	NM_004045	ATOX1	TGCTTGCAACCCTGAAGAAA	GGACCAGGCCCCTGCTA
	Chemokine (C-C motif) ligand 5	NM_002985	CCL5	TGCATCTGCCTCCCCATATT	AGTGGGCGGGCAATGTAG
	24-dehydrocholesterol reductase	NM_014762	DHCR24	CATGCTGGTGCCCATGAAG	GACGTGGATGTCGTTTTGGAA
	Forkhead box M1	NM_021953	FOXM1	AGGAAACGCTGCCCATCTC	CGTGAGCCTCCAGGATTCAG
	Ferritin, heavy polypeptide 1	NM_002032	FTH1	CTGGCTTGGCGGAATATCTCT	GCCCGAGGCTTAGCTTTCAT
	Glutamate-cysteine ligase, modifier subunit	NM_002061	GCLM	CCGCCTGCGGAAGAAGT	CATTCAAGGTTTTTTGGATACAATCA
	Glutathione synthetase	NM_000178	GSS	GCAGGAAAAGACACTCGTGATG	CATGCTCGATGGCTTTGGT
	Heme oxygenase (decycling) 1	NM_002133	HMOX1	TCCGATGGGTCCTTACACTCA	GCCTGCATTCACATGGCATA
	Heat shock 70kDa protein 1A	NM_005345	HSPA1A	GCTGATTGGCCGCAAGTT	TGGAAAGGCCAGTGCTTCAT
	Mannose-binding lectin (protein C) 2, soluble	NM_000242	MBL2	AGTGAAGGCCTTGTGTGTCAAGT	TCCATTCTCTGCAGCATTCCT
	NAD(P)H dehydrogenase, quinone 1	NM_000903	NQO1	CAGCAGACGCCCGAATTC	TGGTGTCTCATCCCAAATATTCTC
	Ring finger protein 7	NM_014245	RNF7	AAAGGAAAGAGCTCCAAATTGAATC	CATAAGCATGCAAAAAGTTCTCTGA
	Sirtuin 2	NM_012237	SIRT2	GCTGGAACAGGAGGACTTGGT	TGGCGCTGACGCAGTGT
	Sequestosome 1	NM_003900	SQSTM1	GGAAGGTGAAACACGGACACTT	ACGTGGGCTCCAGTTTCCT
Pathway Activity Signature Genes	Aldo-keto reductase family 1	NM_001354	AKR1C2	GATTGCCCTGCGCTACCA	TGTCTGATGCGCTGCTCATT
	BCL2-associated athanogene 2	NM_004282	BAG2	CTCACCGTTGAAGTGTCAGTAGAAA	ATCAATAATCCTTGTGGCATGCT
	Four and a half LIM domains 2	NM_001450	FHL2	CCTGCAGGAAGCAGCTGTCT	AGTTCAGGCAGTAGGCAAAGTCA
	Galactosidase, alpha	NM_000169	GLA	GGATGGCTCCCCAAAGAGAT	GGCGAATCCCATGAGGAAA
	Heat shock protein 90kDa alpha (cytosolic), class A member 1	NM_001017963	HSP90AA1	TTGGCAGTGAAGCATTTTTCAG	GAGCACGTCGTGGGACAAA
	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	NM_022126	LHPP	TGCGCACCGGGAAGTT	CACGTACCCATCAGCCTTCA
	Trafficking protein particle complex 6A	NM_024108	TRAPPC6A	GGTGTTCCAGAAGCAGATGGA	AGCTGTTGTCTTGCAGGACGTA
Mitochondrial dysfunction	Mitochondrial ribosomal protein L43	NM_176794	MRPL43	CAGTTGCACCGCAGATCCT	GGAAGATCGGATGACTGAACTGA
	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3kDa	NM_019056	NDUFB11	GCAGCACCTTTGTGGCCTAT	TCCCATCCCACGCTCTTG
	Polymerase (RNA) mitochondrial (DNA directed)	NM_005035	POLRMT	CACAGGTGCTGGAAGGTTTCA	CCGTACACCACCGTCATCAC
	Sirtuin 1	NM_012238	SIRT1	TGAGCCTGATGTTCCAGAGAGA	AGCTTCATTAATTGCCTCTTGATCAT
	Sirtuin 3	NM_012239	SIRT3	CCAGTGGCATTCCAGACTTCA	GATCGTACTGCTGGAGGTTGCT
