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Enhancement of nucleic acid delivery to hard-to-transfect human colorectal cancer cells by magnetofection at laminin coated substrates and promotion of the endosomal/lysosomal escape

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Despite a great diversity of nanomaterials, such as cationic lipid, polymers or inorganic nanoparticles, has been developed in order to carry nucleic acids across plasma membrane, these methodologies have still insufficient efficacy in cells named hard-to-transfect cells, as the colorectal HT29 and Caco-2 cell lines. This paper describes the improvement of plasmid DNA (pDNA) and small interfering RNA (siRNA) transfer in these cells through the combination of magnetofection, a simplified extracellular matrix of laminin and endosomal/lysosomal escape promotion using the endosome-disruptive peptide INF-7. Magnetofection of pDNA complexes using selected vector formulations resulted in up to 2-fold enhancement in luciferase expression, as compared to lipofection. Further enhance in pDNA transfer in HT29 cells was obtained when magnetofection was applied on cells grown on laminin coated substrates, increasing 6-fold the luciferase expression compared to lipofection at uncoated substrates. This technique was also applied to siRNA delivery in cells expressing stably luciferase (Caco-2Luc and HT29Luc), selected magnetic vector formulations resulted in 61±5% and 50±5% of luciferase silencing in HT29Luc and Caco-2Luc, respectively. Further improvement in reporter gene silencing was obtained when the magnetic complexes were modified with INF-7, reaching more than 95% of luciferase silencing in Caco-2Luc cells, while pre-treatment of HT29Luc cells by chloroquine resulted in 80±4% of down regulation of luciferase expression. Thus, magnetofection applied on cells grown over laminin coated substrates and the optimization of endosomal escape of magnetic complexes would be a good alternative to enhance nucleic acid transfer in hard-to-transfect colorectal cancer cells.

Introduction

Gene down regulation and gene overexpression are potent tools to study the role of certain gene in cellular process and, in these sense, efficient nucleic acid delivery into cells is a prerequisite to obtain optimal results. A great diversity of synthetic nanomaterials, such as those formulated from cationic lipids, polymers or inorganic nanoparticles has been developed in order to carry nucleic acids across plasma membrane. However, these methods has still poor efficiency,[1,2] especially in some types of cells named hard-to-transfect cells, like primary cells, leukemia cells[3] and certain cancer cells lines as the colorectal HT29 cells.[4,5] Particularly, the HT29 cell line is a very interesting model of study due to its known resistance to chemotherapeutic and radiation treatment,[6-8] however this cell line is very refractory to nucleic acid transfer.[4,5] Therefore, the generation of efficient methods to deliver nucleic acids would allow to study cellular process in the adequate models not limiting the studies to the easy-to-transfect cells.

The combination of different nanomaterials could improve nucleic acid cell transfer in order to obtain efficient alternatives as gene transfer tools. An example of such approach is magnetofection, that can be defined as a method for nucleic acid delivery under the influence of magnetic field acting on nucleic acid vectors that are associated with magnetic nanoparticles and comprise an enhancer such as cationic lipids.[9] This technology allows overcome the diffusion limitation, transfection is synchronized and the vector dose requirement for efficient transfection is considerably reduced.[10] Despite magnetic particles used in magnetofection are nanosized, the magnetic complexes usually form aggregates with sizes of several hundred nanometers to microns.[11-13] For *in vitro* applications the influence of sizes of transfection complexes is still controversial,[14-17] however large size complexes would decrease the transfection efficiency *in vivo* applications. The main reason is the fast clearance by the reticuloendothelial system of large particles,[18] in this regard some groups have developed magnetic nanosized formulations as efficient delivery system for *in vitro* and *in vivo* applications.[19,20] The potential of magnetofection to efficiently deliver nucleic acid *in vitro* has been emphasized by numerous recent scientific publications, for example in primary human gastric myofibroblasts,[21] in COS7 (monkey kidney) cell line[22] and, in suspension cells Jurkat (Human T cell leukemia),[12] however magnetofection method have not been reported in colorectal cancer cells.

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Even though magnetofection has improved gene delivery over standard transfection methods, other aspects of gene delivery process could be considered to further enhance this technique. In this sense, the influence of the extracellular matrix microenvironment has not been explored in order to arrive at optimal efficiencies in gene cellular transfer by magnetofection. It have been demonstrated that specific components of extracellular matrix were able to improve gene transfer by lipofection in some cells types through the stimulation of specific internalization pathways.[23] For example, gene delivery in NIH/3T3 fibroblast was enhanced when the cells were plated on fibronectin coated substrates compared to other ECM protein coating[24] and gene transfer in PC12 cells was enhanced in cells grown over collagen IV coating compared to collagen I, laminin, fibronectin, or polylysine.[25] Particularly, laminins are the most abundant glycoproteins present in basement membranes in the human epithelium and therefore in colorectal cancer tumors originating from glandular epithelium.[26] Hereby we have selected laminin to evaluate the influence of the extracellular microenvironment in the magnetofection process in hard-to-transfect colorectal cancer cells.

Endosomal/lisosomal escape of magnetic complexes is another aspect that should be taken account in order to optimize nucleic acid intracellular delivery. The addition of peptides that can disrupt membranes in an acidic cellular microenvironment such as INF-7 peptide derived from the influenza virus[27] have substantially improved the activity of several non-viral vectors.[27,28] This approach is based on the fact that these peptides are pH-sensitive molecules and can shift from inactive state to a membrane-disruptive state when the pH of endocytic organelles changes from neutral to acidic during endosome maturation.[29]

In this study we not only reported the improvement of nucleic acid transfer in hard-to-transfect colorectal cancer cells by magnetofection but also the optimization of this technique by cultivation of cells over laminin coated substrate as simplified extracellular matrix, and by the modification of magnetic complexes with the INF-7 fusogenic peptide or by cells pre-treatment with a lysosomotropic agent such as chloroquine.

Experimental

Reagents

Polyethyleneimine 25-kD branched (PEI), Pluronic F-127, 1,9-Nonanedithiol, fluorinated surfactant ZONYL FSA, tetraethyl orthosilicate 3-(trihydroxysilyl) propylmethyl-phosphonate (TEOS), 3-(trihydroxysilyl) propylmethyl-phosphonate 50 wt % in water (THPMP), laminin and methylthiazoyldiphenyl-tetrazolium (MTT) were purchased from Sigma-Aldrich (Schnelldorf, Germany). D-luciferin and liposomal transfection reagent X-TremesiRNAReagent (X-Treme) was obtained from Roche Diagnostics. The luciferase reporter plasmid p55pCMV-IVS-luc+ containing firefly luciferase cDNA under the control of the cytomegalovirus (CMV) promoter was purchased from PlasmidFactory (Bielefeld, Germany). Small interfering RNA (siRNAs) against luciferase was purchased from Ambion (Kaufungen, Germany). Liposomal transfection reagent DreamFectGold (DFGold) was acquired from OZ Biosciences (Marseille, France), HiPerfect from Quiagen (Hilden, Germany) and FugeneHD from Promega (Mannheim, Germany). Endosome-disruptive peptide INF-7 was synthesized and purified as described.[27]

