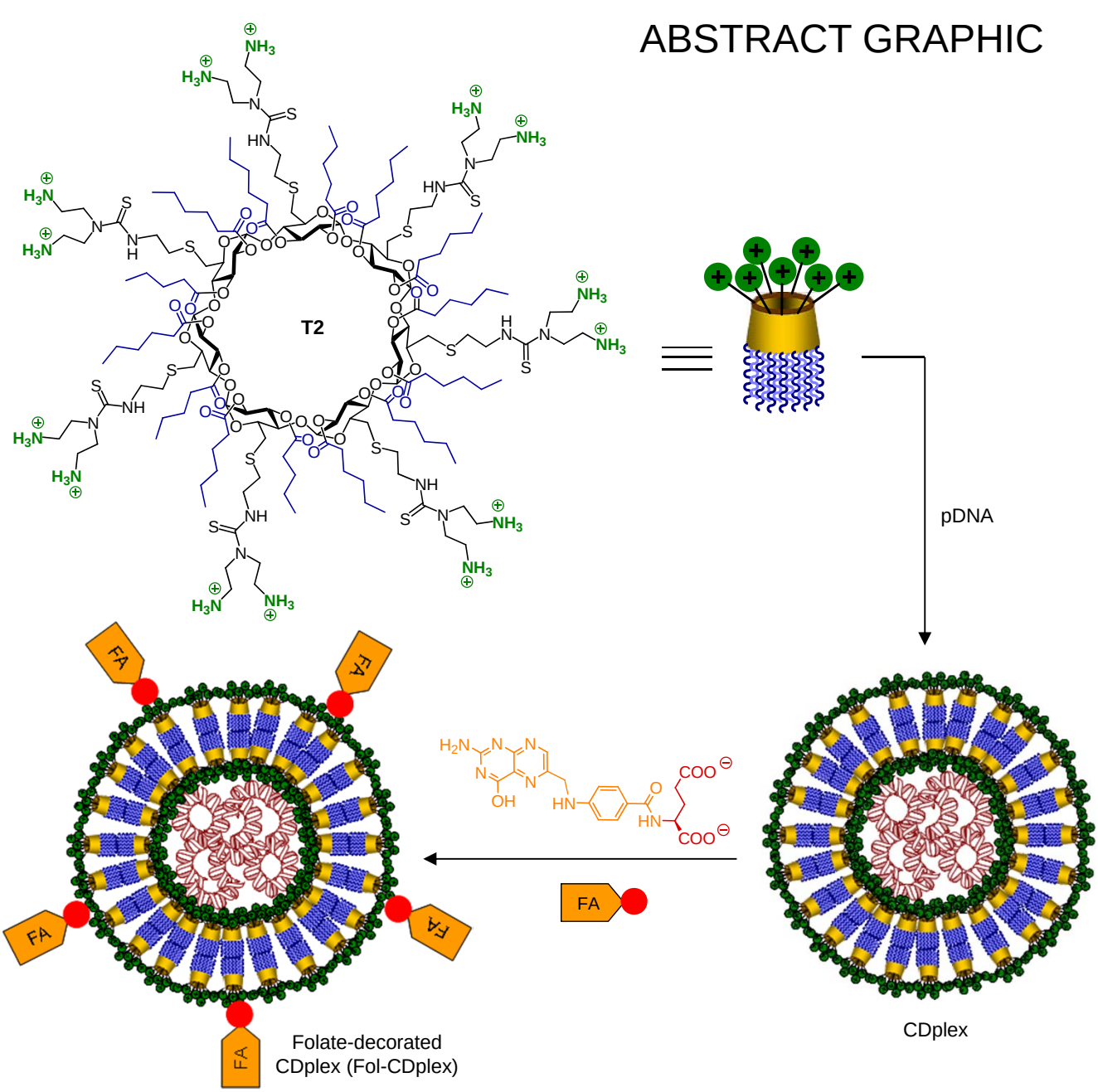

Schematic representation for preparation of the folate-decorated nanocomplexes (Fol-CDplexes) from paCD T2, plasmid DNA and folic acid.

TITLE PAGE

Title: Targeted gene delivery by new folate-polycationic amphiphilic cyclodextrin-DNA nanocomplexes in vitro and in vivo.

Authors:

Cristina Aranda[a], Koldo Urbiola[a], Alejandro Méndez Ardoy[b], José M. García Fernández[c], Carmen Ortiz Mellet [b*], Conchita Tros de Ilarduya [a*]

Affiliations:

[a] Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, 31080 Pamplona,Spain.

[b] Department of Organic Chemistry, Faculty of Chemistry, University of Seville, C/ Prof. García González 1, E-41002, Seville, Spain.

[c] Institute for Chemical Research (IIQ), CSIC – University of Seville, Avda. Américo Vespucio 49, E-41092, Seville, Spain

Full postal address of coauthors

- Aranda C. School of Pharmacy, University of Navarra, 31080 Pamplona,Spain.
- Urbiola K. School of Pharmacy, University of Navarra, 31080 Pamplona,Spain.
- Méndez-Ardoy A. Faculty of Chemistry, University of Seville, C/ Prof. García González 1, E-41002, Seville, Spain.
- García Fernández J. M. Institute for Chemical Research (IIQ), CSIC – University of Seville, Avda. Américo Vespucio 49, E-41092, Seville, Spain

***Corresponding authors:**

Dr. Conchita Tros de ILarduya

Department of Pharmacy and Pharmaceutical Technology. School of Pharmacy.

University of Navarra. 31080 Pamplona, Spain

Phone: 34-948/425600 ext. 80-6375

Fax: 34-948/425740

e-mail: ctros@unav.es

Dr. Carmen Ortiz Mellet

Department of Organic Chemistry, Faculty of Chemistry, University of Seville, C/ Prof. García González 1, E-41002, Seville, Spain.

Fax: 34-954/624960

e-mail: mellet@us.es

Running Title: Cyclodextrin-DNA nanocomplexes

Conflict of interest disclosure: There are no disclosures or any conflict of interest with commercial associations. The authors declare no competing financial interest.

