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RESEARCH PAPER

# Inulin-Ethylenediamine Coated SPIONs Magnetoplexes: A Promising Tool for Improving siRNA Delivery

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## ABSTRACT

***Purpose*** An inulin based polycation (Inu-EDA) has been synthesized by the grafting of ethylenediamine molecules onto inulin backbone. The obtained inulin copolymer has been though to coat SPIONs (IC-SPIONs) and obtain stable magnetoplexes by complexation of IC-SPIONs with a model duplexed siRNA, for improving oligonucleotide transfection efficiency.

***Methods*** The physical-chemical characteristics of IC-SPIONs and IC-SPIONs/siRNA magnetoplexes have been investigated by scanning and transmission electron microscopies, dynamic light scattering, FT-IR and qualitative surface elementary analysis. Cell compatibility and internalization *in vitro* of IC-SPIONs have been evaluated by MTS and fluorescence microscopy respectively on cancer (HCT116) and normal human (16HBE) cells. The efficiency of gene silencing effect of magnetoplexes was studied on both tumoral (JHH6) and non tumoral (16HBE) cell lines also by applying an external magnet.

***Results*** IC-SPIONs showed dimension of 30 nm and resulted cytocompatible on the tested cell lines; in the presence of an external magnet, the magnetic force enhanced the IC-SPIONs uptake inside cells. Magnetically improved transfection was observed in 16HBE cells under magnetofective conditions, in accordance with the IC-SPIONs uptake enhancement in the presence of an external magnet.

***Conclusions*** These findings support the potential application of this system as a magnetically targeted drug delivery system.

**KEY WORDS** inulin · magnetoplexes · polycation · siRNA · SPIONs

## ABBREVIATIONS

| | |
|---|---|
| IC-SPIONs | Inulin coated superparamagnetic iron oxide nanoparticles |
| SPIONs | Superparamagnetic iron oxide nanoparticles |

## INTRODUCTION

Nucleic acids are unique substances exploited as drugs in last decades. This new class of drugs may produce peculiar cellular effect upon their uptake into target cells, such as induce cells to produce a desired protein or to shut down the expression of endogenous genes or even to repair defective genes. In particular, use of short interfering RNA (siRNAs) represents an emerging paradigm for the treatment of many human diseases such as cancer (1), cardiovascular, neurodegenerative, metabolic diseases and viral infections (2). siRNAs are double stranded RNA molecules (19–27 bp) that can specifically bind to mRNA in the cytoplasm of cells via complementary base pairing and induce mRNA degradation (3). The use of siRNA has several advantages over widely used plasmidic DNA (pDNA) in conventional gene therapy: siRNA delivery, infact, avoids the nuclear barrier as its site of action is in the cytosol,

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unlike pDNA and, importantly, siRNA duplexes are readily available from various sources via custom or predesigned siRNAs targeting a particular mRNA of interest. Key to the potential of RNAi as a therapy in humans was also the discovery that, unlike pDNA, that are composed by several hundred base pair, small dsRNAs less than 30 bp, were able to bypass the mammalian immune response to facilitate gene-specific silencing (4). Although, according to some authors already 21 base-pair long dsRNA, can lead to a sequence independent-interpherone response (5).

A siRNA's drawback respect to pDNA is the transient gene knockdown in rapidly dividing cell lines because of the dilution of the siRNAs below a therapeutic threshold level with repeated cell division (6). Overall, in many cases, transient gene expression and silencing are often preferred as it allows for a better control of the therapeutic effect.

However, to exploit their potential they need to be delivered into cells, and this goal is actually a challenging task in many respects. Firstly, owing to their hydrophilicity and negative charges, siRNAs are not readily taken up by cells; secondly, they are susceptible to nuclease degradation that limits the possibility to effectively administer these biomolecules through most of the routes.

Among methods designed for siRNA delivery, one of the most recent and promising is magnetofection, i.e., nucleic acid cell uptake under the influence of a magnetic field acting on magnetic nanoparticles that are associated with nucleic acid vectors (7). The major advantages of magnetofection are: (1) improvement of the dose–response relationship in nucleic acid delivery; (2) strong improvement of the kinetics of the delivery process and (3) the possibility to localize nucleic acid delivery to an area where the magnetic field is applied (8). Moreover, the addition of a delivery vehicle, such as polymer coated magnetic nanoparticles, can help to prolong the serum and intracellular half-lives of siRNA by improving pharmacokinetics and nuclease resistance, thus making the RNAi effect more long-lasting and therapeutically viable (9).

In a recent work carried out by S.Prijic and Prosen *et al.* (10) new surface modified SPIONs with a combination of polyacrylic acid and endosomolytic polyethylenimine proved to be effective for magnetofection of murine mammary adenocarcinoma cells and tumors in mice.

During the last years, our group has optimized different polymer-coated super-paramagnetic iron oxide nanoparticles (SPIONs) proposed as carrier for magnetic drug delivery and targeting (11, 13) and recently, an inulin based polymer-coated magnetic nanocarrier has been studied as system able to improve the accumulation of doxorubicin into cancer cells in the presence of an external magnet, displaying a good magnetic targeting (14). Comparing with other polysaccharides, to date inulin has been insufficiently explored as starting biomaterial for the development of drug delivery systems, although it shows peculiar advantageous properties, namely low molecular weight (~5000 Da), hydrophilicity, biocompatibility, and also the possibility to be easy functionalized in the side chain, obtaining derivatives with different potentialities (14), Thanks to these characteristics, inulin copolymers bearing primary amine groups in the side chain were recently synthesized by using the enhanced microwave synthesis (EMS) in order to combine the employment of this abundant and biocompatible macromolecules with the possibility to obtain safety polycations useful for siRNA delivery (15). Inulin may be an optimal candidate as starting biomaterial for the production of polycations for siRNA delivery, since low-molecular-weight polycations show less cytotoxicity and better nucleic acid unpackaging once inside cells (16). Herein, the inulin based polycation named Inu-EDA, synthesized by the grafting of ethylenediamine molecules onto inulin backbone by EMS (17) has been used with a double goal: prepare Inu-EDA coated SPIONs (IC-SPIONs); obtain stable magnetoplexes by complexation of IC-SPIONs with siRNA.

Then, the physical-chemical characteristics of IC-SPIONs and IC-SPIONs/siRNA magnetoplexes have been investigated and the cellular compatibility and internalization *in vitro* was evaluated by transfection assays. In particular, the efficiency of gene silencing effect was studied on both tumoral and non tumoral cell lines also by applying a static magnet below cell culture during uptake and transfections experiments.

## MATERIALS AND METHODS

### Materials

Inulin (Inulin from dahlia tubers, Sigma Aldrich, Milan, Italy) ethylenediamine (EDA) (Ethylenediamine, Sigma Aldrich, Milan, Italy), Bis(4-nitrophenyl)carbonate (BNPC) (Bis(4-nitrophenyl) carbonate, Sigma Aldrich, Milan, Italy), Sephadex G-15 (Sephadex® G-15, Fluka Switzerland), anhydrous dimethylformamide (a-DMF) (N,N-Dimethylformamide, Fluka Switzerland), ethanol (Ethanol, Fluka Switzerland), dimethyl sulfoxide (DMSO) (Dimethyl sulfoxide, Fluka, Switzerland). Iron oxide ($Fe_3O_4$) superparamagnetic nanoparticles (SPIONs) (average particle size specified by the supplier = 20 ± 1 nm) in toluene (magnetization >20 emu/g, at room temperature under 4500 Oe) (Iron oxide(II,III), magnetic nanoparticles solution, Sigma Aldrich, Milan, Italy), sodium dodecyl sulphate (SDS) (Sodium dodecyl sulfate, Sigma Aldrich, Milan, Italy), sodium hydrogen carbonate ($NaHCO_3$) (Sodium hydrogencarbonate, Sigma Aldrich, Milan, Italy), iron(III)chloride (Iron(III) chloride, Sigma Aldrich, Milan, Italy), ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate, Sigma Aldrich, Milan, Italy), neocuproine (2,9-dimethyl(1,10-phenanthroline) (Neocuproine, Sigma Aldrich, Milan, Italy), L-Ascorbic acid

sodium salt ((+)-Sodium L-ascorbate, Sigma Aldrich, Milan, Italy). Alexa Fluor 647® N-hydroxysuccinimide ester (Alexa Fluor® 647 NHS Ester (Succinimidyl Ester, Invitrogen, Monza, Italia). All reagent were of analytic grade, unless otherwise stated. SpectraPor dialysis tubing (SpectraPor dialysis, Spectrum Laboratories, Inc, Rancho Dominguez, California).