Magnetic nanoparticles

Core/shell-type iron oxide magnetic nanoparticles (MNPs) were synthesized by precipitating Fe(II)/Fe(III) hydroxide from an aqueous salt solution, followed by transformation into magnetite in an oxygen-free atmosphere with immediate spontaneous adsorption of the shell components, as described elsewhere.[30] PEI-Mag2 MNPs have a surface coating composed by the fluorinated surfactant ZONYL FSA (lithium 3-[2-(perfluoroalkyl)ethylthio] propionate) combined with PEI, and SOMag5 MNPs have a surface coating formulated of TEOS and THPMP.[31]

The mean magnetite crystallite size (<d>) was calculated from the broadening of the X-ray diffraction peaks using the Scherrer formula.[32] The mean hydrodynamic diameter and the zeta (ξ) potential of the MNP suspension in water were determined by Electrophoretic Light Scattering (ELS) and Dynamic Light Scattering (DLS), respectively, using a Malvern 3000 HS Zetasizer (UK). Size and morphological aspects were observed by transmission electron microscopy using a Philips CM200 (Philips, The Netherlands) transmission electron microscope with EDAX microanalysis and 160 kV of accelerating voltage (Fig. S1). The magnetization and hysteresis loop measurements were performed at 298 K using a vibrating sample magnetometer (Oxford Instruments Ltd.) in a ± 1.0-T applied field. Table 1 summarizes characteristics of magnetic nanoparticles.

Cell culture and plating cells for transfection

Human colorectal adenocarcinoma cells Caco-2 (ATCC, cat No HTB-37) and HT29 (ATCC, cat No HTB-38) and the human cervix adenocarcinoma cells HeLa (ATCC, cat No CCL-2) were maintained in DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. Caco-2, HT-29 and HeLa cells were stably transduced with Luciferase (Luc) gene (HeLaLuc, Caco-2Luc and HT29Luc) using lentiviral vectors and maintained as described above. For pDNA transfection experiments, 150 µl of cell suspension in complete medium containing $12x10^3$ for Caco-2 or HeLa cells and $15x10^3$ for HT29 cells were seeded in 96-wells plates 24h before transfection experiments. For siRNA transfection experiment, 150 µl of cell suspension in complete medium containing $5x10^3$ for Caco-2Luc or HeLaLuc cells and $8x10^3$ for HT29Luc cells were seeded per well into 96-wells plates 24h before transfection experiments. In both cases cell number was calculated in order to reach 70% and 50% of cell confluence at 24h post seeding for pDNA and siRNA experiments, respectively.

To perform laminin coated substrates, 96-well plates were pre-incubated with laminin solutions in PBS to result in different laminin surface densities (0.6 or 1 µg/cm2). Briefly, 75 µl of protein solution at specific initial concentrations was added in each well, the plates were incubated at 37°C for 6 hours and afterwards were washed twice with PBS to remove unbound proteins.

Preparation of magnetic complexes and magnetofection

Transfection complexes were prepared by mixing 20 µl of MNPs solution in water (90 µg Fe/ml), 40 µl of the enhancer solution in water, followed by addition of 300 µl solution of 3.6 µg luciferase

gene plasmid p55pCMV-IVS-luc+ (pDNA) in DMEM without additives. The enhancer solution was prepared by mixing 14.4 µl of the DFGold and 25.6 µl of water. To prepare lipoplexes, the 20 µl of the MNP suspension was substituted with water. This resulted in 360µl of complex having an iron-to-plasmid ratio of 0.5:1 and an enhancer-to-nucleic acid ratio of 4 to 1 (v/w). The mixture was incubated for 15 min at room temperature to allow complex assembling, thereafter 180 µl were used to make serial dilution 1:1 in medium without supplements and 50 µl of each dilution were added per well. A magnetic field of 130–240mT and gradient of the field of 70–120 T/m was applied at the cell monolayer for 20 min using 96-Magnets Magnetic Plate (OZ Biosciences, France). Incubation was continued for 24 h at 37 ºC without medium change until luciferase gene expression analysis was performed in cell lysate.

Experimental setup to prepare siRNA transfection complexes was the same as for pDNA complexes, MNPs and enhancer were mixed first and then complexed with siRNA, but using different final volume: 10 µl of the MNPs solution (43 µg Fe/ml), 20 µl of the enhancer solution, and 864 pg of siRNA against luciferase in 150 µl of DMEM without additives were used for preparation of the siRNA transfection complexes. This resulted in 180 µl of complex having an iron-to-siRNA ratio of 0.5:1. To test higher iron-to-siRNA ratios, 1:1 and 2:1, the complexes were prepared with MNPs solutions with 86 and 172 µg Fe/ml, respectively. As enhancer, DFGold, X-Treme and FugeneHD were used in an enhancer-to-siRNA ratio of 4 to 1 (v/w), and HiPerfect in a ratio of 8 to 1 (v/w), according to manufacturers' specifications. 90 µl of the complexes were used to make serial dilution 1:1 in medium without supplements and 25 µl of each dilution were added per well. Luciferase expression in cell lysate was quantified 72 h post-transfection. Complexes prepared with siRNA control were used as a reference (100%) to calculate the percentage of gene expression for evaluation of silencing efficacy.

Modification of the magnetic siRNA complexes with INF-7 fusogenic peptide and chloroquine experiments

To test the silencing efficiency of the magnetic complexes modified with INF-7 fusogenic peptide, 4.3, 2.2, 1.1 or 0.54 µl of the INF-7 stock solution (10 mg/ml in 20 mM HEPES, pH 7.4.) were added to the 180 µl of siRNA complexes, resulting in INF-7 peptide-to-siRNA w/w ratio of 100:1, 50:1, 25:1, 12:1 and 6:1, respectively.

For chloroquine experiments, transfections were performed as described above, except that HT29Luc cells were pre-incubated with 100 µM of chloroquine (Sigma, Argentina) for 15 min at 37 °C before the addition of the siRNA magnetic complexes and for 4h after magnetofection.

Quantification of luciferase gene expression

In order to quantify luciferase expression, transfected cells were washed with PBS and incubated with 100 µl lysis buffer (0.1% Triton X-100 in 250 mM Tris pH 7.8) per well for 10 min at room temperature, then culture plates were placed on ice. 50 µl cell lysate were mixed with 100 µl luciferase buffer (35 mM D-luciferin, 60 mM DTT, 10 mM magnesium sulphate,1 mM ATP and 25 mM glycyl-glycine-NaOH buffer, pH 7.8) and chemiluminescence was recorded using a Microplate Scintillation & Luminescence Counter (Canberra Packard, Groningen, The Netherlands). To quantify total protein of the samples 10 µl of cell lysates were used in BioRad protein assay (BioRad, Munich, Germany). To calculate the amount of luciferase in the transfected cell samples a calibration curve was used as described elsewhere.[30]