ABSTRACT

Aim: Development and evaluation of a new targeted gene delivery system by first preforming self-assambled nanocomplexes from a polycationic amphiphilic cyclodextrin (paCD) and pDNA and then decorating the surface of the nanoparticles with folic acid (FA)**. Experimental Section:** The cyclodextrin derivative (T2) is a tetradecacationic structure incorporating 14 primary amino groups and 7 thioureido groups at the primary face of a cyclomaltoheptaose (β-CD) core and 14 hexanoyl chains at the secondary face. **Results and Conclusions:** T2 complexed and protected pDNA (luciferase-encoding plasmid DNA, pCMVLuc) and efficiently mediated transfection *in vitro* and *in vivo* with no associated toxicity. The combination of folic acid with CDplexes afforded ternary nanocomplexes (Fol-CDplexes) that enhanced significantly the transfection activity of pCMVLuc in human cervix adenocarcinoma HeLa cells, especially when formulated with 1 μg FA/μg DNA. The observed transfection enhancement was associated to specific folate receptor (FR)-mediated internalization of Fol-CDplexes, as corroborated by employing a receptor-deficient cell line (HepG2) and an excess of free folic acid. The *in vivo* studies, including luciferase reporter gene expression and biodistribution, indicated that 24 h after intravenous administration of the T2-pDNA nanocomplexes, transfection takes part mainly in the liver and partially in the lung. Interestingly, the corresponding Fol-CDplexes lead to an increase in the transfection activity in the lung and the liver compared to non-targeted CDplexes. Folate-CDplexes developed in this study have improved transfection efficiency and although various methods have been used for the preparation of ligand-DNA-complexes, covalent binding is usually needed and insoluble aggregates are formed unless the concentration of the components are minimized. However, the complexes developed by first time in this work were prepared by simple mixing. The synthetic

nature of this formulation provides the potencial of flexibility in terms of composition, and the capability of inexpensive and large-scale production of the complexes. These nanovectors may be an adequate alternative to viral vectors for gene therapy in the future.

KEYWORDS: Gene delivery, nanomedicine, pharmaceutics, polycationic amphiphilic cyclodextrins, folate receptors, folic acid.

INTRODUCTION

Gene therapy provides great strategies for treating different diseases as cancer [1], genetic disorders [2] and infectious diseases [3]. Gene delivery systems should be developed to save genetic material from premature degradation in systemic blood stream and to efficiently transfer the therapeutic genes to target cells. That's why, recently, research of new gene therapy vectors has been increased. In general, gene delivery vectors are divided into two principal groups: recombinant virus [4] and synthetic vectors [5]. Viral-based delivery systems like retroviruses, adenoviruses and adeno-associated viruses show high transfection efficiency. However, their limited DNA carrying capacity, expensive cost and safety concerns such as immunogenic response, toxicity or oncogenicity [6], as well as the possibility of conversion of benign virus into wild virus, are major limitations inherent to these vectors. Compared to viral vectors, non-viral (synthetic) systems are attractive alternatives for improved safety, greater flexibility and easier manufacturing. Because of that, the design of efficient and biocompatible non-viral vectors has been developed. Most of the current examples comprise two categories, namely cationic polymers and cationic lipids. However, the main problem is that these types of vectors have difficulty in obtaining high levels of expression and high selectivity in some cell lines.

Cyclodextrins (CDs) are naturally occurring cyclic oligosaccharides composed of α(1→4)-linked glucose units arising from enzymatic degradation of starch that hold a privileged position as drug delivery and controlled drug release systems. Apart from their inherent properties as nanometric containers, CDs ability to improve drug bioavailability has been suggested to benefit from two additional features: (i) their membrane absorption enhancing properties and (ii) their ability to stabilize biomolecules in physiological media by shielding them from nonspecific interactions [7]. CDs interaction with biological membranes results in the release of certain membrane components (e.g. cholesterol or phospholipids) and consequently their destabilization and permeabilization [8]. Their unique molecular inclusion properties is also an important advantage.

Although the effectiveness of the best cyclodextrin-based gene delivery systems currently developed, as for other nonviral gene vectors, remains orders of magnitude poorer compared with viral vectors, the possibility to combine covalent and supramolecular approaches offers new venues for the design of tailor-made artificial viruses. Actually, some CD-based vector formulations have reached clinical phase studies [9,10], particularly in the very active field of RNA nanotechnology for the delivery of therapeutic nucleic acids [11-13]. The role of CDs as transfection enhancers in formulations containing cationic lipids, cationic polymers or even viral vectors has been known for a long time [14-18]. However, the real potential of CDs in gene therapy has only been revealed by exploiting selective chemical functionalization tools [19]. Noteworthy, polycationic CD-based vectors were shown to self-organize in the presence of pDNA to promote compaction and safe delivery to cells [20-22]. Threading CD polycations by linear polymeric chains affords polyrotaxanes that likewise complex nucleic acids into transfectious nanoparticles [23-27].

Monodisperse polycationic amphiphilic cyclodextrins (paCDs) have been further shown to exhibit better self assembling properties in the presence of plasmid DNA (pDNA) and the resulting nanocomplexes (CDplexes) exhibited enhanced cell-membrane crossing capabilities [28,29].

In a previous work, a set of paCDs with various architectures was evaluated with a focus in cancer therapy [30]. Although some candidates exhibited promising features, the lack of selectivity of the resulting CDplexes between cancerous and healthy cells was an anticipated disadvantage. To achieve active targeting, the CDplexes should be equipped with functional molecules, which can recognize and adhere to the biomarkers on the surface of the corresponding cancer cells.

Because folic acid (FA) is essential for the biosynthesis of nucleotide bases, the vitamin is consumed in elevated quantities by proliferating cells. The attractiveness of folate has been further enhanced by its high binding affinity (Kd ≈10-10), low immunogenicity, ease of modification, small size (Mw 441,4), stability during storage, compatibility with a variety of organic and aqueous solvents, low cost and ready availability [31]. Folate receptors (FR) are frequently overexpressed on cancer cells, identifying the receptor as a potential target for a variety of ligand and antibody-directed cancer therapeutics [32,33]. These receptors are elevated in malignant tissues of the ovary, uterus, endometrium, brain, kidney, head and neck and skin, among others, but they are also present in some healthy tissues [34,35]. Folate binding to different non-viral systems for drug and gene delivery has been studied by some authors [31,36-40].

Targeted cyclodextrin-containing vectors equipped with lactose [41], mannose [42], folate [43] and transferrin ligands [44] have been studied in gene delivery. However, in all these formulations the cyclodextrin material had to be combined with another

polymer, usually PEI, to be efficient. In the absence of any helper, only the polycationic amphiphilic cyclodextrins synthetized in our group have shown to mediate site-specific gene delivery. It required the incorporation of mannosyl [45] or galactosyl [46] glycotopes onto the cyclodextrin scaffold through chemical ligation methods prior to CDplex assembly.