The $^1$H-NMR spectra were recorded in $D_2O$ using a Bruker Avance II 300 spectrometer operating at 300 MHz. Centrifugations were performed using a Centra MP4R IEC centrifuge. Size exclusion chromatography (SEC) was carried out using a PolySep-GFC-P3000 column (Phenomenex, California, USA) connected to a Water 2410 refractive index detector. Phosphate buffer pH 6.5/methanol 9:1 (*v*/*v*) solution was used as eluent at 35°C with a flux of 0.6 mL/min, and pullulan standards (112.0–0.18 kDa, Polymer Laboratories Inc., USA) were used to set up calibration curve.

## Cell Lines Culture and Reagents

Human bronchial epithelial (16HBE), human colon cancer (HCT116) and human hepatocellular carcinoma (JHH6) cells were purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Italy). 16HBE and HCT116 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin (100 U/mL penicillin and 100 mg/mL streptomycin), 1% glutamine and 0.5% of amphotericin B, at 37°C in 5% $CO_2$ humidified atmosphere. JHH6 were grown in Williams' medium E with 10% FBS, 1% penicillin/streptomycin and 1% glutamine. DMEM, Williams' medium E and other constituents were purchased by Euroclone. Opti-MEM® I Reduced-Serum Medium was purchased from Life Technologies. Cell Titer 96 Aqueous One Solution (MTS reagents for cell proliferation assay) and pGL3 promoter vector were purchased from Promega (Milan, Italy). TurboFect™ Transfection Reagent (TF) was purchased from Thermo Scientific (Milan, Italy).

## siRNAs

Duplexed siRNAs, with and without Cy5 linked to the 5′ end of the sense strand, were purchased from Eurofins MWG operon (Ebersberg, Germany). The gene target sequence (5′→3′) is reported below: Luciferase GL3; CUUACGCU GAGUACUUCGA(dTdT).

## Preparation of (IC-SPIONs)/siRNA Magnetoplexes

### *Synthesis of Inulin-(2-aminoethyl)-Carbamate (Inu-EDA) Copolymer via "Enhanced Microwave Synthesis" (EMS)*

Inu-EDA was synthesized as previously reported (14). Briefly, inulin dissolved in a-DMF, was activated by reacting with BNPC in a CEM Discover Microwave Reactor, for a 1 h with a power of 25 W. The reaction temperature was monitored and maintained at 60°C by cooling with external compressed air. Then the reaction mixture was added drop-wise to an EDA solution in a-DMF. The reaction mixture was kept under stirring for 1 h at 25°C. After this reaction time the obtained product was precipitated in a diethyl ether–dichloromethane mixture (2: 1 *v*/*v*) and the solid residue was recovered and washed with acetone to remove excess of unreacted EDA and BNPC. Then, the solid product was purified by gel permeation chromatography using Sephadex G-15 as the separating gel. The pure product was obtained with 100% yield based on the starting inulin. The obtained copolymer Inu-EDA was characterized by $^1$H NMR (300 MHz, $D_2O$) and spectroscopic data were in agreement with attributed structure: δ 2.74–3.15 ($4H_{EDA}$, -NH-**$CH_2$-$CH_2$**-$NH_2$), 3.55- 4.10 (5 $H_{INU}$, -**$CH_2$**-OH; -**CH**-$CH_2$-OH; -C-**$CH_2$**-O-), 3.92-4.30 ($2H_{INU}$, -C-**CH**-OH; -**CH**-OH).

### *IC-SPIONs' Preparation*

Two mL of superparamagnetic nanoparticles (SPIONs, 5 mg/mL) superparamagnetic nanoparticles (Aldrich) (20 ± 1 nm, magnetization > 20 emu/g, at room temperature under 4500 Oe) were dried by toluene evaporation under vacuum from an azeotrope mixture with ethanol (11). The dried pellet was dispersed in 2 mL of SDS solution (1.33 mg/mL) as stabilizer (14). The dispersion of SPIONs stabilized with SDS was added with gentile stirring to 50 mg of copolymer Inu-EDA and pH value was adjusted to 5.5 using HCl 0.1 N. The obtained mixture was sonicated in an ultrasonic bath for 10 min, thus diluted to 5 mL with double distilled water and incubated overnight. Finally, the product was purified by using a dialysis tube (Spectra Por, Float-A-Lyzers) with a nominal molecular weight cut-off (NMWCO) 25 kDa. After 48 h Inu-EDA coated SPIONs (IC-SPIONs) were freeze-dried from water obtaining a brown powder with a yield of 70 *w*/*w*%.

### *IC-SPIONs/siRNA Magnetoplexes Preparation*

IC-SPIONs/siRNA complexes were prepared as follows: 10 μL of siRNA solution at a concentration of 0.2 mg/mL in nuclease free DPBS was added to an equal volume of IC-SPIONs dispersion in the same dispersant medium at a precise concentration (2, 4, 6, 8, 10 mg dry weight nanomaterial/mL) in order to obtain the desired IC-SPIONs/siRNA weight ratios (R = mg dry weight IC-SPIONs/mg siRNA) equal to 10, 20, 30, 40 and 50. The mixture was gently pipetted, and fresh prepared complexes were used for each experiments after 3 h incubation at room temperature.

## CHARACTERIZATION OF IC-SPIONS/SIRNA MAGNETOPLEXES

### Scanning Electron Microscopy

For the morphology studies, freeze-dried IC-SPIONs were visualized using a scanning electron microscope, ESEM Philips XL30. Samples were dusted on a double sided adhesive tape previously applied on a stainless steel stub and examined when dried.

### Trasmission Electron Microscopy

IC-SPIONs were visualized using 120 keV TEM (JEOL 1010, Japan) equipped with GATAN US1000 CCD camera (2 k×2 k). Ten microliter droplets of the sample were drop casted onto a piece of ultrathin Formvar-coated 200-mesh copper grid (Ted-pella, Inc.) and left to dry in air before examination.

### Dynamic Light Scattering (DLS) Analysis and Zeta-Potential Measurement

DLS studies and Zeta-potential measurements (mV) were performed at 25°C using a Malvern Zetasizer NanoZS instrument fitted with a 532 nm laser at a fixed scattering angle of 173°, using the Dispersion Technology Software 7.02. Aqueous dispersion of SPIONs, IC-SPIONs or IC-SPIONs/siRNA complexes prepared in bi-distilled water were analysed. The intensity-average hydrodynamic diameter and polydispersity index (PDI) were obtained by cumulant analysis of the correlation function. The zeta-potential (mV) was calculated from the electrophoretic mobility using the Smoluchowsky relationship and assuming that $K \times a >> 1$ (where $K$ and $a$ are the Debye-Hückel parameter and particle radius, respectively).

### FT-IR Analysis

Investigation of the general and quantitative composition of polymer coated iron oxide nanoparticles were carried out via infrared spectroscopy (FT-IR). The pure solid Inu-EDA copolymer, IC-SPIONs and Iron(II,III) oxide nanopowder (637106 ALDRICH) were analyzed in the frequency range of 4000–400 $cm^{-1}$ by using a Brucker Spectrum FT-IR ATR System spectrophotometer. Spectra were recorded in reflectance scale with a resolution of 1 $cm^{-1}$ and a number of scans = 100.

### Qualitative Surface Elementary Analysis

Qualitative elementary composition of freeze-dried Inu-EDA coated SPIONs was obtained using a scanning electron microscope, ESEM Philips XL30. Samples were dusted on a double sided adhesive tape previously applied on a stainless steel stub prior to chemical elementary analysis.

### Total Iron Determination

The iron content in IC-SPIONs was measured using the ferrozine-based spectrophotometric iron estimation method (11). For this method freeze-dried IC-SPIONs (5 mg) were dispersed in 1.4 N hydrochloric acid (0.1 dry weight nanomaterial/mL) and kept for 2 h at 60°C in a water bath to allow the mineralization of nanoparticles and the complete dissolution of the SPIONs. After that, 0.5 mL of iron-detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine and 1 M acid ascorbic dissolved in acetate buffer at pH 4.5) were added to 0.5 mL of the obtained iron solution and incubated for 30 min at room temperature. Then, the optical density of the sample was measured at 560 nm using a Shimadzu UV-2401PC spectrophotometer, and the concentration of $Fe_3O_4$ was calculated by comparing this value with a calibration curve obtained by recording the absorbance of $FeCl_3$ standard solutions in HCl 0.01 N (ranging from 3 to 33 μg/mL). HCl 0.01 N with iron-detection reagent was used as blank.