	Transcription factor B1, mitochondrial	NM_016020	TFB1M	GCCATCGAGGGCTCAGAA	CAGCCTGCCCGTGCTTT
	Transcription factor B2, mitochondrial	NM_022366	TFB2M	AAGGCGTCTAAGGCCAGCTT	TTTGCGCCAGGGTCTCA
	Copper chaperone for superoxide dismutase	NM_005125	CCS	GCCGCGCCATCTTCAG	ATCAGGCTGCGGCCAAT

Function	Description	GeneBank	Symbol	Forward 5'-3'	Rewerse 5'-3'
	Selenoprotein P, plasma, 1	NM_203472	SELENOS	CTGAAACGGAAATCGGACAGA	CGCCTCCTTCACCAGACAAC
Anti Apoptotic	B-cell CLL/lymphoma 2	NM_000633.2	BCL2	CTGGGATGCCTTTGTGGAACT	AGACAGCCAGGAGAAATCAAACAG
	aculoviral IAP repeat containing 3	NM_001165.4	BIRC3	GGACAGGAGTTCATCCGTCAAG	TCTCCTGGGCTGTCTGATGTG
	myeloid cell leukemia 1	NM_021960.4	MCL1	CACGAGACGGCCTTCCAA	CACTCGAGACAACGATTTCACATC
	TNF receptor-associated factor 2	NM_021138.3	TRAF2	GGCCGTCTGTCCCAGTGAT	TTCGTGGCAGCTCTCGTATTC
Autophagy	autophagy related 3	NM_022488.4	ATG3	CCATTGAAAATCACCCTCATCTG	CACCTCAGCATGCCTGCAT
	autophagy related 12	NM_004707.3	ATG12	CCCGGGAACAGAGGAACCT	GGAGTGTCTCCCACAGCCTTT
	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_003998.3	NFKB1	GGCTACACCGAAGCAATTGAAG	CAGCGAGTGGGCCTGAGA
	ribosomal protein S6 kinase, 70kDa, polypeptide 1	NM_003161.3	RPS6KB1	TGGCATAGAGCAGATGGATGTG	AGAGTTCGGCTGTCGTATTGGA
Necrosis	coiled-coil domain containing 103	NM_213607.2	CCDC103	GCTGCAAGGGCTTGTTTCAG	GCCCCTCCTTCACGGATCT
	forkhead box I1	NM_012188.4	FOXI1	CGCCTCACTCTCAGCCAGAT	CCGGCCTTGCTCTTGTTGTA
	junctophilin 3	NM_020655.3	JPH3	CCAGGATCACTGCCAAAGAGTT	CGCTTCGGCCTCTGGTACT
	RAB25, member RAS oncogene family	NM_020387.2	RAB25	TGTCTTCAAGGTGGTGCTGATC	CGCGTGAATCGGGAGAGTAG
Pro apoptotic	BCL2-associated X protein	NM_004324.3	BAX	GTGGCAGCTGACATGTTTTCTG	GCAAAGTAGAAAAGGGCGACAA
	CD40 molecule, TNF receptor superfamily member 5	NM_001250.4	CD40	ACACTGCCACCAGCACAAATACT	CTGTTTCTGAGGTGCCCTTCTG
	CASP8 and FADD-like apoptosis regulator	NM_003879.5	CFLAR	GTGTGTATGGTGTGGATCAGACTCA	GGCATGAATCTCCCATGAACA
	Fas cell surface death receptor	NM_000043.4	FAS	GAATCATCAAGGAATGCACACTCA	AAAGCCACCCCAAGTTAGATCTG
	Tumor necrosis factor receptor superfamily, member 10a	NM_003844.3	TNFRSF1	CTGGCGCTTGGGTCTCCTA	TGCGTTGCTCAGAATCTCGTT
House keeping	glyceraldehyde-3-phosphate dehydrogenase	NM_002046.5	GAPDH	GTGGAAGGACTCATGACCACAGT	GCCATCACGCCACAGTTTC
	ribosomal protein, large, P0	NM_001002.3	RPLP0	ATGCAGCAGATCCGCATGT	TTGCGCATCATGGTGTTCTT
	beta-actin	XM_006715764.1	Actin	TCGTGCGTGACATTAAGGAGAA	AGCAGCCGTGGCCATCT