Aiming at developing paCD-DNA nanocomplexes with improved tumour cell-discriminating abilities for cancer gene therapy, we have now explored an alternative strategy consisting in the post-decoration of preformed CDplexes with folic acid. The β-cyclodextrin derivative T2, having a tetradecacationic structure incorporating 14 primary amino groups and 7 thioureido groups at the primary face of a cyclomaltoheptaose (β-CD) core and 14 hexanoyl chains at the secondary face, was chosen as the paCD prototype. T2 has been previously shown to efficiently complex and compact pDNA into CDplexes with positive surface potential that promoted transfection in several cell lines [30]. We hypothesized that these CDplexes would interact electrostatically with folic acid to form ternary T2:pDNA:FA nanocomplexes (Fol-CDplexes) in which the FA ligands would be exposed at the nanoparticle surface and available for molecular recognition by folate receptors at the membrane of cancer cells (Figure 1). The capabilities of the new system to promote FR-mediated transfection have been investigated in comparison with PEI formulated polyplexes.

MATERIALS AND METHODS

Materials

The polycationic amphiphilic cyclodextrin T2, synthetized as previously reported 20, was used for CDplex formulation. Polyethylenimine 25 (bPEI, MW 25 kDa, branched) was purchased from Aldrich. The plasmid pCMV-Luc VR1216 (6934

bp) encoding luciferase (Clontech, Palo Alto, CA, USA) used for transfection experiments was amplified in E. coli, isolated, and purified using Qiagen Plasmid Giga Kit (Qiagen GMBH, Hilden, Germany). Folic acid dehydrate, HEPES and D(+)-glucose were purchased from Sigma (Madrid, Spain). Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA).

Cell culture

HeLa (human cervix adenocarcinoma) and HepG2 (human hepatoblastoma) cells were obtained from American Type Culture Collection (Rockville, MD, USA) and were maintained at 37 ºC under 5% CO2 in complete medium constituted by Dulbecco's modified Eagle's medium-high glucose + glutaMAX® (Gibco BRL Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were passaged by trypsinization twice a week.

Preparation of plain and Fol-CDplexes

Plain- or targeted CDplexes were prepared by mixing the plasmid pCMVLuc and the cyclodextrin derivative T2 at protonable nitrogen/phosphate (N/P) ratio of 5. The N/P ratio refers to the number of protonable nitrogens in the CD derivative per phosphates in DNA. Complexes were prepared in the absence (plain-CDplexes) or in the presence of a variable amount of the ligand folic acid (FA) (Fol-CDplexes). They contained 5 µg/mL of DNA and were prepared in HEPES 10 mM containing 5 % (w/v) of glucose at pH 7.4. The preparations were orbitally stirred for 2 h prior to nano complex characterization or transfection experiments. Polyplexes used as control were formulated with bPEI (25 kDa) at N/P ratio of 5.

Particle size and zeta-potential measurements

The particle size of plain- and targeted nanocomplexes was measured by dynamic light scattering (DLS), and the overall charge by zeta-potential measurements, using a particle analyzer (Zeta Nano Series, Malvern Instruments, Spain). Samples of the prepared complexes were diluted in distilled water and were measured at least three times for 2 h after preparation and incubation of the complexes. Size results are given as volume distribution of the major population by the mean diameter with its standard deviation.

In vitro transfection activity

The procedure for *in vitro* transfection assays was the same for HeLa and HepG2 cell lines. Cells were seeded in medium (100.000 cells/well) in 48-well plates (Iwaki Microplate, Japan), and incubated for 24 h at 37 ºC in 5% CO2. After this, the medium was removed and 0.3 mL of complete medium (with 10% serum) and 0.2 mL of complexes (containing 1 µg of pDNA) were added to each well. After 4 h incubation the medium was replaced by complete medium and the cells were further incubated for 48 h. Cells were washed with phosphate-buffered saline (PBS) and lysed with 100 µL of Reporter Lysis Buffer (Promega, Madison, WI, USA) at room temperature for 10 min, followed by a freeze-thaw cycle. 20 µL of the supernatant was assayed for total luciferase activity using the luciferase assay reagent (Promega), according to the manufacturer's protocol. A luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Diagnóstica y Terapéutica, Barcelona, Spain) was used to measure luciferase activity. The protein content of the lysates was measured by de DC protein Assay Reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the

standard. The data were expressed as nanograms of luciferase (based on a standard curve for luciferase activity) per milligram of protein.

Cell viability

Cell viability was quantified by a modified Alamar Blue Assay. Briefly, 2 ml of 10% (v/v) Alamar blue dye in DME-HG supplemented with 10% (v/v) FBS medium were added to each well 48 h post-transfection. After 2.5 h of incubation at 37 ºC, 200 µl of the supernatant was assayed by measuring the absorbance at 570 and 600 nm. Cell viability, expressed as a percentage of control cells, was calculated according to the formula (A570 - A600) of treated cells X 100/ (A570 - A600) of control cells.

In vivo transfection studies

Female Balb-c mice (6-8 weeks of age, 20-25 grams weigh) were purchased from Harlan Ibérica Laboratories. All animals were studied in accordance with guidelines established by Directive 86/609/EEC and with the approval of the Committee on Animal Research at the University of Navarra (Pamplona, 033/00). Individual mice in groups of six were injected via the tail vein with 200 µL of Fol-CDplexes containing 60 µg of pCMV-Luc and prepared at N/P 5. Naked DNA was injected as control. Twenty four hours after injection the mice were sacrificed. The liver, heart, lungs and spleen were collected and washed with cold PBS. The organs were homogenized with 1 mL lysis buffer using an homogenizer at 5000 rpm (Mini-Beadbeater; BioSpec Products, Inc., Bartlesville, OK, USA) and centrifuged at 10000 rpm for 3 min. 20 µL of the supernatant were analysed for luciferase activity following the same procedure as for *in vitro* assays. For *in vivo* imaging over time, the plasmid pCMV-Luc and D-Luciferin (Promega, Mannhein, Germany) were used. Images were taken with an IVIS CCD camera system (Xenogen) and analyzed with the Living Image 2.6 software

package (Xenogen). Mice were anesthetized with a mixture of Xylacine and Ketamine (at a dose of 8 mg/kg and 60 mg/kg, respectively) and 150 mg/kg of D-luciferin were injected intraperitoneally. Ten minutes later, animals were placed in the dark chamber for light acquisition.

Statistical Analysis

Results are reported as the mean values ± standard deviation. Statistical analysis was performed with SPSS 15.0 (SPSS®, Chicago, IL, USA). The different formulations were compared with ANOVA (Tukey post-hoc adjust). Differences were considered statistically significant at $p<0.05$.

RESULTS

Characterization of CDplexes

The relevance of the size and surface charge in gene delivery by non-viral vectors is known. A small particle size is specially required for *in vivo* gene delivery in order to allow systemic delivery.