### Gel Retardation Assay

IC-SPIONs/siRNA magnetoplexes were formed in nuclease free ultrapure water. In particular, 10 μL of siRNA solution (0.2 mg/mL) were added to 10 μL of IC-SPIONs dispersions at various concentration. After 3 h samples were then loaded in a 1.5*w*/*v*% agarose gel, containing 0.5 μg/mL ethidium bromide, and run at 100 V in tris-acetate/EDTA (TAE) buffer pH 8 for 30 min. Gels were visualized against an UV transilluminator and photographed using a digital camera.

### Cytotoxicity Assay and Uptake Studies of IC-SPIONs/siRNA Magnetoplexes

#### *IC-SPIONs MTS Assay on HCT116 and 16HBE Cells*

The cytotoxicity was assessed by the MTS assay on HCT116 and 16HBE cells using MTS cell proliferation assay. Cells were seeded in 96 well plate at a density of $2.5 \times 10^4$ cells/well and grown as reported above. After 24 h the medium was replaced with 200 μl of fresh DMEM containing IC-SPIONs at a concentration equal to 0.001, 0.025, 0.05, 0.1, 0.25 and 0.5 mg dry weight nanomaterial/mL. Moreover, the same experiment was performed applying a permanent magnet (4 mm diameter/1 mm h) at the bottom of the wells (the magnets were fixed at the bottom of each wells by adhesive tape), using a concentration of IC-SPIONs of 0.1 mg dry weight nanomaterial/mL. Additionally, cells were treated with the same magnet for 24 and 48 h, to evaluate the effect

of the exposition to external magnetic field on cell viability (11). After 24 and 48 h, DMEM was replaced with 100 μL of fresh medium, and 20 μL of a MTS solution was added to each well. Plates were incubated for additional 2 h at 37°C. Then, the absorbance at 490 nm was measured using a microplate reader (Multiskan, Thermo, UK). Pure cell medium was used as a negative control. Results were expressed as percentage reduction of the control cells. All culture experiments were performed in triplicates.

## IC-SPIONS UPTAKE STUDIES BY FLUORESCENCE MICROSCOPY

### Conjugation of Alexa Fluor-647® dye to IC-SPIONs (IC-SPIONs-Alexafluor-647®)

Alexa Fluor 647® N-hydroxysuccinimide ester (0.5 mg) was dissolved in 100 μl of DMSO and this solution was added to a colloidal dispersion of IC-SPIONs (5 mg dry weight nanomaterial/mL) in 0.1 M sodium bicarbonate buffer, pH 8.3. The reaction was carried out at room temperature, overnight. The product was purified by several washes in distilled water and then freeze dried. The pure product was obtained with a yield of 88% based on the starting IC-SPIONs.

### IC-SPIONs uptake on HCT116 and 16HBE cell lines

HCT116 and 16HBE cell lines were seeded at a density of $2.5\times10^4$ cells/well and grown as reported above. After 24 h the medium was replaced with 200 μl of fresh DMEM containing IC-SPIONs-Alexafluor-647® at the concentration of 0.1 mg dry weight nanomaterial/mL, thus cells were incubated for 4, 24 and 48 h. This experiment was performed with and without an external magnet (13). For the experiments in the presence of an external magnetic field, permanent magnets as already used for cytotoxicity studies, were fixed to the bottom of the wells containing the cells. After each incubation period the magnet and medium were removed and the cell monolayer was washed twice with DPBS and fixed with 4% formaldehyde for 30 min. Subsequently, the formaldehyde solution was removed, the cells washed with DPBS and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). After 20 min incubation, DAPI solution was removed, the cells were washed three times with DPBS and observed by a Axio Vert.A1 fluorescence microscope (Zeiss). The images were recorded using an Axio Cam MRm (Zeiss).

### Quantitative IC-SPIONs/Cy5-siRNA Magnetoplexes Uptake by Fluorescence Analysis

$1.2\times10^5$ JHH6 cells per well were seeded in 24-well plates and incubated for 24 h. Then, medium was replaced with 1.08 mL of fresh OPTI-MEM I medium and 120 μL of IC-SPIONs/Cy5-siRNA complexes at various nanoparticles/siRNA weight ratios (R 10, 20, 30, 40), obtaining a final siRNA concentration of 200 nM. After 4 h, cells were washed three times with DPBS and lysed in 300 μL lysis buffer (1% Triton X-100, 2% SDS in DPBS) at −20°C. 275 μL of lysates were transferred to disposable cuvettes and diluted to 1 mL with nuclease free water, for fluorescence intensity measurements ($\lambda_{EX}$: 650 nm; $\lambda_{EM}$: 670 nm) on a Shimadzu RF-5301 PC spectrofluorophotometer. 25 μL of lysates were used to determine the protein content using the bicinchoninic acid kit for protein determination (Sigma Aldrich), according to the protocol of the manufacturer. For determination of the mean fluorescence intensity, fluorescent signals were corrected for the amount of protein in the samples. The experiment was carried out in triplicate.

In addition, after incubation time, cells were fixed with formaldehyde and treated with DAPI for nuclear staining as previous reported. Cells were visualized with an Axio Vert.A1 fluorescence microscope equipped with an Axio Cam MRm (Zeiss).

### Luciferase Gene Down Regulation Assay

For transfection assays, JHH6 or 16HBE cells were seeded in a 96 well plate at a density of $2\times10^4$ cells/well and incubated 24 h prior to transfection. Then, pGL3 promoter vector

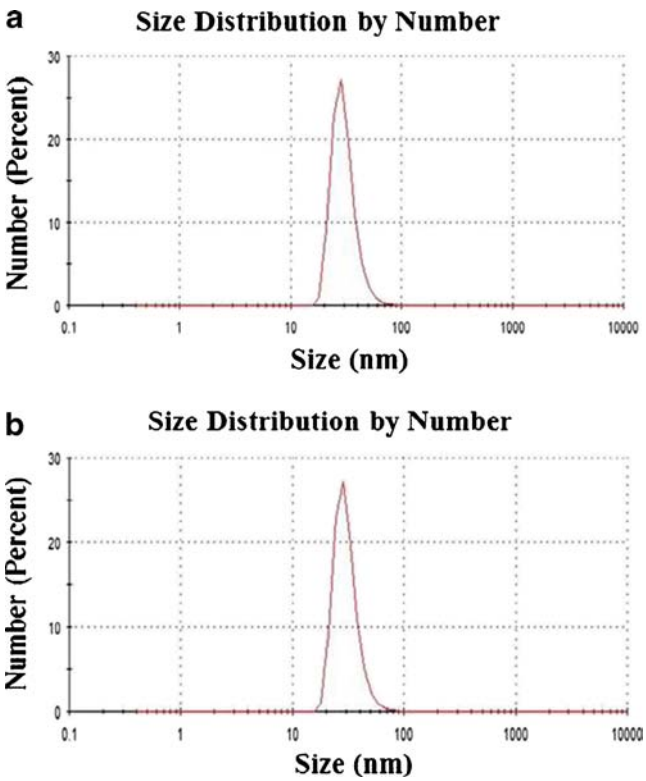


**Fig. 1** DLS size distribution histogram of IC-SPIONs before (**a**) and after (**b**) freeze-drying, in aqueous medium at concentration of 0.2 mg/mL.

**Table I** DLS Data and Zeta Potential Values of IC-SPIONs Samples Before and After Freeze Drying, in Aqueous Medium at a Concentration of 0.2 mg/mL

| Sample | Hydrodynamic diameter (nm) | PDI | Zeta potential (mV) |
|---|---|---|---|
| IC-SPIONs Before freeze drying | 30.3 | 0.4 | 30.5 ± 6 |
| IC-SPIONs After freeze drying | 31.86 | 0.4 | 34 ± 3 |

(luciferase encoding plasmid) was transfected using TF. pGL3/TF complexes were prepared as follows: 0.6 µl of TF reagent were added to 20 µl pGL3 stock solution in OPTI-MEM I, at the concentration of 0.01 mg/mL. The mixture was immediately mixed by pipetting and incubated 20 min at room temperature. After this time pGL3/TF mixture was diluted to 200 µL with OPTI-MEM I media and added to well. After 4 h cells were washed several times with sterile DPBS and then incubated overnight with complete DMEM.