Characterization of siRNA complexes

The mean hydrodynamic diameter and the ξ-potential of the complexes were determined by ELS and DLS using a Malvern Zetasizer 3000 (UK). The magnetically induced velocity (magnetic responsiveness) of the magnetic complexes in a gradient magnetic field was evaluated as described elsewhere.[33,13] Briefly, a gradient field was generated by positioning two mutually attracting packs of four quadrangular Ne-Fe-B permanent magnets symmetrically on each side of a cuvette holder and parallel to a light beam for optical density measurements (average magnetic field and field gradient in the measuring window of 213 mT and 4 T/m, respectively). The 180 µl of the siRNA complexes were diluted to 500 µl with DMEM medium without additives and were placed into a spectrophotometer and exposed to the gradient magnetic field. The change in optical density or turbidity was recorded at 360 nm. The efficient velocity υ under a magnetic field gradient is evaluated as $\upsilon = L/t_{0.5}$, where L is the average path of the complex movement perpendicular to the measuring light beam and $t_{0.5}$ is the time required for a two-fold decrease in optical density. The experimental data were fitted to an exponential decay equation to calculate $t_{0.5}$. Turbidity of the complexes suspension was a linear function of concentration allowing data representation as cumulative distribution function $\Phi(\upsilon)$ showing the probability to found a complex with magnetophoretic mobility less than or equal to $\upsilon = L/t$. Further calculation of the average magnetic moment *M* of the magnetic complex was performed using an approach described in detail by Wilhelm et al, accounting for the hydrodynamic diameter and core size of the complexes and magnetization of the nanoparticles.[34]

Quantification of transfection complex internalization into cells using radioactively labelled siRNA

To quantify transfection complex internalization into cells, the transfection complexes were prepared with solution of ^{125}I-labeled siRNA and magnetofection of the cells was performed. After different time points of incubation at 37 °C in a 5% CO_2 atmosphere, the cells were washed with PBS, detached with Trypsin–EDTA (0.25%) and collected with supplemented medium. The radioactivity was measured in each sample using a gamma counter. The applied dose of the radioactively labeled siRNA complexes was used as a reference. The results were recalculated in terms of the cell associated/internalized siRNA weight per seeded cell.

Cell viability

The MTT assay, based on reduction of the MTT reagent into formazan by superoxide anions produced in the mitochondrial respiratory chain, was carried out to assess the cytotoxicity of the complexes. After 24h of magnetofection of pDNA complexes or 72 h post magnetofection with siRNA complexes, cells were washed with PBS and incubated in 100 µl of 1 mg/ml MTT solution prepared in PBS with 5 mg/ml glucose for 2 h. Afterwards, 100 µl solubilisation

solution (10% Triton X-100 in 0.1 N HCl in anhydrous isopropanol) was added to each well and incubated at 37°C with shaking overnight to dissolve the formazan. The optical density was measured at 590 nm. Untreated cells were used as a reference. Cell viability in terms of cell respiration activity normalized to the reference data (%) is expressed as: *Cell viability (%)=(D_{sample}-D_{blank})/($D_{reference}$-D_{blank})·100%*

Statistics

All values are expressed as the mean ± standard deviation. Statistical differences between the mean values of different groups were evaluated by unpaired student's t-test. A p-value≤0.05 was considered significant.

Results and discussion

Improvement of pDNA cell transfer by magnetofection and laminin coated substrates

First, we have compared the efficiency of pDNA and siRNA transfer in Caco-2 and HT29 cells versus the easy-to-transfect HeLa cells by standard lipofection. Figure 1A shows that the luciferase transgene expression was 10 times less in Caco-2 cells and more than 100 times less in HT29 compared to HeLa at 24 h post lipofection with DFGold/pDNA lipoplex (Fig. 1A). On the other hand, lipofection performed with the complex DFGold/siRNA achieved till 60% of silencing of luciferase expression in HeLaLuc cells, while lipofection with this complex in both Caco-2Luc and HT29Luc cells resulted in very low luciferase silencing (Fig. 1B), which highlight that these cell are hard-to-transfect cell lines.

Therefore, in order to increase transfection efficiency in these cells, we have tested magnetofection with the magnetic complex PEI-Mag2/DFGold/pDNA. As shown in figure 2, magnetofection improved significantly the expression of luciferase up to 1.5 times in Caco-2 cells and 2 times in HT29 cells in a wide range of applied plasmid dose respect to standard lipofection with DFGold/pDNA complex. No improvement in transfection efficiency was found in cells exposed to magnetic triplexes when no magnetic field was applied (data not shown). To progress in the optimization of pDNA transfer in HT29 cells we explore different kinds of MNPs (PEI-Mag2 and SOMag5) to perform the complexes and also the influence of a simplified extracellular matrix of laminin over gene transfer efficiency followed by magnetofection. Magnetofection of HT29 cells grown at laminin pre-coated substrates improved the efficiency of pDNA transfection up to 2 times with PEIMag2/DFGold/pDNA complex and 4 times with SOMag5/DFGold/pDNA complex compared to magnetofection of cells grown at uncoated substrates (Fig. 3A and 3B). Indeed the optimum of luciferase expression was 6-fold enhanced by magnetofection with SOMag5/DFGold/pDNA at laminin pre-coated plated (0.6 μg/cm^2) without toxicity compared to lipofection with DFGold at uncoated substrates, achieving 320±66 and 52±5 ng luciferase/mg total protein when 33.3 pg pDNA/cell was applied, respectively. Further increase of laminin surface density (1ug/cm^2) did not result in an enhancement of transgene expression, which can be in part attributed to toxicity increase (Fig. 3C and 3D). So, silencing assays in HT29Luc cells described in the next section were performed on substrates coated at 0.6 μg/cm2 of laminin. Garcia Nieto et al have described that cultivation of dendritic cells at laminin coated substrates generates cells with superior endocytic capacity.[35] Although we can suggest that a similar phenomenon could also appear in colorectal cancer cells, future studies need to be done to determine the specific routes of internalization of the magnetic complexes to understand the potential mechanisms of the influence of microenvironment on transfection cell efficacy.

The optimization of plasmid transfection in HT29 cell has previously been explored by Saleh A et al using the active peptide LK15 covalently attached to the Tat-derived peptide. However, the complex LK15-Tat/pDNA was still less efficient than lipofection and polyfection with PEI[5], while our experiments have demonstrated that magnetofection improved the pDNA transfer in this cell line with respect to standard lipofection in a wide range of applied pDNA doses. It is important to mention that in that work results have been expressed in relative light units (RLUs) normalized to total protein content, that makes difficult to compare transfection efficiency since RLU depend on luminescence reader sensitivity and is not lineal with concentration.[30] So, to facility future comparisons we have used a calibration curve to express the results in absolute units as ng luciferase normalized to total protein in cell lysates.

Improvement of siRNA cell transfer by magnetofection and endosomal/lysosomal escape promotion

First we have evaluated the improvement of siRNA transfection in Caco-2Luc and HT29Luc cell lines using magnetofection compared to standard lipofection. Transfections were performed using DFGold/siRNA lipoplex (4:1 v/w) and this complex modified with PEI-Mag2 MNPs at an iron-to-siRNA of 0.5:1 w/w. In Caco-2Luc cells magnetofection enhanced silencing of luciferase until 50±5% at siRNA concentration of 60 ng/well compared to lipofection that showed very low luciferase down-regulation (20±3% of silencing at 120 ng siRNA/well) (Fig. 4A). In order to obtain further improvement in luciferase silencing, magnetic complex were modified with the fusogenic peptide INF-7 at different INF7 to siRNA ratio (w/w). The optimum ratio of INF-7 to siRNA was 50:1 (Fig. 4B), this magnetic complex was able to down-regulate efficiently luciferase expression, reaching more than 95% of luciferase silencing in Caco-2Luc, even at very low dose of 15 ng siRNA/well (or 3 pg siRNA/cell) with low toxicity in the tested dose range (Fig. 4C).