Plain- and Fol-CDplexes prepared at a DNA concentration of 5 µg/mL and containing different amounts of folic acid ligand were characterized in terms of size and surface charge prior to *in vitro* transfection assays. Table 1 shows the values of the particle size and the zeta-potential of complexes at N/P ratio of 5, in the absence or presence of increasing amounts of folic acid. The size and the overall charge of plain CDplexes were 254 nm and 34 mV, respectively. No significant differences were observed in the size of complexes when the amount of folic acid was 2 µg or lower. Complexes were

stable and the polydispersity index was lower than 0.3 in all cases. CDplexes formulated with 5 µg FA/µg DNA aggregated significantly. The zeta-potential of CDplexes showed clearly positive values. By increasing the amount of the ligand, lower values of zeta-potential were obtained, as expected, given that folic acid is negatively charged.

In vitro transfection activity by Fol-CDplexes

Transfection experiments in HeLa and HepG2 cells were carried out in the presence of 10% (v/v) FBS. Polyplexes prepared with bPEI were used as control. Plain (non-targeted) and Fol-CDplexes were prepared at N/P 5 and contained 1µg of pCMVLuc and different amounts of folic acid.

Figure 2 shows that Fol-CDplexes formulated with 0.5 and 1 µg of folic acid significantly enhanced transfection activity in HeLa cells, compared to the non-targeted CDplexes and to the control polyplexes prepared with bPEI ($p<0.001$, $p<0.001$, $p<0.01$ respectively). Nanocomplexes containing higher amounts of the ligand (2 µg) were slightly more effective in transfecting HeLa cells than non-targeted CDplexes. Transfection activity was maximal by Fol-CDplexes containing 1 µg FA/µg DNA, reaching a 1.7-fold higher efficiency as compared with plain-CDplexes ($p<0.001$).

Specificity of targeting to the folate receptor

In order to investigate whether or not the uptake of Fol-CDplexes is mediated via specific interaction with the folate receptor, additional experiments were performed using the FR(-) HepG2 cell line and adding an excess of free folic acid previous to the addition of folate-complexes. In HepG2 cells, the addition of the FA ligand decreased transfection performance, in agreement with the lack of folate receptors at their surface (Figure 3A). On the other hand, by blocking the receptors with an excess (5 mg/mL) of

folic acid a decrease in the transfection levels is observed by Fol-CDplexes (Figure 3B). No transfection activity was observed by naked DNA in HeLa or HepG2 cells.

Cell viability

This assay was done in order to evaluate whether plain- and Fol-CDplexes formulated with different amounts of folic acid were toxic to HeLa or HepG2 cells. The Alamar Blue Assay showed good cell viability, higher than 80% in all transfected wells, independently of the amount of the ligand and the cell line used. Plain- and Fol-CDplexes turned to be less toxic compared to polyplexes prepared with bPEI (Figure 4).

In vivo transfection activity

In order to know if Fol-CDplexes could be used for *in vivo* gene delivery, complexes were injected systemically into mice and their activity was compared with control and naked DNA. Figure 5 shows the results of transfection activity in different organs by injecting intravenously plain- and Fol-CDplexes. By increasing the amount of the FA ligand, an increase in gene expression was observed in the lung and the liver compared to plain CDplexes. Maximal transfection activity is observed in the liver. In this organ, gene expression by CDplexes containing 1µg FA/µg DNA was 2 times higher as compared with results obtained using plain-CDplexes. In the lung, a 4.6-fold increase is observed with Fol-CDplexes (1 µg FA/µg DNA) compared to nontargeted complexes. No transfection activity was detected in mice injected with naked DNA or control PBS.

In vivo biodistribution studies

High gene expression levels by CDplexes containing 0.5 and 1 µg FA/µg DNA are observed in the liver compared to control mice injected with PBS or naked DNA, where no bioluminiscence is detected in any organ (Figure 6).

DISCUSSION

A crucial limiting factor in gene therapy is the low efficiency of gene transfer with the currently available vectors, especially *in vivo*. Lack of cell discriminating capabilities is another important limitation that becomes particularly relevant in cancer-directed therapies. In an attempt to solve these problems, we have engineered targeted self-assembled nanocomplexes intended to deliver genetic material into cancerous cells. For that purpose, we examined the association of folic acid with CDplexes formulated with the *C*7-symmetric βCD derivative T2, which has been selected for this study on the basis of previous results on pDNA complexing abilities and transfection capabilities [30,47,48]. The polyamino-thioureido amphiphilic CD derivative T2 is readily accessible from commercial βCD after installation of the cysteaminyl spacer at the primary positions and multiple amine-isothiocyanate coupling as previously reported. This paCD, presenting a dendritic display of the cationic elements (Figure 1), has been shown to form stable complexes with pDNA (CDplexes), where the genetic material is fully protected from the environment that exhibit positive surface potential. We envisioned that electrostatic interactions with folic acid could be exploited to build ternary FA-T2-pDNA nanocomplexes without the need of further synthetic efforts.

The folate receptor is highly over-expressed in a variety of carcinomas and has also expression in normal tissues [35]. Actually, gene delivery via folate receptor-mediated endocytosis has been shown to be a powerful method for the specific delivery of genes

to certain cell types of tissues [37,49]. Nevertheless, one of the major limitations of folate-targeted gene therapy lies in the low rate of vector escape from intracellular compartments following folate receptor-mediated endocytosis. Since T2-formulated CDplexes have shown good cell internalization and endosome escaping abilities, assaying folate-decorated versions seemed very appealing. Additional favourable features are expected in view of the membrane absorption enhancing properties of CDs and their ability to stabilize biomolecules in physiological media by shielding them from non-specific interactions. CDs have also proven to be beneficial for increasing the stability of oligonucleotides against endonucleases or even modulating undesirable side effects such as immune stimulation [50].

The size of the complexes is a crucial parameter for efficient transfection of genes. The nanocomplexes developed in this work, formulated with 1-to-5 μg Fol/μg pDNA, were found to have nanometric size, with the exception of Fol-CDplexes containing 5 μg Fol/μg DNA, where aggregation is detected. For this formulation, the lowest value of the series in the zeta-potential is observed, which probably contribute to this result. In the range 0-1 μg an increase in the amount of folic acid resulted in no significant increase in the particle size of the targeted complexes. The small size of folic acid can contribute to this fact, making it an ideal ligand for targeted delivery (Table 1). Comparing to classical liposome systems, no extrusion process is needed to homogenize the particles. On the other hand, zeta-potential measurements showed that high concentrations of folic acid in the CDplexes are associated with a decrease in the overall charge of the Fol-CDplexes. This is due to the presence of two (α and γ) carboxyl groups in the molecular structure of the ligand. In any case, the targeted Fol-CDplexes evaluated in this work (containing 0.5, 1, 2 or 5 μg Fol/μg DNA) had a net positive zeta-potential, which may facilitate the interaction with the negatively charged outer

face of the cells. Nevertheless, previous work has demonstrated that cell internalization through specific membrane receptors can favourably compete with unspecific mechanisms for CDplexes bearing functional elements provided that they are formulated at N/P 5 [45].