Cells were than treated with 200 µL of IC-SPIONs/siGL3 siRNA, which encodes a complementary sequence for modified firefly luciferase gene in pGL3 promoter vector, in OPTI-MEM I medium and incubated for 4 h followed by overnight incubation in complete medium. siRNA final concentration of 200 nM was used. Luciferase gene expression was analyzed after 24 h from IC-SPIONs/siGL3 incubation measuring luminescence of cell lysates by the Luciferase Assay System (Promega), according to the product manual, using a GloMax 20/20 Luminometer (Promega). 25 µL of lysates were used to determine the protein content using the bicinchoninic acid kit. Transfection efficiencies of IC-SPIONs/siGL3 at weight ratios of 10, 20 and 30 were compared to each other and to naked siRNA as negative control. Results are shown as relative mean values ± standard deviation (% of cells with full luciferase expression) corrected for the amount of protein in the samples. Results are normalized using pGL3/TF as 100% RLU/mg protein. Naked siRNA at the same concentration used in IC-SPIONS/siRNA magnetoplexes was used as negative control. The experiment was carried out in triplicate.

### IC-SPIONs/siRNA MTS Assay on 16HBE and JHH6 Cells

Cells were seeded in 96 well plate at a density of $2 \times 10^4$ cells/well and grown as reported above. After 24 h the medium was replaced with 200 µl of fresh OPTI-MEM I medium containing IC-SPIONs/siGL3 magnetoplexes at weight ratios of 10, 20 and 30 (siRNA final concentration of 200 nM was used). After 48 h, medium was replaced with 100 µL of fresh DMEM, and MTS assay was performed as reported above. All culture experiments were performed in triplicates. Moreover, the same experiments were performed applying a permanent magnet.

### Statistical Analysis

A *T* Test was applied to compare different groups. Data were considered statistically significant with a value of $p < 0.05$. All values are the average of three experiments ± standard deviation.

## RESULTS AND DISCUSSION

### Preparation and Characterization of IC-SPIONs

Superparamagnetic iron oxide nanoparticles (SPIONs) are recently emerging as excellent vehicles for targeted drug delivery, but their potential application in nanomedicine is strictly influenced by their physical stability in the aqueous physiological media and by the characteristics of their surface. Actually, the presence of a polymeric coating is crucial to disperse the SPIONs in water and prevent their agglomeration and to reduce non-specific cell interactions (18). On the other hand,

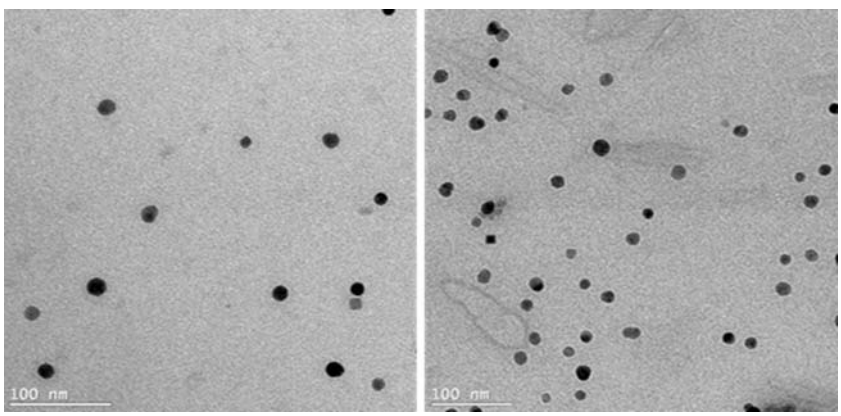


**Fig. 2** TEM Images of IC-SPIONs showing well separated nanoparticles with a diameter of about 30 nm. The scale bare is 100 nm. The magnification is 40000X.

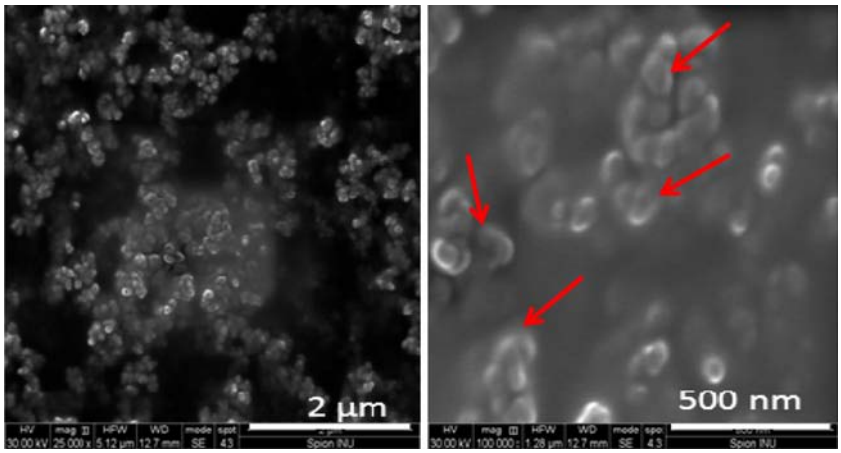


**Fig. 3** SEM images of IC-SPIONs.

the coating should provide the possibility to make chemical reaction or conjugation with biomolecules or targeting ligands. All the above considerations justified the idea to use the inulin polycation (Inu-EDA) as coating material. In a previous published article (14), a similar copolymer carrying squalenoyl and $PEG_{2000}$ tails have been already studied for the preparation of coated SPIONs acting as drug delivery systems for targeted cancer therapy. In this system the squalenoyl motieties conferred the appropriate hydrophobicity to the Inulin backbone useful to stabilize the polymeric coating by London dispersion forces maintaining a good citocompatibility. This amphiphilic copolymer was obtained by different synthetic steps, conjugating squalenoyl derivative and aldehyde terminated PEG chains to the previously functionlized Inu-EDA copolymer. Differently, in the present study we investigated the capability of the starting Inu-EDA copolymer to stabilize SPIONs for therapeutic applications. Herein, the Inu-EDA copolymer was used with double purpose: to obtain stable inulin coated iron oxide nanoparticles (IC-SPIONs) in the aqueous phase, acting as hydrocolloidal coating; to bind negatively charged siRNA for improving transfection efficiency.

The Inu-EDA copolymer employed in this study has a degree of functionalization equal to 25 mol%, calculated by $^{1}H$ NMR as reported in (17). This percentage means that on average 25 mmoles of EDA are bound per each 100 mmoles of fructose repeating units of inulin. The molecular weight, obtained by aqueous SEC analysis and Pullulan calibration, resulted equal to 2.5 KDa with a polydispersity (Mw/Mn) of 1.8.

To bind negatively charged siRNA for improving transfection efficiency IC-SPIONs were simply prepared by mixing inulin-EDA (50 mg) and SPIONs (10 mg Iron oxide(II,III) magnetic nanoparticles), the last previously stabilized with SDS (1.33 mg/mL), simultaneously in an adequate volume of water at pH 5.5. These conditions lead to the spontaneous assembling of the copolymer on the SPIONs surface upon few