Although, PEI-Mag2/DFGold/siRNA and PEI-Mag2/DFGold/siRNA/INF7 complexes resulted in efficient luciferase silencing in Caco-2Luc cells, in HT29Luc cells these complexes resulted in very low silencing effect; achieving until 15±4% and 38±5% of reporter gene down-regulation, respectively (Fig. S2). Therefore, in order to improve gene silencing in this cell line we have compared the luciferase silencing efficiency of others commercial lipid reagents, Fugene, X-Treme and HiPerfect, and these lipoplexes modified not only with PEI-Mag2 but also with SOMag5 MNPs, at an iron-to-siRNA ratio of 0.5:1 (w/w). The magnetic complex SOMag5/HiPerfect/siRNA was the only one from all tested magnetic and non-magnetic complexes that improved the luciferase silencing (Fig. S3). In order to obtain the optimum SOMag5/HiPerfect/siRNA complex different SOMag5 to siRNA w/w ratios were tested. As shown in figure 5A, increasing of the ratio SOMag5 to siRNA from 0.5:1 to 1:1 resulted in an improvement of

the down regulation of luciferase expression, reaching a 61±5% of reporter gene silencing at the applied dose of 60 ng siRNA/well, without causing toxicity (Fig. 5B), however further increase of MNP to siRNA ratio from 1:1 to 2:1 caused no further change in silencing efficacy. The addition of INF-7 peptide to the magnetic complex SOMag5/HiPerfect/siRNA did not produce any further improvement in down regulation of the reporter gene expression (data not shown), suggesting that negatively electrokinetic potential of the magnetic complex (Table 2) could not allow the association with INF-7 peptide that has a negative charge at physiological pH.[18] To evaluate if the magnetic complexes would end in lysosomes compartments in HT29 cells, we have evaluated the endosomal escape ability of the magnetic complex SOMag5/HiPerfect/siRNA using the weak base chloroquine (lysosomotropic agent). Figure 5C shows the percentage of luciferase expression in the presence/absence of 100 µM chloroquine in HT29Luc cells. A significant decrease (21±5%) in the level of gene expression (enhancement of silencing) was observed in presence of 100 µM chloroquine (p-value<0.05), suggesting that the magnetic complexes could end in lysosome compartment and their transfection capacity could be enhanced by using lysosomotropic and lysosome-disruptive agents.

The research combining magnetofection and lysosome-disruptive agents reported in this work contributes for the study of cellular processes in hard-to-transfect cells and also to further improvement in non-viral delivery systems, adding new tools to previous contributions in this research area.[36-38] However, despite the optimization of endosomal escape is a critical issue in nucleic acids delivery,[39,40] other aspect should be consider in order to enhance the siRNA therapeutic effectiveness, for instance siRNAs design and chemical modification, biodegradability, toxicity, target specificity and novel siRNA nanovehicles developtment.[41-44]

Physicochemical characteristics of the selected complexes

The mean hydrodynamic diameter and ξ-potential of the lipoplexes and magnetic complexes were measured immediately after preparing the complex in DMEM medium without additives. The average size of the magnetic and non-magnetic complexes varied from about 500 nm to almost 1500 nm. The ξ-potential of the complexes varied from a positive net charge (+17.0±1.7 mV) to slightly negatively charged (-5.2±1.9 mV) (Table 2). Magnetic complexes performed by a cationic enhancer combined either with the negative SOMag5 MNP or the positive PEI-Mag2 MNP have showed similar gene transfection efficiency and cellular toxicity (Fig. 3), demonstrating that net charge of the transfection complex is not a critical point for nucleic acid cell transfer by the magnetofection technique.[45] This result could be due to the fact that the magnetic attraction overcomes the electrostatic repulsion between negative complexes and negative plasmatic membrane. On the other hand, we performed magnetofection under serum conditions, which turns complexes charges to slightly negative due to the adsorption of the negatively serum protein,[46,11] which indeed did not affect the transfection efficiency. While the presence of serum had an inhibitory effect in non-magnetic gene delivery systems,[47,48] other researchers have also reported efficient magnetofection in serum conditions and have reported that higher saturation magnetization value (Ms) of magnetic nanoparticles improve magnetofection efficiency in this conditions.[20]

The time course of the turbidity of the magnetic complexes is plotted in figure 6, the experimental data were fitted to exponential decay equation and it was calculated that 50% of the complexes SOMag5/HiPerfect/siRNA, PEI-Mag2/DFGold/siRNA and PEI-Mag2/DFGold/siRNA/INF-7 were sedimented in 2.5 min, 13.9 min and 10.9 min, respectively, in the applied magnetic fields. The derived magnetophoretic mobility of the complexes and the average hydrodynamic diameter of the complexes allowed estimate the average magnetic moment of the complex (Table 2). This data could be useful for choosing the proper parameters of the magnetic field and time exposition necessary to achieve full sedimentation of the complex in *in vitro* experiments and targeting efficacy in *in vivo* experiments.

Finally, we have also tested the nucleic acid condensation in lipoplex and magnetic complexes by gel retardation assay and nuclease protection, both magnetic and non-magnetic complexes were able to protect nucleic acid from nuclease degradation (Fig. S4)

Evaluation of cell association and internalization of the complexes

To assess the kinetics of cell association/internalization of the magnetic and non-magnetic vectors, the complexes were prepared using ^{125}I-labelled siRNA. The data presented in figure 7 show the kinetic of siRNA-cell association/internalization in Caco-2Luc and HT29Luc cells at different time points after lipofection or magnetofection with different complexes. In Caco-2Luc cells, no significant difference was revealed in the vectors-cell association between magnetic complexes and INF-7 modified magnetic complexes. Therefore, the enhancement of luciferase down-regulation after magnetofection with magnetic complex modified with INF-7 is not due to a higher siRNA-cell association/internalization compared to the unmodified magnetic complexes suggesting that endosome escape of the INF-7 modified complex was indeed facilitated. In addition, the results showed no significant difference between siRNA-cell association/internalization of lipoplexes (DFGold/siRNA) and magnetic complexes (PEI-Mag2/DFGold/siRNA), but at the same time, magnetofection improved target silencing, suggesting that magnetic complex comprising MNPs PEI-Mag2 could also stimulate endosomal escape due to the known endosomolitic properties of the polyetylenimine[49] that is a component of the coating of PEI-Mag2. Similar results have been reported by Sanchez-Artequeda et al,[12] in that work magnetofection has resulted in a 1.5-fold increase in the percentage of Jurkat T cells that have internalized the complexes, but led to a 3 to 4.5-fold enhancement in transgene expression levels, depending on the type of particles used to formulate the complexes. On the other hand, magnetofection performed with SOMag5/HiPerfect/siRNA complex in HT29Luc cells resulted in up to 2 times higher internalization levels of siRNA compared to lipofection with HiPerfect/siRNA. In this case, it could be suggested that magnetic field facilitate the association of negative complex (Table 2) with the typical negative charge of plasmatic membrane.