Transfection data for targeted Fol-CDplexes formulated with T2 and 0.5 or 1 μg of FA per μg of DNA and the luciferase-encoding reporter gene (pCMV-Luc VR1216) at N/P 5 in HeLa cells (FR+), compared with plain (non-targeted) CDplexes and bPEI derived polyplexes, indicated a noteworthy increase in efficiency (Figure 2). Some authors have compared non-targeted cationic cyclodextrins (CDs) as gene delivery vectors for transfection to standard transfection agents (Lipofectamine or Superfect), concluding that polycationic CDs showed higher transfection efficiencies [20,51]. Further decoration with folic acid appears to facilitate the internalization of the ternary nanocomplex due to the ability of the ligand to stimulate endocytosis. It is also interesting to note that Fol-CDplexes promoted efficient transfection even in the presence of 10% serum. At this respect, it is known that negatively charged compounds present in serum often inhibited transfection activity [52] and represent a serious limitation for the use of nonviral vectors *in vivo*.

The enhancement in gene delivery by using CDplexes could be due, in part, to the membrane disturbing and macromolecule shielding effect of CDs. The stabilizing effect that CDs impart to gene delivery systems is also an important factor. Also, the cell membrane-disturbing effect of CDs can play a role, due to their capability to extract membrane components without lysing the cell, thereby making it more permeable to DNA, as put forward in a report by Aachmann and Aune describing successful gene delivery into bacteria [53]. In any case the similar particle size of plain CDplexes and Fol-CDplexes obtained by adding 0.5 or 1 μg of folic acid to the CDplexes indicates

that the increase in transfection levels obtained in HeLa cells cannot be ascribed to an increase in the particle size of the nano complexes.

Another important aspect for *in vivo* applications studied in this work is the toxicity of the CDplexes. It is known that polycationic macromolecules in general may cause destabilization of the cell membrane leading to cell lysis, although the exact mechanism of this process has not yet been fully revealed. Taking this into account, cell viability following transfection was assessed to evaluate whether CDplexes formulated with folic acid at N/P 5 were toxic to HeLa and HepG2 cells. No toxicity was observed for either plain or Fol-CDplexes, in contrast to that observed for bPEI polyplexes, which represents an important advantage for the new systems. The viability values were very similar in all cases, indicating that the differences in transfection activity between these sets of complexes are not due to different toxicities of the formulations (Figure 4).

The specificity of FR-mediated gene transfer by Fol-CDplexes was illustrated by using the cell line HepG2 (FR -) and an excess of free folic acid previous to addition of Fol-CDplexes in order to block the folate receptors. In HepG2 cells, a decrease in transfection activity is observed by Fol-CDplexes, as compared to non-targeted ones (Figure 3A). Differences in transfection in groups "0.5 µg folic acid/ µg DNA" and "1 µg folic acid /µg DNA" could be due to the bigger size and lower zeta potential of complexes formulated with 1 µg of folic acid, which lead to smaller values in transfection efficiency. In the case of blocking the folate receptors a decrease by Fol-CDplexes is observed (Figure 3B).These results support the hypothesis that the targeted CDplexes are recognized by folic acid receptors on the cell surface, which in turn facilitates receptor-mediated endocytosis. Our observations are also in accordance with previous evidence showing that folate conjugates are taken up non-destructively by mammalian cells via receptor-mediated endocytosis [39].

The extent of nanoparticle uptake *in vivo* is variable and also dependent on the size, charge, rigidity and other physicochemical properties of the particles. The *in vivo* studies based on luciferase reporter gene expression indicated that 24 h after intravenous administration of the paCD-based nanocomplexes transfection occurs mainly in the liver and only partially in the lung (Figure 5), which is in accordance with our previous data [30]. We think that, due to the small size of the particles, the capture by macrophages and Kupffer cells is avoided, so that the liver cells which are mainly transfected are hepatocytes. The presence of folic acid in the CDplexes lead to an increase in the transfection efficiency. Maybe, FR-independent processes may also contribute to this effect, e,g, the easier intracellular dissociation of the ternary FA-T2-pDNA nanocomplex in the cytoplasm, the protective effect of folate around the complex in Fol-CDplexes or a more efficient CD-membrane permeation enhancement in the lung and liver tissues. Results from the biodistribution studies showed in Figure 6, further evidenced the marked tropism of the nanocomplexes formulated with the paCD T2 to the liver.

CONCLUSIONS

In this work we have prepared self-assembled targeted transfectious nanocomplexes using a hierarchical strategy consisting in (i) complexation and compaction of the plasmid pCMVLuc with the polycationic amphiphilic cyclodextrin T2 and (ii) surface decoration of the preformed CDplexes with folic acid through supramolecular electrostatic interactions. Similarly to T2-pDNA CDplexes, the ternary FA-T2-pCMVLuc nanocomplexes (Fol-CDplexes) are efficient gene vectors for gene therapy purposes. Fol-CDplexes efficiently protected pDNA by forming monodisperse and

stable nanoparticles that efficiently promoted transfection in HeLa cells, with no associated toxicity. The new Fol-CDplexes were also efficient vectors for gene delivery *in vivo* in a mouse model, leading to relatively high transfection levels in the lung and, especially, in the liver. Interestigly, the presence of the FA ligand translates into enhanced transfection efficiency. All these features, together with the easy and relatively low synthesic cost of the appropriate derivatives, facile purification, robustness and stability, biocompatibility, lack of immunogenicity and safety, make the presented Fol-CDplexes optimal candidates for gene delivery and an adequate alternative to viral vectors in the future.

ACKNOWLEDGEMENTS

This work was financially supported by the University of Navarra Foundation (FUN), the Spanish Ministerio de Economía y Competitividad (contract numbers CTQ2010-15848 and SAF2010-15670; cofinanced with the Fondo Europeo de Desarrollo Regional FEDER) and the Junta de Andalucía. A.M.-A. was a FPU fellow (Spanish Ministerio de Economía y Competitividad – Fondo Social Europeo). The CITIUS (University of Seville) is also acknowledge for technical support.

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Table(s)

Table 1. Influence of the amount of the ligand on the particle size and zeta potential of CDplexes. Complexes were prepared at N/P 5 with 5 μg/mL of pCMVLuc. Results are expressed as the mean ± s.d. of three independent experiments.