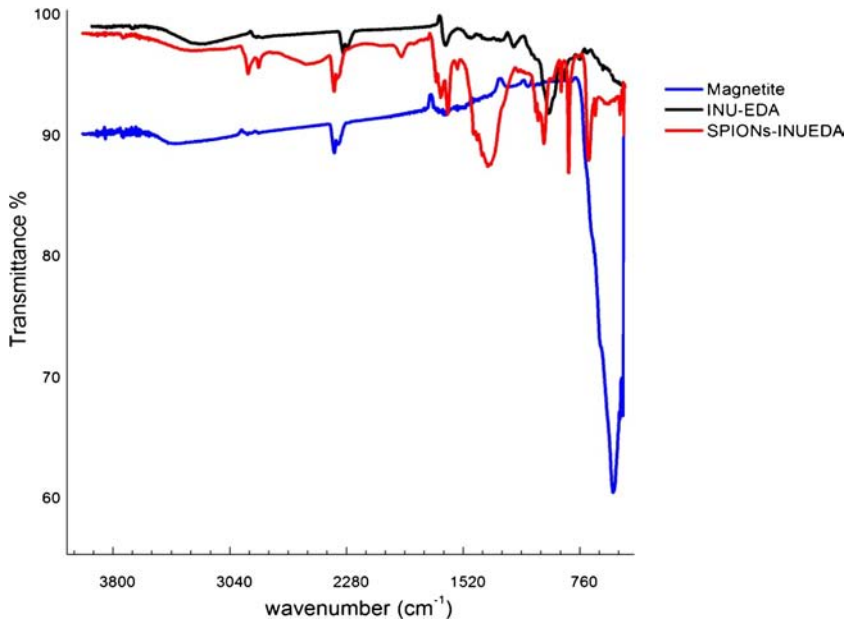


**Fig. 4** FTIR spectra of Iron(II,III) oxide, Inu-EDA copolymer and IC-SPIONs.

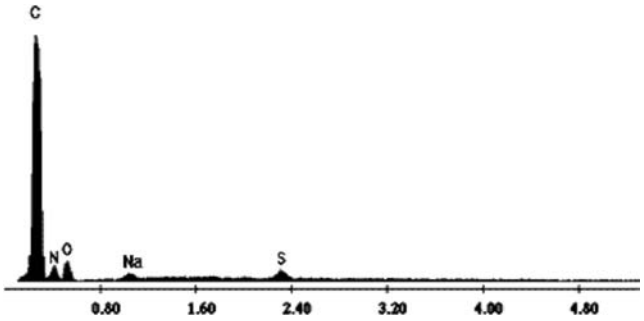


**Fig. 5** Elementary chemical analysis of IC-SPIONs surface.

hours of equilibration. The driving force which allows the adsorption of the copolymer on the surface of the iron oxide nanoparticles to form IC-SPIONs is the electrostatic interaction established between SPIONs which are negatively charged, and the cationic Inu-EDA copolymer. On the contrary, magnetoplexes were obtained by complexation of positively charged IC-SPIONs with the negatively charged siRNA.

The adsorption phenomena above described are in turn responsible of the changes of nanoparticles' surface charge and a measure of the variation of surface composition. For this reason, the measurement of zeta potential values can be used to assess the coating of magnetic nanoparticles with polymeric materials. Zeta-potential is a crucial parameter that influence the stability and the aggregation state of colloidal systems (19). In principle, Zeta-potential measurements showed that SPIONs were characterized by a negative charged surfaces (Zeta-potential = −29.2 ± 4 mV). The electrostatic interaction of SPIONs with Inu-EDA, not only generated the formation of a polymer coating, but also changed the surface charge into positive, probably due to the presence of protonated amines of Inu-EDA onto the nanoparticle surface (Zeta-potential = +30.5 ± 6 mV) (12) . These high value of surface charge guarantees a good shelf stability of the system and permitted the electrostatic loading of siRNA (20).

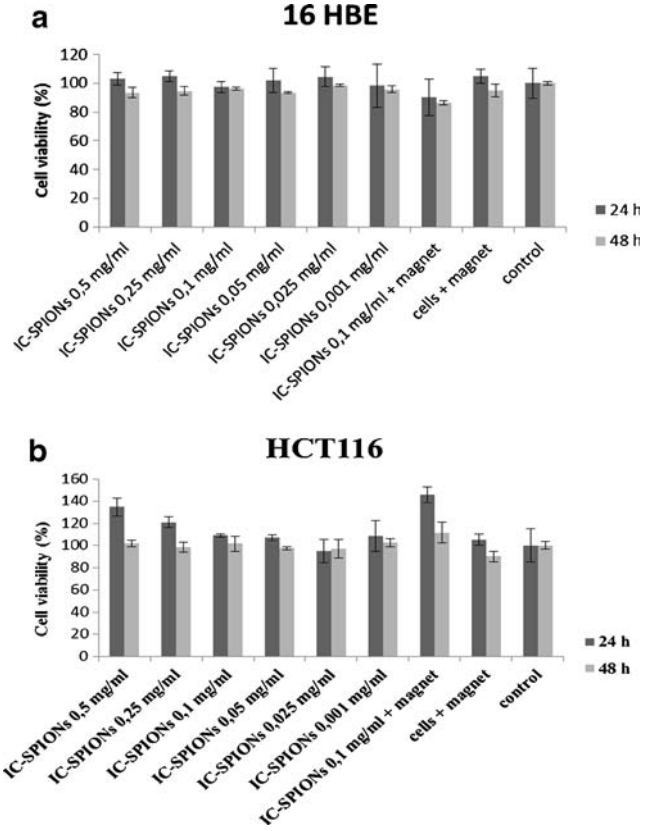


**Fig. 7** Cell viability % (MTS assay) of IC-SPIONs on human bronchial epithelial (16HBE) cells (**a**) and human colon cancer (HCT116) cells (**b**), at the concentration of 0.5, 0.25, 0.1 (with and without magnet), 0.05, 0.025 and 0.001 mg/mL, after 24 and 48 h of incubation.

Differently from a previous polyaminoacidic coated superparamagnetic nanoparticles based system (11) in which an amphiphilic PHEA copolymer used as the coating agent conferred negative zeta potential values to the colloidal system, here a positive superficial charge, which was still present after siRNA loading, is

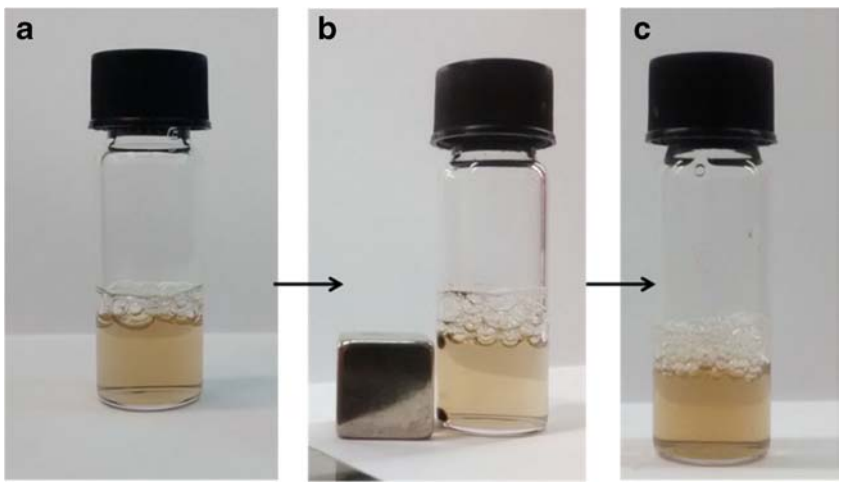


**Fig. 6** Photographs of IC-SPIONs water dispersion before (**a**) during (**b**) and after (**c**) the application of an external magnetic field.

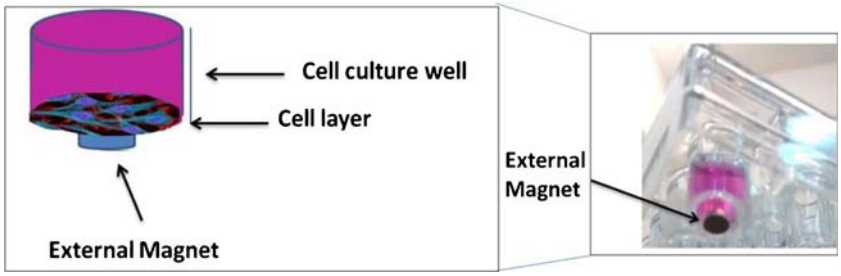


**Fig. 8** Photograph of cell wells used for cell viability and uptake studies in presence of an external permanent magnet.

helpful for the cellular uptake, which usually requires positively charged systems (12).

The hydrodynamic diameter of these IC-SPIONs in water was measured by DLS measurements (Fig. 1). The average hydrodynamic diameter before freeze drying was of 30.3 nm (Table I). This value was confirmed by TEM analysis (Fig. 2) that showed well dispersed nanoparticles with a diameter of 30 ±2.7 nm in accordance with DLS results. Negligible differences in size and zeta potential values were detected after freeze drying without the use of any cryoprotectant. This is advantageous with regards to storage and stability of dried system.

Figure 3 shows the morphology of dried IC-SPIONs obtained by high resolution SEM analysis. SEM micrographs showed a homogeneous population of nanoparticles with an almost round shape and in which is clearly visible the presence of an irregular polymeric coating (red arrows).