Conclusions

In this work we have demonstrated that magnetofection improves pDNA and siRNA delivery into hard-to-transfect human colon cancer

cells respect to standard lipofection. Further significant improvements on nuclear acid transfer were achieved by magnetofection at laminin coated substrates and by the promotion of the endosomal escape by the modification of magnetic complexes with INF-7 peptide or chloroquine cell pre-treatment. This work highlights the need to optimize different aspects of nucleic acids transfer by magnetofection, not only the vector formulation, but also other aspect such as the endosomal escape and cellular microenvironment have to be considered to develop effective ways to delivery nucleic acids using non-viral approaches. Thus, this work contributes for studying cellular process in the adequate models not limited to the easy-to-transfect cells.

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References

1. Wang W, Li W, Ma N, Steinhoff G. Curr Pharm Biotechnol. 2013; 14: 46-60.
2. Nayerossadat N, Maedeh T, Ali PA. Adv Biomed Res. 2012; 1: 27.
3. Zhao N, Qi J, Zeng Z, Parekh P, Chang CC, Tung CH, Zu Y. J Control Release. 2012; 159: 104-10.
4. Alameh M, Jean M, Dejesus D, Buschmann MD, Merzouki A. Int J Nanomedicine. 2010; 5: 473-481.
5. Saleh AF, Aojula H, Arthanari Y, Offerman S, Alkotaji M, Pluen A. J Control Release. 2010; 143: 233-242.
6. Akhdar H, Loyer P, Rauch C, Corlu A, Guillouzo A, Morel F. Eur J Cancer. 2009; 45: 2219-2227.
7. Dunne AL, Price ME, Mothersill C, McKeown SR, Robson T, Hirst DG. Br J Cancer. 2003; 89: 2277-2283.
8. Chapman JD. J Radiat Res. 2014; 55: 2-9.
9. Scherer F, Anton M, Schillinger U, Henke J, Bergemann C, Kruger A, Gansbacher B, Plank C. Gene Ther. 2002; 9: 102-109.
10. Plank C, Zelphati O, Mykhaylyk O. Drug Deliv Rev. 2011; 63: 1300-1331.
11. Arsianti M, Lim M, Marquis CP and Amal R, Langmuir. 2010; 26: 7314–7326.
12. Sanchez-Antequera Y, Mykhaylyk O, Thalhammer S, Plank C. Int J Biomed Nanosci Nanotechnol. 2010; 1: 202-229
13. Mykhaylyk O, Zelphati O, Rosenecker J, Plank C. Curr Opin Mol Ther. 2008; 10: 493-505.
14. Ross PC and Hui SW. Gene Ther. 1999; 6: 651-659.
15. Ogris M, Steinlein P, Kursa M, Mechtler K, Kircheis R, Wagner E. Gene Ther. 1998; 5: 1425-1433.
16. 16 . X. Wang, L. Zhou, Y. Ma, X. Li and H. Gu, Nano Res. 2010; 2: 365–372.
17. Ang D, Tay CY, Tan LP, Preiser PR and Ramanujan RV. Mater Sci Eng. C. 2011; 31: 1445–1457.
18. S. Guo and L. Huang. J Nanomater. 2011; Article ID 742895
19. Kievit FM, Veiseh O, Bhattarai N, Fang C, Gunn JW, Lee D, Ellenbogen RG, Olson JM and Zhang M. Adv. Funct Mater. 2009; 19: 2244–2251.
20. Xie L, Jiang W, Nie Y; He Y; Jiang Q; Lan F; Wu Y; Gu Z. RSCAdv.2013; 3, 23571-23581
21. McCaig C, Duval C, Hemers E, Steele I, Pritchard DM, Przemeck S, Dimaline R, Ahmed S, Bodger K, Kerrigan DD, Wang TC, Dockray GJ, Varro A. Gastroenterology. 2006; 130: 1754-1763.
22. Huang P, Senga T, Hamaguchi M. Oncogene. 2007; 26: 4357-4371.
23. Dhaliwal A, Maldonado M, Han Z, Segura T. Acta Biomater. 2010; 6: 3436-3447.
24. Bengali Z, Rea JC, Shea L. D. J Gene Med. 2007; 9: 668-678.
25. Uchimura E, Yamada S, Uebersax L, Yoshikawa, T, Matsumoto K, Kishi M, Funeriu DP, Miyake M, Miyake J. Neurosci Lett. 2005; 378: 40-43.
26. Teller IC, Beaulieu JF. Expert Rev Mol Med. 2001; 3: 1-18.
27. 27 Plank C, Oberhauser B, Mechtler K, Koch C, Wagner E. J Biol Chem. 1994; 269: 12918-12924.
28. El-Sayed A, Masuda T, Akita H, Harashima H. J Pharm Sci. 2012; 101: 879-882.
29. Erazo-Oliveras A, Muthukrishnan N, Baker R, Wang TY, Pellois JP. Pharmaceuticals (Basel). 2012; 5: 1177-1209.
30. Mykhaylyk O, Antequera YS, Vlaskou D, Plank C. Nat Protoc. 2007; 2: 2391-2411.
31. Mykhaylyk O, Sobisch T, Almstatter I, Sanchez-Antequera Y, Brandt S, Anton M, Doblinger M, Eberbeck D, Settles M, Braren R, Lerche D, Plank C. Pharm Res. 2012; 29: 1344-1365.
32. Krill CE and Birringer R. Philos Mag A. 1998; 77 (3): 621-40.
33. Mykhaylyk O, Zelphati O, Hammerschmid E, Anton M, Rosenecker J, Plank C. Methods Mol Biol. 2009; 487: 111-146.
34. Wilhelm C, Gazeau F, Bacri JC. Eur Biophys J. 2002; 31: 118-125.
35. Garcia-Nieto S, Johal RK, Shakesheff KM, Emara M, Royer PJ, Chau DY, Shakib F, Ghaemmaghami AM. PLoS One.2010; 5: e10123.
36. Ladeira MS, Andrade VA, Gomes ER, Aguiar CJ, Moraes ER, Soares JS, Silva EE, Lacerda RG, Ladeira LO, Jorio A, Lima P, Leite MF, Resende RR, Guatimosim S. Nanotechnology. 2010; 21: 385101
37. Tezgel AÖ, Gonzalez-Perez G, Telfer JC, Osborne BA, Minter LM, Tew GN. Mol Ther. 2013; 21:201-209.
38. Tietz SM, and Berghoff M. J Biomol Tech. 2012; 23: 47-50.
39. 39. Margus H, Padari K and Pooga M.,Mol Ther. 2012; 20: 525–533.
40. Wang F, Wang Y, Zhang X, Zhang W, Guo S, Jin F. J Control Release. 2014; 174:126-36.
41. De Paula D, Bentley MV and Mahato RI. RNA. 2007; 13: 431-56.
42. Gao Y, Liu XL and Li XR. Int J Nanomedicine. 2011; 6: 1017–1025
43. Qi R, Liu S, Chen J, Xiao H, Yan L, Huang Y, Jing X. J Control Release. 2012;159: 251-60.
44. Qin B, Chen Z, Jin W and Cheng K. J Control Release. 2013; 172: 159–168.