μg FA/ μg DNA	Size (nm)	Zeta potential (mV)
0	254 ± 23	34 ± 3
0.1	204 ± 10	28 ± 2
0.5	215 ± 8	20 ± 2
1	263 ± 12	14 ± 1
2	308 ± 15	12 ± 1
5	3554 ± 870	7 ± 1

Figure 1

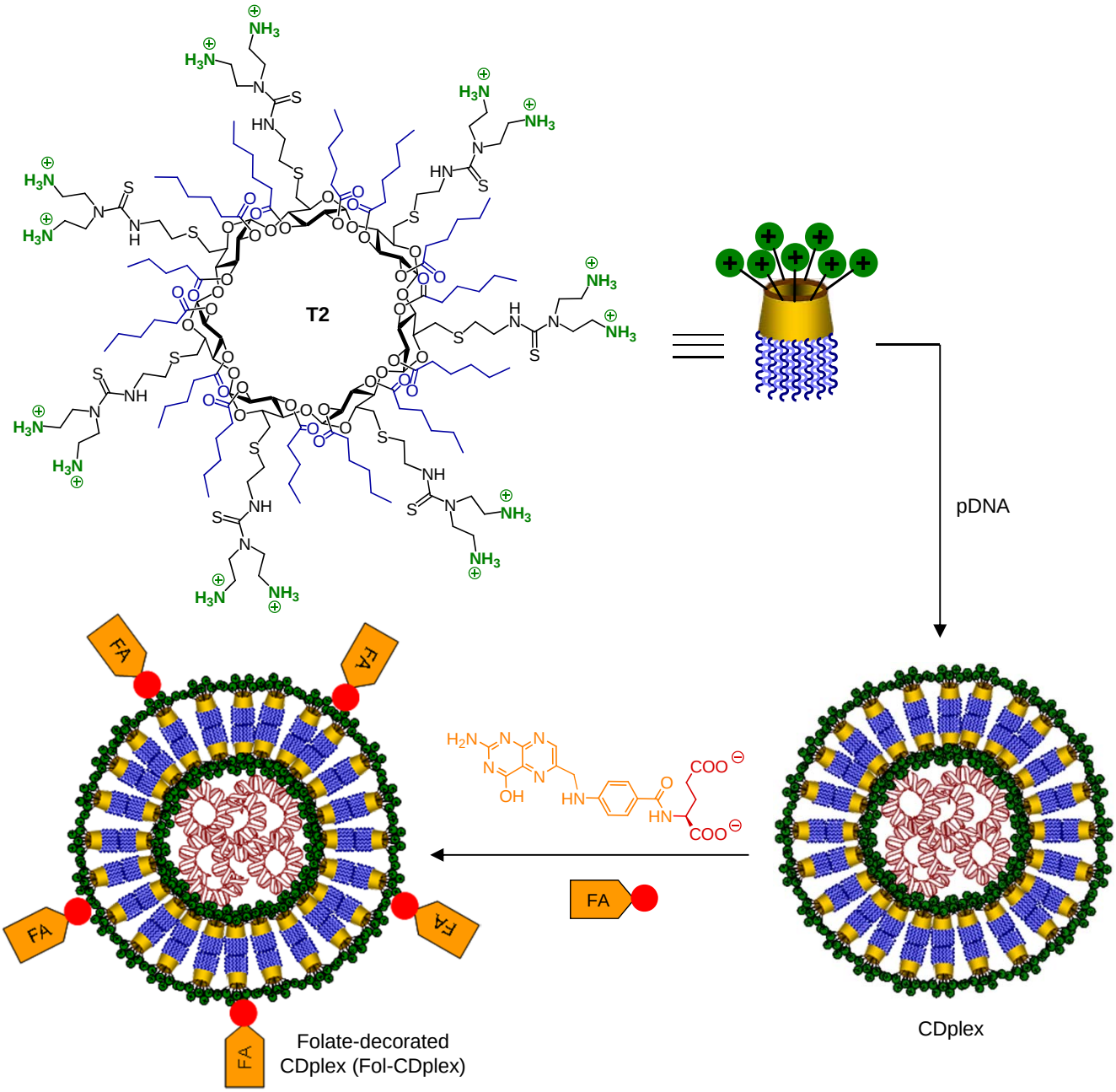


Figure 1. Schematic representation for preparation of the folate-decorated nanocomplexes (Fol-CDplexes) from paCD T2, plasmid DNA and folic acid (FA).

Figure 2

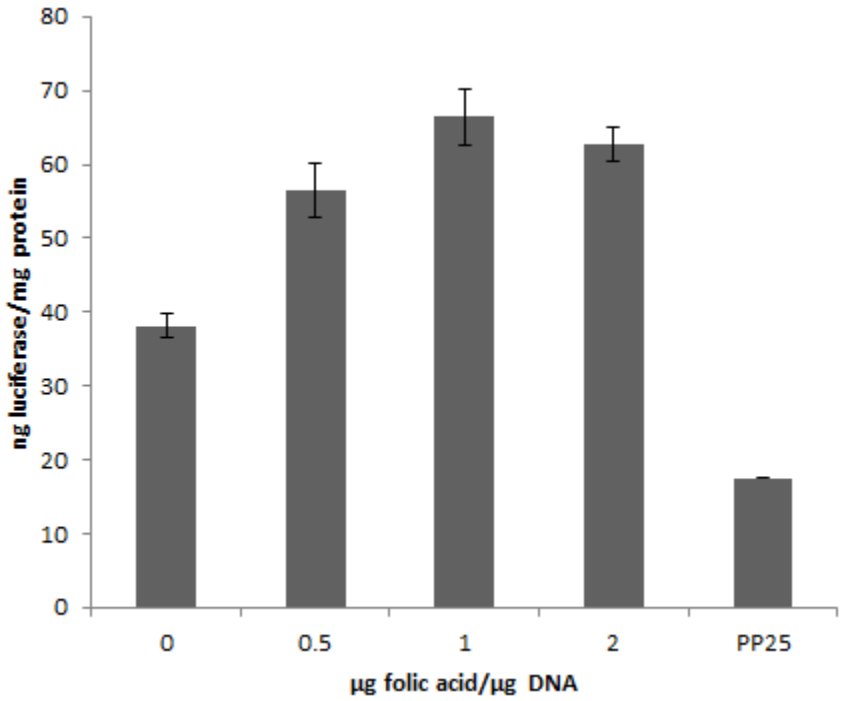
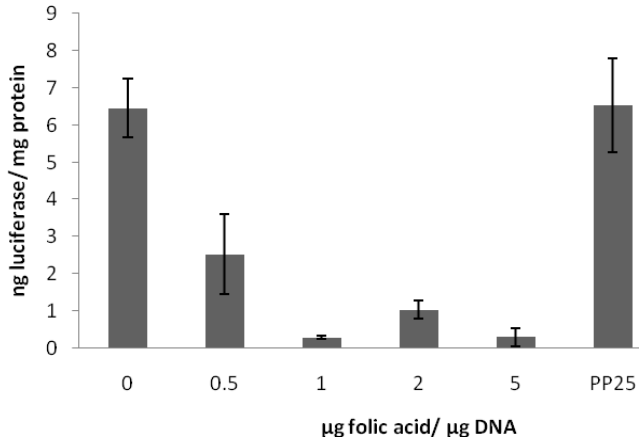


Figure 2. Transfection of HeLa cells with plain- and Fol-CDplexes prepared at N/P 5 containing 1 µg of DNA. PEI25-pCMVLuc polyplexes were used as control (PP25). The data represent the mean ± s.d. of three wells and are representative of three independent experiments.