The chemical composition of the hybrid nanoparticles IC-SPIONs, was assessed by ferrozine colorimetric quantitative iron determination assay, by FTIR spectroscopy and by qualitative elementary chemical analysis. The iron oxide and the organic coating weight percentages were estimated assuming magnetite structure of the iron oxide. The total iron oxide

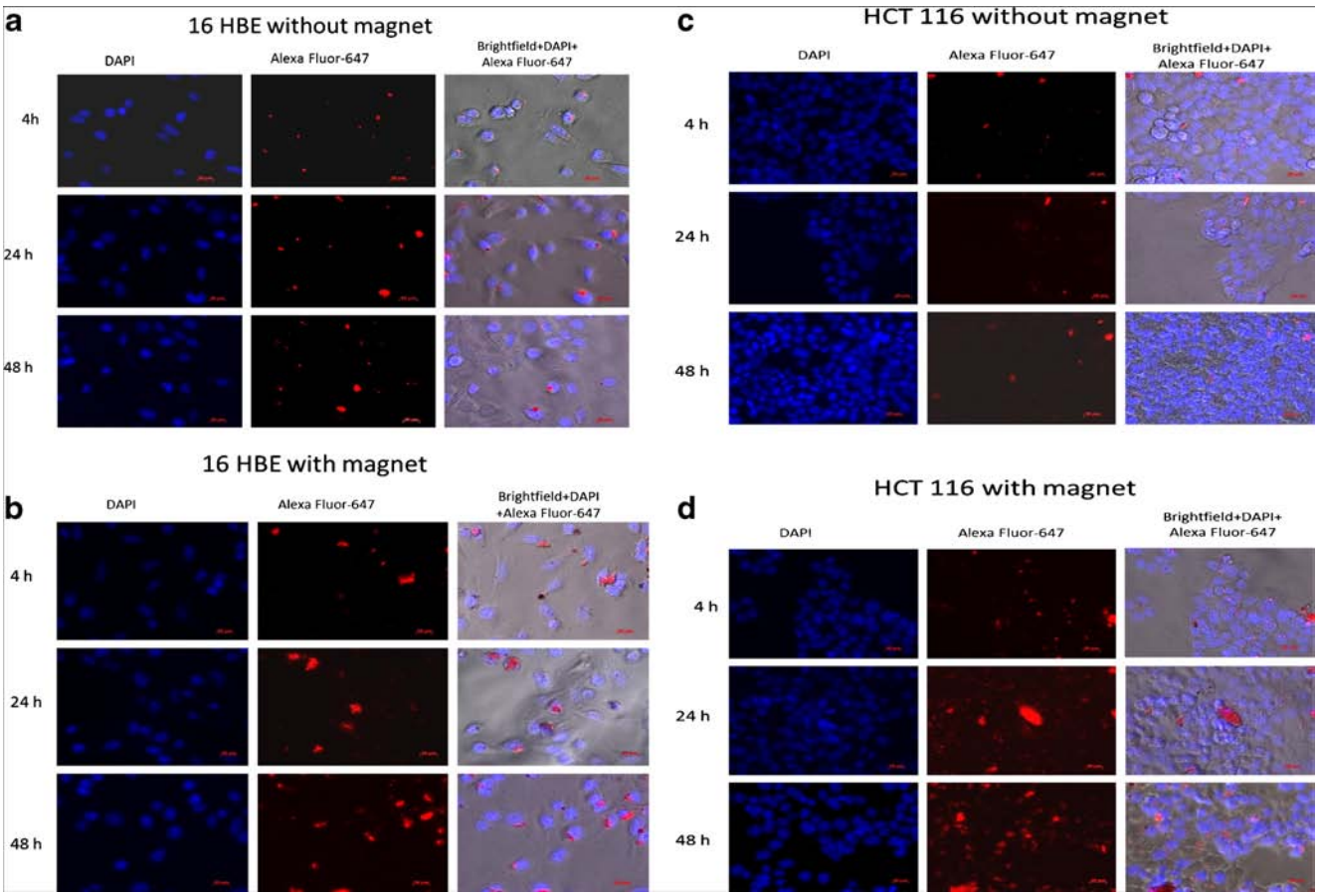


**Fig. 9** Fluorescence microscopy images of 16 HBE and HCT116 cells incubated with IC-SPIONs with (panels **b** and **d**) and without (panels **a** and **c**) the application of the external magnet. Incubation times were 4, 24 and 48 h (*lines A*, *B* and *C*). IC-SPIONs-Alexa Fluor-647 is visualized in *red*; Cell nuclei were stained with DAPI (*blue*). Magnification is 40X for all images.

amount in IC-SPIONs was found to be equal to 12.5±1.5% on a weight basis. The existence of the polymeric coating on the IC-SPIONs surface was further confirmed by FTIR and by elementary chemical analysis and it can be estimated that the organic coating content is about the 87.5 wt.% of the nanoparticles composition. In Fig. 4 the FTIR spectra are reported in comparison with Iron(II,III) oxide and Inu-EDA copolymer alone. The IR spectrum of Iron(II,III) oxide showed only the characteristic broad band of Fe–O stretching at 579 $cm^{-1}$, whereas the Inu-EDA copolymer displays characteristic stretching bands of carbonyl functions at about 1650 and 1740 $cm^{-1}$ and a broad band at 3300 $cm^{-1}$ of hydroxyl and amine groups. The spectrum of IC-SPIONs clearly shows the vibrating bands of the polymer consistent with the existence of a polymer coating on magnetite core. The elementary chemical analysis spectrum (Fig. 5) showed the characteristic peaks of carbon, oxigen and sulphur, assuming the presence of a polymeric coating with a minimum thickness of 5 nm, which is the medium resolution depth of the instrument.

The superparamagnetic behaviour of prepared IC-SPIONs was evidenced macroscopically by the attractive effect of an external magnet on the nanoparticles water dispersion. Figure 6a clearly displays IC-SPIONs dispersion in water; whereas, after the application of an external magnet for few minutes, nanoparticles were easily recovered and accumulate near the magnet, Fig. 6b. Anyway, it was noticed that, a homogeneous dispersion was re-obtained after the removal of the magnetic stimulus (Fig. 6c) thus indicating the superparamagentic behaviour of the nanoparticles and the good physical stability of the polymer coating.

### *In Vitro* Biological Evaluations of IC-SPIONs

Cytocompatibility of IC-SPIONs was evaluated by the MTS assay on two different cell lines: one, 16HBE, is a non tumoral cell line extensively used as model normal cells to screen cytotoxicity of novel compounds or carriers (11,21); the other one, HCT116, is a cancer cell line used to investigate the anticancer activity of drugs and the associated mechanism of action, by MTS assay (22,14). These cells were incubated with IC-SPIONs at concentrations of 0.001, 0.025, 0.05, 0.1, 0.25 and 0.5 mg/mL (mg of IC-SPIONs refers always to dry weight nanomaterial), corresponding to 8, 200, 400, 800, 2000, 4000 pg dry weight nanomaterial/cell respectively, for 24 and 48 h. Results, in term of cell viability (%) as a function of samples concentration, are shown in Fig. 7a and b. The same experiment was performed applying a permanent magnet at the bottom of the wells as shown in Fig. 8, in order to evaluate the effect of the magnetic field on cells viability (14).

The data shown that cell viability was always above 90% of untreated cells viability, for all the tested concentrations on normal and cancer cells, both after 24 and 48 h. In particular, cells treated with IC-SPIONs at concentration of 0.1 mg/mL

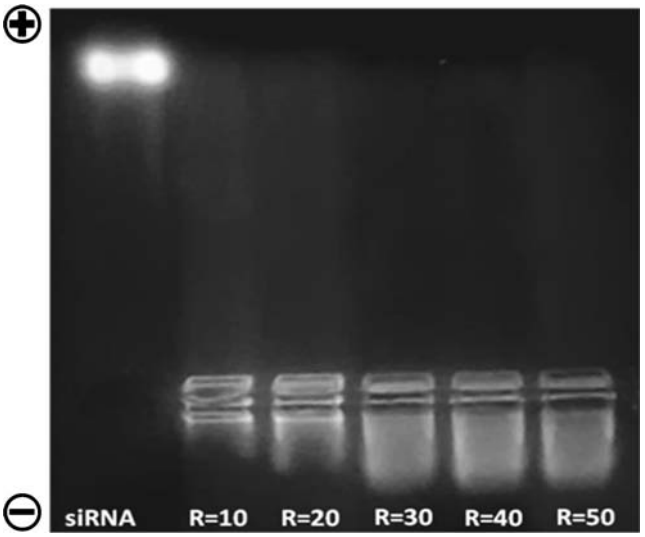


**Fig. 10** Gel electrophoresis of IC-SPIONs/siRNA magnetoplexes.

and untreated cell culture were incubated for 24 h and 48 h in the presence of the magnet. As shown in Fig. 7a and b, the magnetic field applied both to the untreated cell culture and the cells treated with the IC-SPIONs did not affect cell viability, since the cell viability was definitely comparable to the control, indicating a good cytocompatibility of IC-SPIONs.