45. Mykhaylyk O, Vlaskou D, Tresilwised N, Pithayanukul P, Moller W, Plank C. J Magn Magn Mater. 2007; 275–281
46. Xie L, Jiang Q, He Y, Nie Y, Yue D and Gu Z Biomater Sci. 2015; 3, 446- 456
47. Farhood H, Bottega R, Epand RM and Huang L. Biochem. Biophys. Acta. 1992; 1111: 239–246
48. Yang JP and Huang L. Gene Ther. 1997; 4: 950–960
49. Akinc A, Thomas M, Klibanov AM, Langer R. J Gene Med. 2005; 7: 657-663.

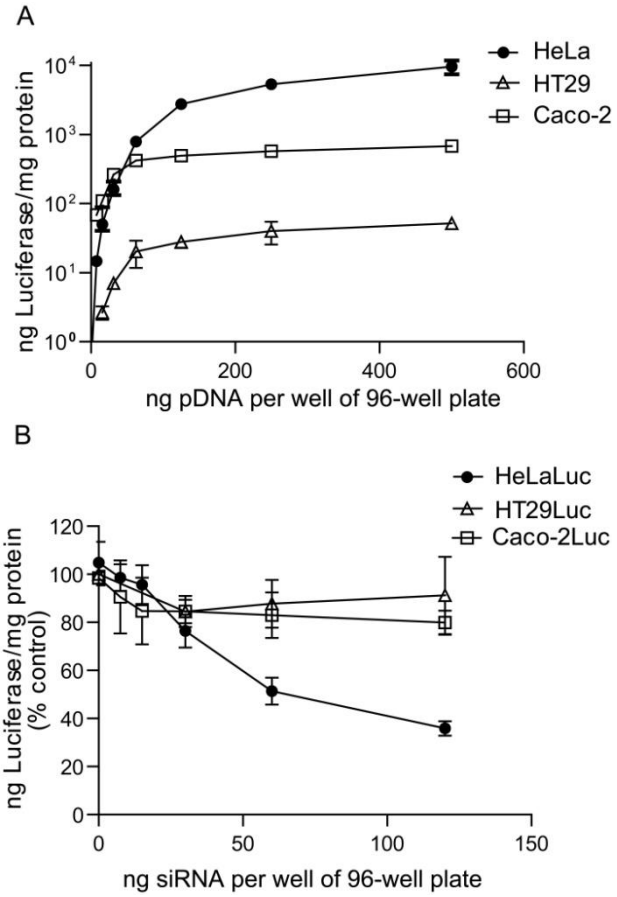

Figure 1. pDNA and siRNA transfection by lipofection in HeLa and hard-to-transfect colorectal cancer cells. (A) Luciferase expression in HeLa, Caco-2 and HT29 cells at 24h after transfection with DFGold/pDNA lipoplex, expressed as ng luciferase/mg total protein versus applied pDNA dose per well of 96-well plate; (B) Luciferase silencing in constitutive luciferase cell lines (HeLaLuc, HT29Luc and Caco-2Luc) at 72h after transfection with DFGold/siRNA lipoplex. The values represent the mean ± SD, n=3.

Abbreviations: pDNA, plasmid DNA; siRNA, small interfering

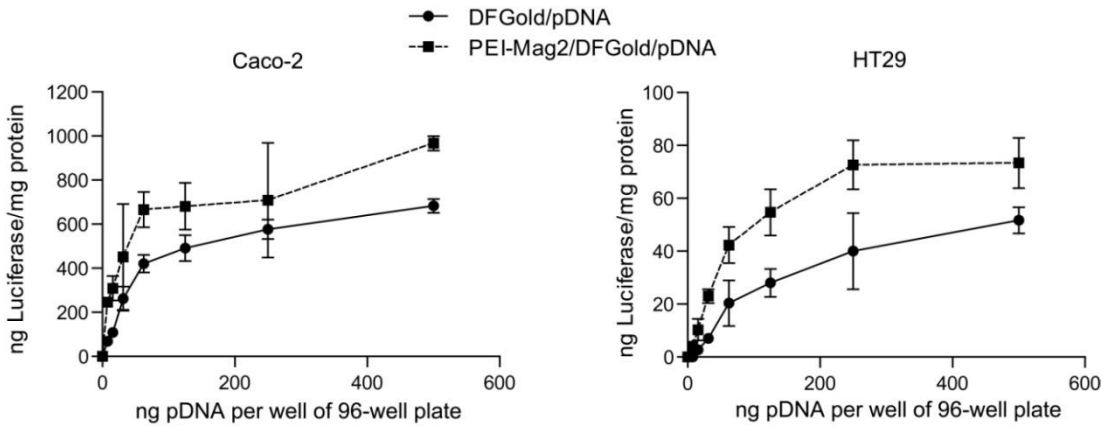

Figure 2. Improvement of pDNA expression by magnetofection in hard-to-transfect colorectal cancer cells. Luciferase expression in Caco-2 and HT29 cells 24h after lipofection with DFGold and magnetofection with the magnetic lipoplex formed with PEI-Mag2 MNPs, expressed as ng luciferase/mg total protein versus pDNA dose per well of 96-well plate. The w/w ratio of iron-to-pDNA was 0.5:1 and DFGold was used at 4µl/µg pDNA. The values represent the mean ± SD, n=3.
Abbreviations: pDNA, plasmid DNA; DFGold, DreamFect Gold reagent; MNPs, magnetic nanoparticles.