FIGURE 3

A)



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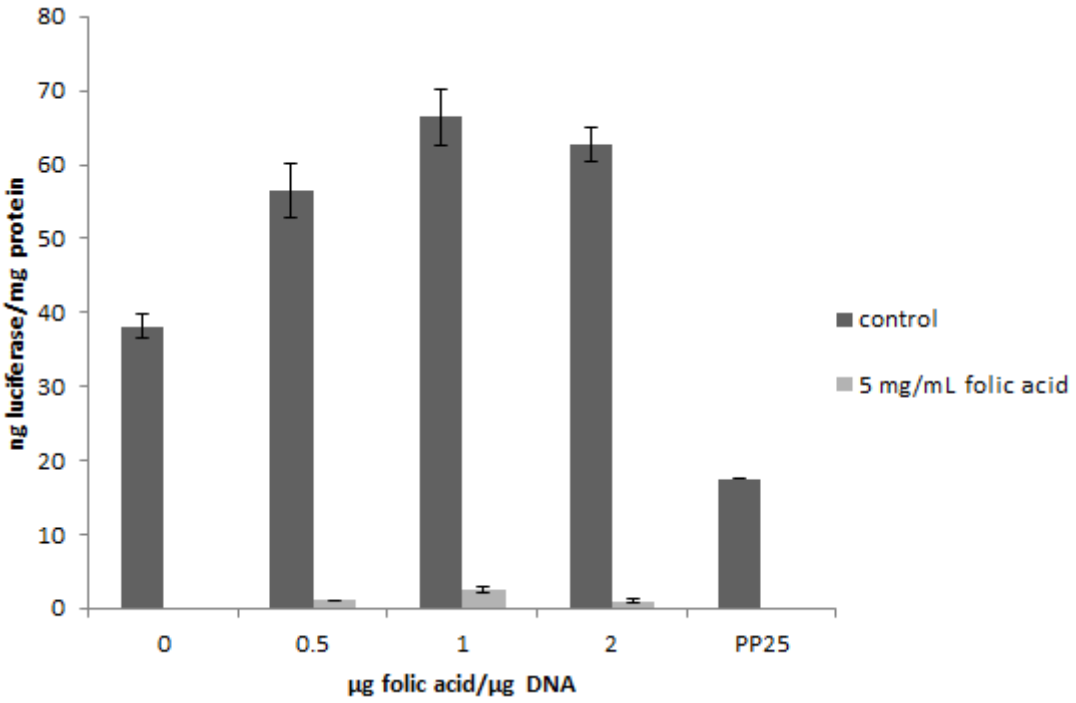

Figure 3. Transfection of HepG2 (FR-)(A) and HeLa cells in the presence of an excess of folic acid (B) with plain- and Fol-CDplexes prepared at N/P 5 containing 1 µg of DNA. PEI25-pCMVLuc polyplexes were used as control (PP25). The data represent the mean ± s.d. of three wells and are representative of three independent experiments.

Figure 4

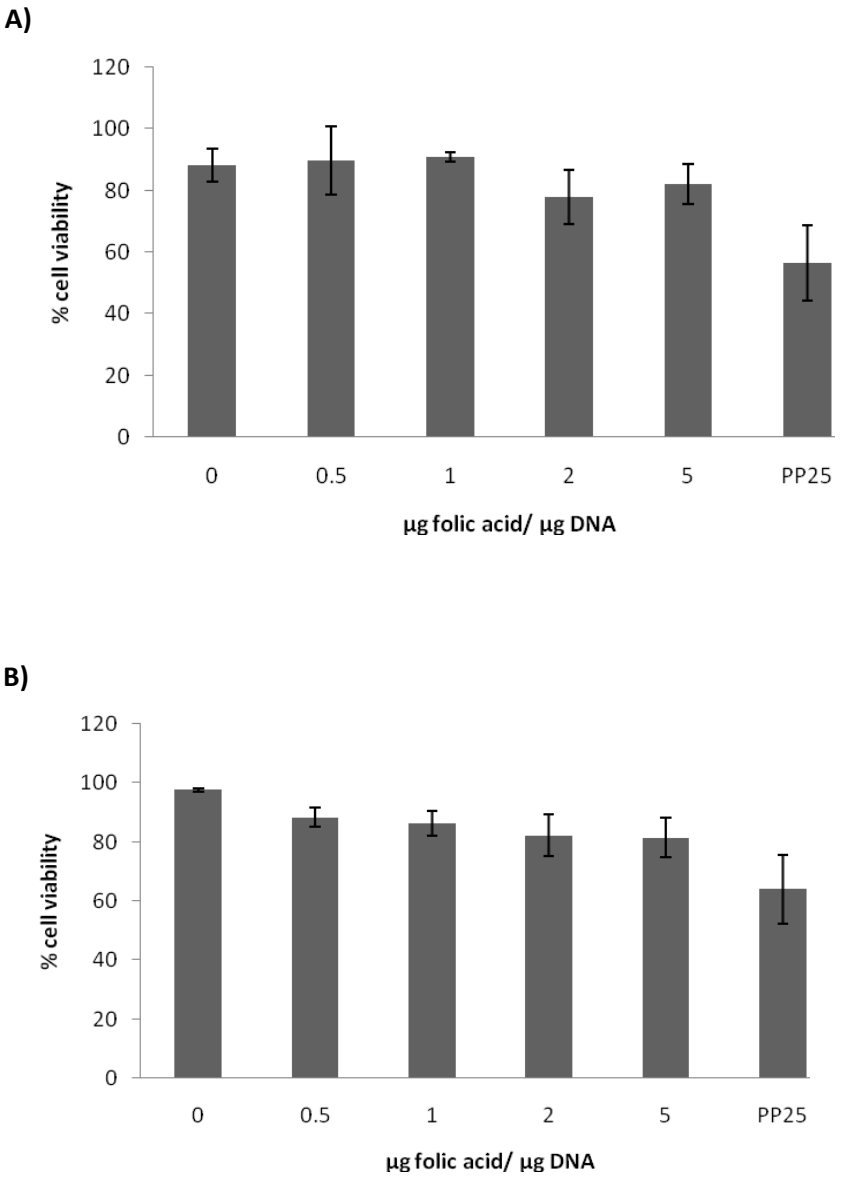

Figure 4. Cell viability. Effect of plain- and Fol-CDplexes on the viability of HeLa (A) and HepG2 (B) cells. PEI25-pCMVLuc polyplexes were used as control (PP25). The dose of the gene carrier per well was 1 µg of plasmid DNA.The data represent the mean ± s.d. of three wells and are representative of three independent experiments.