Furthermore, considering the cytocompatibility of IC-SPIONs, in order to evaluate the ability of the nanosystem to cross cell membrane, cell uptake studies of IC-SPIONs labelled with the fluorescence probe Alexa Fluor-647® were performed. HCT116 and 16HBE cells were incubated with IC-SPIONs, at a concentration of 0.1 mg/mL. After 4, 24 and 48 h, nuclei were stained with DAPI and wells observed with a fluorescence microscope. The same experiment was performed applying the permanent magnet at the bottom of the wells in order to evaluate the effect of the magnetic field on cell uptake. Figure 9 shows the fluorescence images of cells after 4, 24 (lines A, B) and 48 h (line C) of incubation with (panel b and d) and without (panel a and c) the external magnet. These studies demonstrated that IC-SPIONs were rapidly

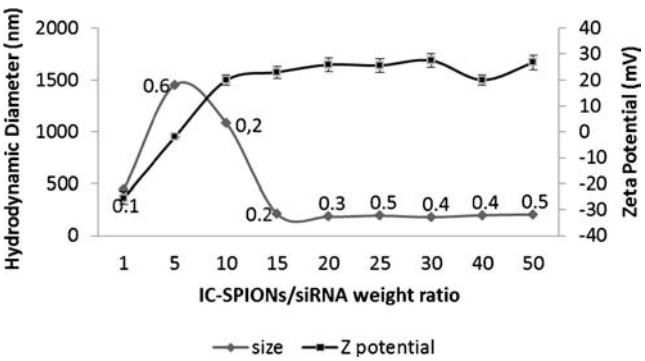


**Fig. 11** Size and Z-potential curve of magnetoplexes at various IC-SPIONs/siRNA weight ratios.

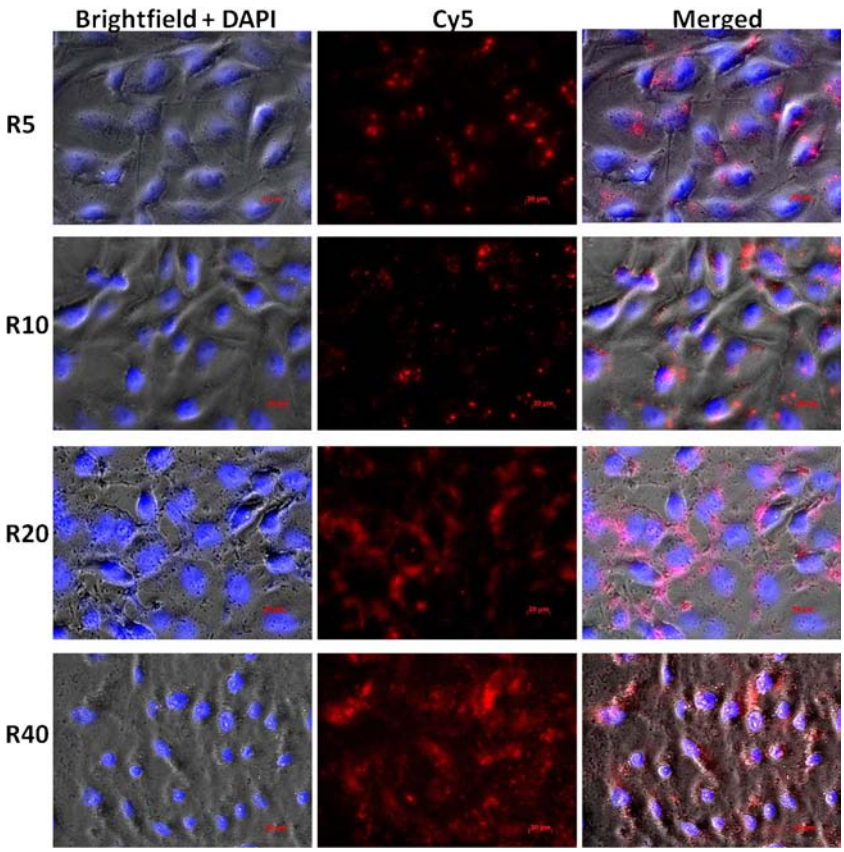


**Fig. 12** Fluorescence microscopy images of IC-SPIONS/siRNA magnetoplexes in JHH6 cells at various weight ratios (R).

accumulated in cell cytoplasm just after 4 h of incubation (see line A of Fig. 9), and no substantial differences in intracellular trafficking were observed after 24 and 48 h for both the cell lines. Nevertheless, it is interesting note that in the case of IC-SPIONs incubated with an external magnet, an increased fluorescence intensity was observed in the cell cytoplasm of both 16HBE and HCT116 cell lines, mainly after 24 and 48 h of incubation (see line B and C of Fig. 9).

This finding suggests the hypothesis that the magnetic force enhanced the IC-SPIONs uptake and created a reservoir of the nanosystem inside cells. Therefore, this system can be potentially exploited as a magnetically targeted drug delivery system, taking the advantage of the powerful accumulation into a specific area of the body, upon the application of an external magnet.

## Characterization of IC-SPIONs/siRNA Magnetoplexes

Biophysical properties of polyplexes such as size, shape and the surface charge are very important to determine their interaction with biological environment. In effect, size and surface properties play a crucial role for the passage across biological barriers, as well as for efficient cell uptake, including the polyplex ability to load the active nucleotides (23). In principle, the ability of IC-SPIONs to electrostatically bind the negatively charged siRNA, was demonstrated by electrophoresis analysis on agarose gel. The outcome of the electrophoresis is visible in the Fig. 10: IC-SPIONs was able to arrest the electrophoresis run of siRNA starting from IC-SPIONs/siRNA weight ratio of 10. Increasing the weight ratio (R) of IC-SPIONs/siRNA the magnetoplexes migrate towards the negative pole of the electric field due to the formation of magnetoplexes with a high positive charge surface. In accordance with Z potential measures, for high complexation's weight ratios, the Z potential of magnetoplexes reaches high positive values up to $25 \pm 4$ mV.

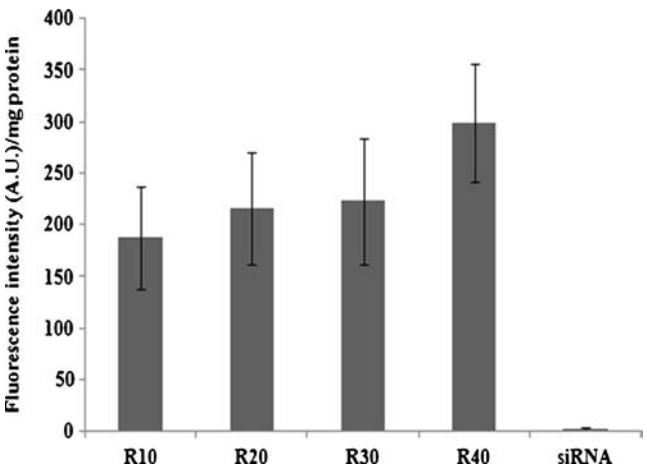


**Fig. 13** Quantitative uptake of IC-SPIONS/siRNA magnetoplexes in JHH6 cells at various weight ratios (R).

On the other hand, the characterization of obtained polyplexes by DLS revealed that IC-SPIONs form stable magnetoplexes at IC-SPIONs/siRNA weight ratios greater than 15, with a size distribution in the range between about 170 and 200 nm (see graph of Fig. 11) and high value of external charge (Zeta-potential = 25 ± 4 mV). In fact, starting from a value of −25 mV, relative to IC-SPIONs/siRNA weight ratio of 1, Z-potential becomes zero at a weight ratio equal to 5 and, consequently, aggregation phenomena of the complexes are well visible.

As IC-SPIONs magnetic nanoparticles showed promising positive attributes for biomedical application, such good physical stability, absence of cell toxicity and high siRNA condensation ability, further investigation were encouraged with the aim to understand the role of IC-SPIONs as siRNA delivery vehicle in achieving enhanced cells penetration and transfection under the effect of a magnetic field as well.

Firstly, uptake studies were performed using undifferentiated hepatocellular carcinoma JHH6 model cell line. This cell line is in fact commonly used to investigate the effect associated to the uptake of the nanocarriers (24,25). The results of this study, are shown in Fig. 12. IC-SPIONs/siRNA magnetoplexes, prepared by using a fluorescent labeled siRNA, are able to be efficiently internalized in the used cells after 4 h incubation and notably, the efficiency of the uptake increase with increasing IC-SPIONs/siRNA weight ratio up to R40. The quantization of siRNA uptake was determined by measuring the fluorescence intensity of the fluorescent labeled siRNA into cells. Obtained results revealed that from R30 to R40 no statistically significant increase of uptake is visible, indicating that approximately at R30 the highest efficiency of the system in deliver the siRNA is already reached (these results are reported in Fig. 13).