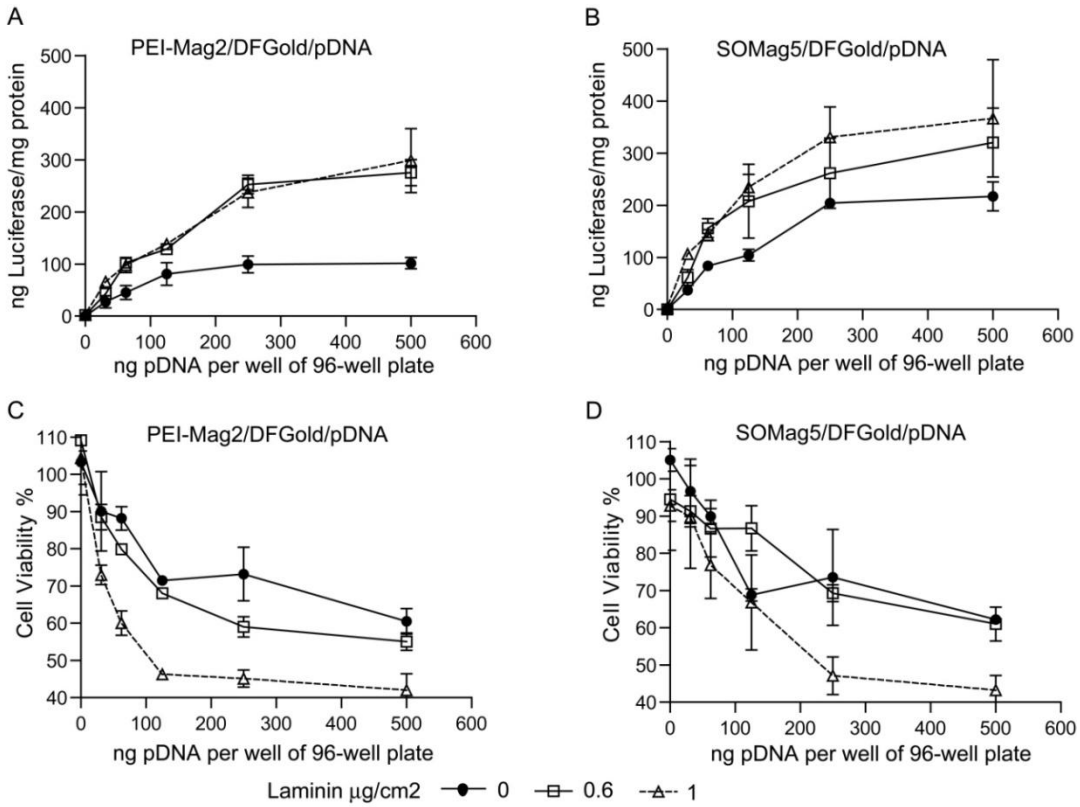

Figure 3. Laminin coated substrates modulates pDNA transfection in HT29 cells. Luciferase gene expression (ng/mg total protein) versus different applied plasmid dose per well of 96-well plate at 24h after magnetofection performed in HT29 cells plated on uncoated or pre-coated laminin coated surfaces (0.6 or 1 µg/cm^2). Magnetic complexes were formed with PEI-Mag2 (A) or SOMag5 (B) at an iron-to-pDNA w/w ratio of 0.5, using DFGold as an enhancer. (C) and (D) show cell viability measure as respiration activity (MTT assay) versus applied plasmid dose 24h after magnetofection with complexes comprising of PEI-Mag2 or SOMag5, respectively. The values represent the mean ± SD, n=3.
Abbreviations: pDNA, plasmid DNA; DFGold, DreamFectGold reagent, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

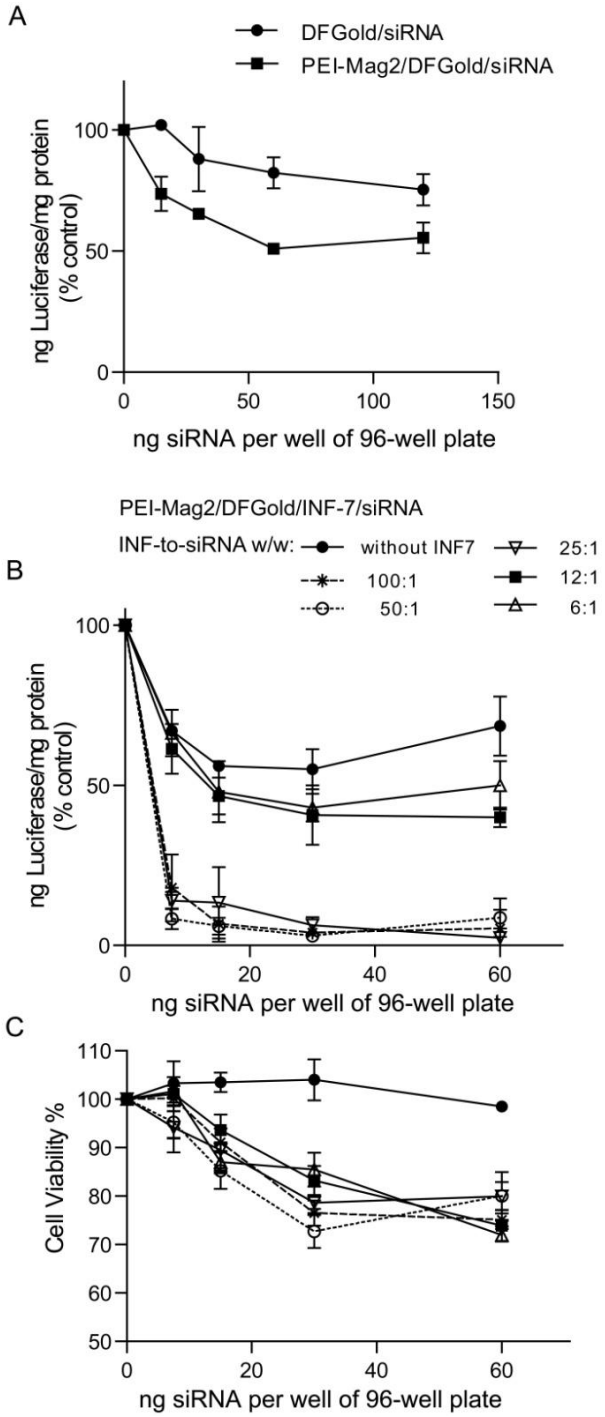

Figure 4. Enhancement of the luciferase down-regulation in Caco-2Luc by magnetic complex modified with INF-7 peptide. (A) The Caco-2-Luc cells were transfected with lipoplex and magnetic complex. Luciferase expression was measure 72 h post-transfection and expressed as ng luciferase per mg total protein normalized to the data for transfection for the complexes with siRNA control (%). (B) Luciferase expression of Caco-2Luc cells transfected with magnetic complexes modified with INF-7 at different INF-7 to siRNA w/w ratios. (C) Cell viability measure as respiration activity (MTT assay) in Caco-2Luc cells treated with magnetic complexes modified with INF-7 at 72 h post transfection. The values represent the mean ± SD, n=3.

Abbreviations: siRNA, small interference RNA; DFGold, DreamFectGold reagent; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