FIGURE 5

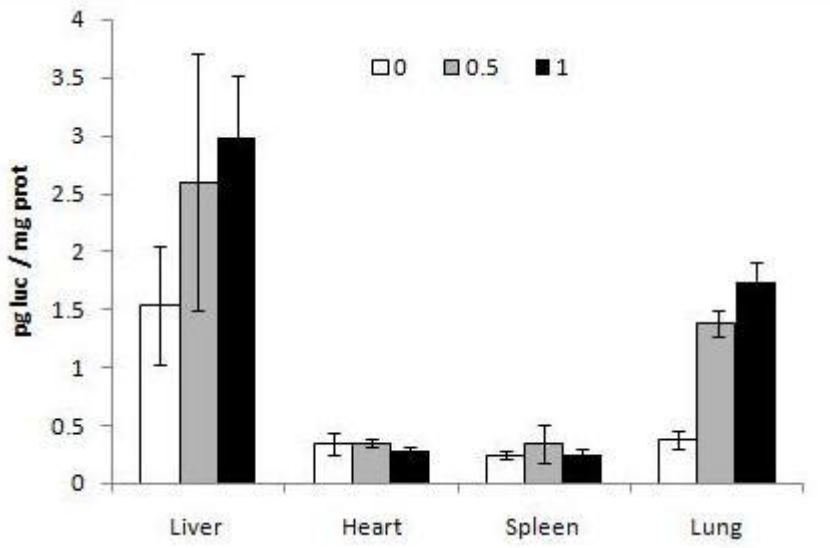

Figure 5. In vivo gene expression of plain- and folate-CDplexes containing 0, 0.5 and 1 µg FA/µg DNA and 60 µg of CMVLuc. Bars represent the mean ± s.d.(n= 6 animals).

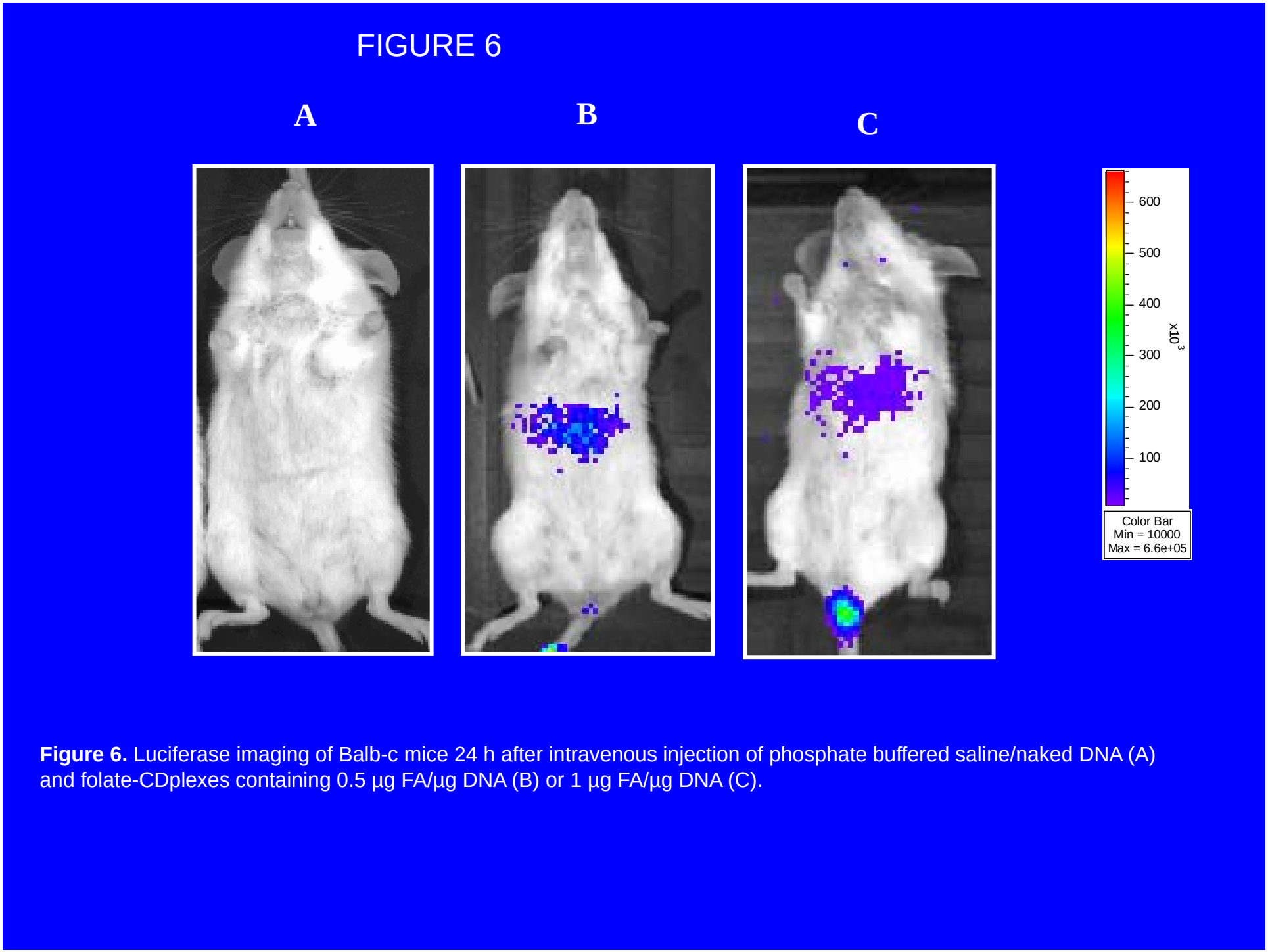

Figure 6. Luciferase imaging of Balb-c mice 24 h after intravenous injection of phosphate buffered saline/naked DNA (A) and folate-CDplexes containing 0.5 µg FA/µg DNA (B) or 1 µg FA/µg DNA (C).

FIGURE LEGENDS

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