To evaluate magnetofection, magnetoplexes (at various weight ratios ranging from 10 to 40) were added to JHH6 and 16HBE luciferase expressing cells, after placing magnets under the wells.

Systems and naked siRNA (the latter used as negative control) at the same concentration used in IC-SPIONs/siRNA magnetoplexes, were incubated with cells for 4 h at 37°C. Subsequently, magnetoplex-containing media was replaced by fresh media and incubated for another 24 h followed by luciferase assay. The same experiment was conducted without magnet at the bottom of the wells. Results (see Fig. 14) show that in JHH6 cells R30 is the best weight ratio to obtain a luciferase down regulation, even if the presence of a magnetic

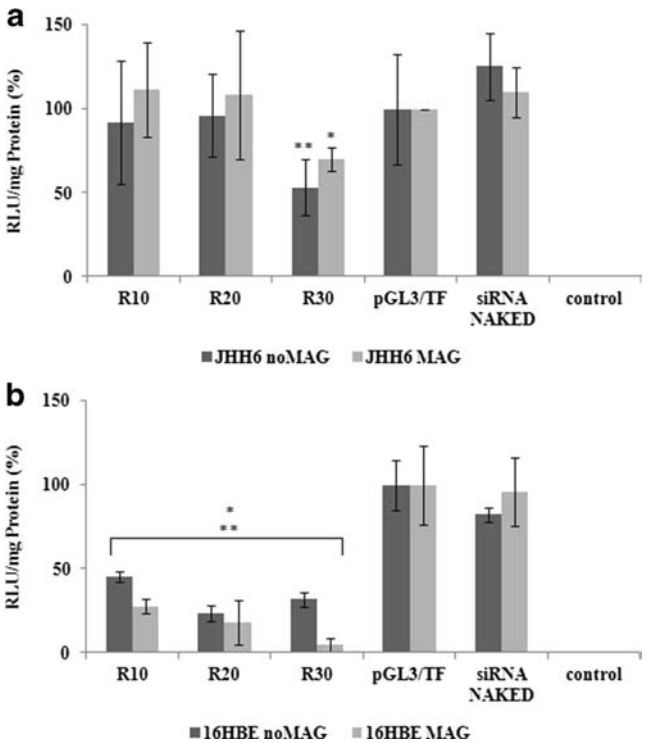


**Fig. 14** Transfection of IC-SPIONs/siRNA magnetoplexes in 16HBE and JHH6 cells at various weight ratios, with (MAG) and without (noMAG) application of magnetic field. Results are reported as relative luciferase expression per unit protein weight % (RLU/mg protein (%)). The cells that received no magnetoplexes but only luciferase encoded plasmid (pGL3) complexed with the commercial transfection reagent Turbofect (TF) were used as a reference 100% (pGL3/TF). Naked siRNA at the same concentration used in IC-SPIONs/siRNA magnetoplexes (200 nM) was used as negative control. *$p < 0.05$ respect to pGL3/TF; **$p < 0.05$ respect to siRNA Naked.

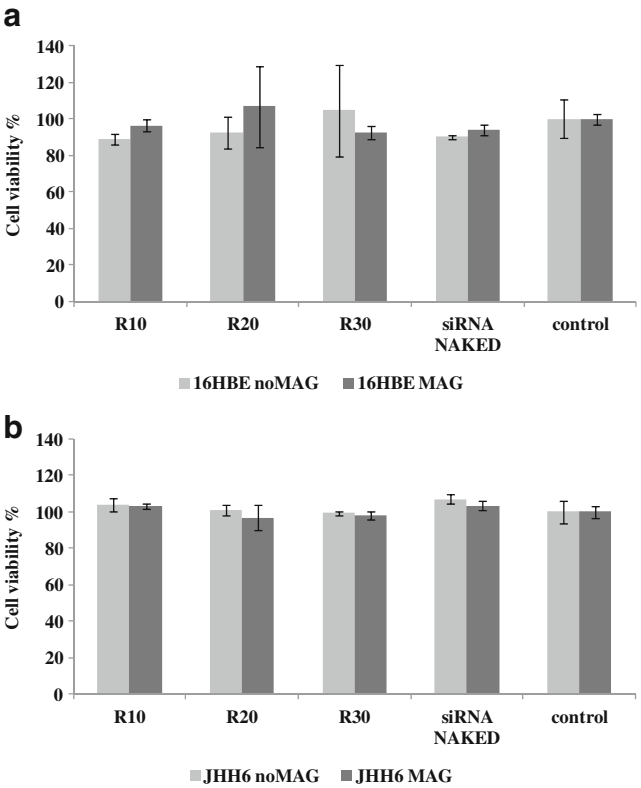


**Fig. 15** Cell viability % (MTS assay) of IC-SPIONs/siGL3 magnetoplexes at various weight ratios (R = mg dry weight IC-SPIONs/mg siRNA) and of naked siRNA at the same concentration used in IC-SPIONs/siRNA magnetoplexes (200 nM), on 16HBE and JHH6 cells after 48 h of incubation, with (MAG) and without (noMAG) application of magnetic field. Untreated cells were used as negative control with 100% viability.

field seems not improve siRNA efficacy. Differently, in 16HBE cells, R30 is still the best weight ratio to obtain a luciferase down regulation, but with an improvement of siRNA efficacy due to the presence of the magnet. Magnetoplexes, at R30 (corresponding to an applied dose of Fe/cell and siRNA/cell of 79.8 pg and 63.8 pg respectively) are able to down regulate luciferase expression up to about 70% without magnetic field, while in the presence of the magnet, transfection considerably increased, leading to a down regulation up to 95%. These results may be considered a consequence of IC-SPIONs uptake enhancement observed on 16HBE cells in the presence of an external magnet.

This could be due to different features like cellular uptake mechanism, intracellular trafficking and intracellular localization of IC-SPIONs/siRNA magnetoplexes in the different cell lines, as it is accepted opinion that this highly dynamic processes strongly depend on nanoparticle physicochemical parameters, such as charge, size, shape, material composition, and surface characteristics, but also on target cell type (25,26). Thus, these differences in the intracellular destiny of IC-SPIONs/siRNA magnetoplexes in the different cell lines could be decisive for their final effectiveness (27).

Finally, since cell uptake and processing as well as cell toxicity effects of nanomaterials can be substantially different for the magnetoplexes compared to the polymer coated MNPs itself, cytocompatibility studies on 16HBE and JHH6 cells of magnetoplexes to assess cell viability after 48 h of complexes incubation have been performed. As can be seen in Fig. 15, cell viability of both cells line is in every case not inferior to 85% respect to untreated cells, confirming that magnetoplexes mantain cytocompatibility of IC-SPIONs in the employed conditions.

## CONCLUSIONS

Polymer coated magnetic nanoparticles (IC-SPIONs) have been prepared by the spontaneous assembling of the cationic copolymer Inu-EDA on the surface of 20 nm negatively charged SPIONs in aqueous media, previously stabilized in SDS water solution. The use of Inu-EDA copolymer as coating material brought a double benefit: obtain stable inulin coated iron oxide nanoparticles in the aqueous phase, acting as hydrocolloidal coating; bind negatively charged siRNA for improving oligonucleotide transfection efficiency. IC-SPIONs here studied shown dimension of 30 nm and resulted cytocompatible on two different cell lines: 16HBE as model non tumoral cell line and HCT116, as model cancer cell line, up to a concentration of 0.5 mg/mL. It was demonstrated that, in the presence of an external magnet, the magnetic force enhanced the IC-SPIONs uptake and created a reservoir of the nanosystem inside cells. This finding support the potential application of this system as a magnetically targeted drug delivery system, taking the advantage of the powerful accumulation into a specific area of the body, upon the application of an external magnet. Moreover, IC-SPIONs was able to efficiently complex a duplexed siRNA (siGL3) down regulating luciferase and improve the oligonucleotide transfection at very low vector dose of 0.63 ng dry weight IC-SPIONs per cell, within very short incubation time of 4 h. Magnetically improved transfection was also observed in 16HBE cells under magnetofective conditions, in accordance with the IC-SPIONs uptake enhancement in the presence of an external magnet.

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