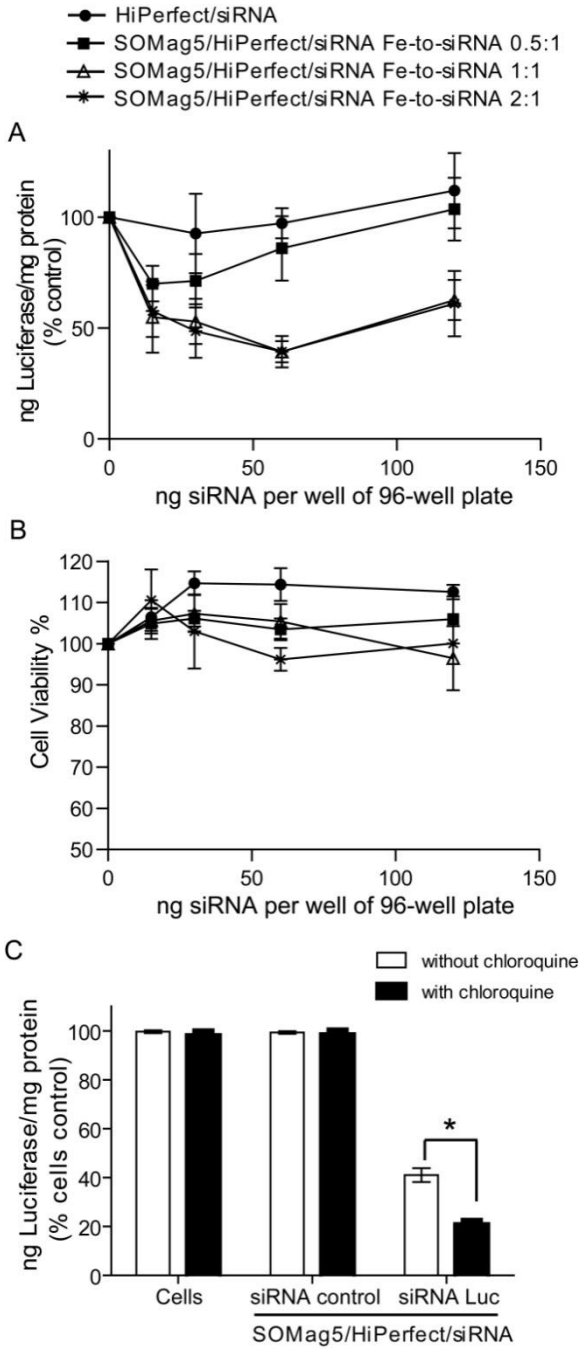

Figure 5. Optimization of siRNA magnetofection in HT29Luc cells. (A) Luciferase expression in HT29Luc cells versus applied siRNA dose after magnetofection with the magnetic complex SOMag5/HiPerfect/siRNA at different ratios of SOMag5 to siRNA (Fe w/w). Luciferase expression was measured 72 h post transfection and expressed as ng luciferase per mg total protein respect to siRNA control (%). (B) Respiration activity measured as respiratory activity (MTT assay) measured at 72 h post transfection. (C) Luciferase silencing efficiency by magnetofection in pre-treated cell by chloroquine. Luciferase expressed as ng luciferase per mg total protein normalized to data of cells without treatment. The values represent the mean ± SD, n=3.
Abbreviations: siRNA, small interfering RNA

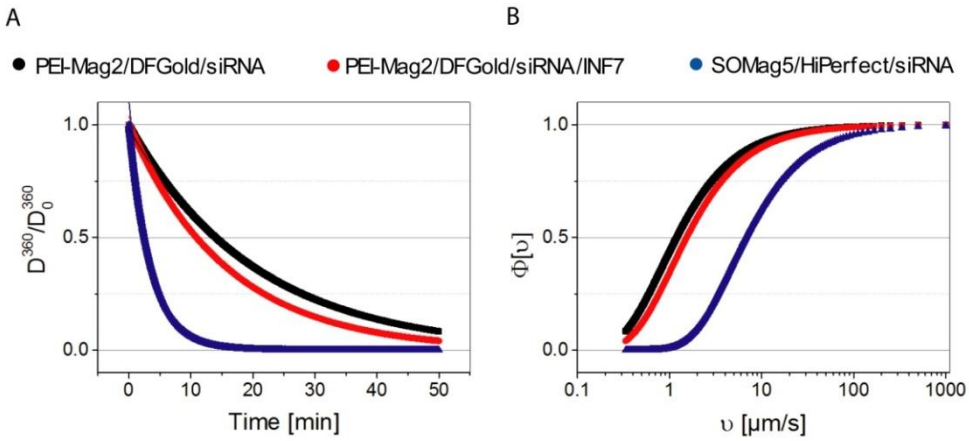

Figure 6. Magnetophoretic mobility of selected magnetic complexes. (A) Time course of the normalized turbidity of the magnetic complexes upon application of the gradient magnetic fields (average field and field gradient of 213 mT and 4 T/m) and (B) cumulative magnetophoretic velocity distribution function Φ (υ).
Abbreviations: siRNA, small interfering RNA; D360, optic density to 360 nm, υ, velocity

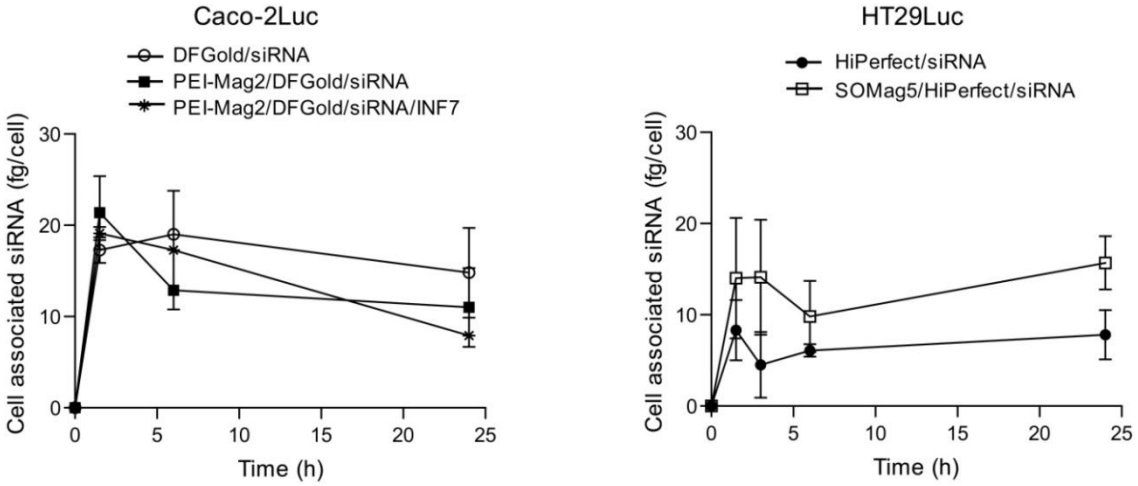

Figure 7. siRNA complexes association/internalization in hard-to-transfect colorectal cancer cells.
The Caco-2Luc and HT29Luc cells were transfected using ^{125}I-labeled siRNA complexes. At indicated time points post-transfection cell associated radioactivity was measured with a gamma-counter. The applied dose of the radioactively labelled siRNA complexes was used as a reference. The results were recalculated in terms of the cell associated/internalized siRNA weight per seeded cell. The values represent the mean ± SD, n=3.
Abbreviations: siRNA, small interfering RNA; DFGold, DreamFectGold reagent

Table 1. Magnetic nanoparticles.

Magnetic Nanoparticles	Mean magnetite crystallite size <*d*>(nm)	Saturation magnetization of the core *Ms* (emu/g iron)	Mean hydrodynamic diameter *D* (nm)	Electrokinetic potential (mV)	Iron content (g Fe / g dry weight)
PEI-Mag2	9	62	28±2	+55.4±1.6	0.56
SOMag5	7	94	40±4	-38.0±2.0	0.52

Table 2. Characterization of siRNA complexes

Complexes	Iron-to-siRNA w/w ratio	ξ-Potential (mV)	Mean hydrated diameter D (nm)	Efficient velocity in magnetic fields[a] υ_z (μm/s)	Average magnetic moment of the complex M [a] (10-15A·m2)
DFgold/siRNA	-	+15.7 ±1.5	479 ± 45	-	-
HiPerfect/siRNA	-	-2.3 ± 0.3	1185 ± 68	-	-
PEI-Mag2/DFgold/siRNA	0.5:1	+17.0 ± 7.0	1436 ± 33	1.2	3.6
SOMag5/HiPerfect/siRNA	1:1	-5.2 ± 1.9	1316 ± 56	6.7	22.5
PEIMag2/DFGold/INF7/siRNA	0.5:1	-1.9 ± 0.7	1560 ± 64	1.5	5.0

[a] Measured at average magnetic field and field gradient of 213 mT and 4 T/m, respectively