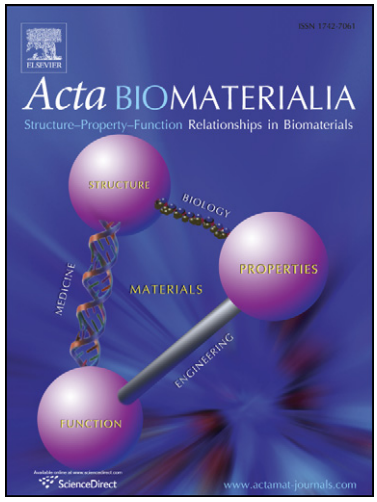

Cationic liposomes-DNA complexes as gene delivery vectors: Development and behaviour towards bone-like cells

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Cationic liposomes-DNA complexes as gene delivery vectors: Development and behaviour towards bone-like cells.

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Abstract

Modulation of biological pathways responsible for fracture repair and osteogenisis may accelerate regeneration. Gene therapy is an alternative method for the release of osteogenisis stimulating proteins into tissues. The development of vectors for gene release is still a problem in terms of ethics and techniques. In this work it was evaluated whether cationic liposomes constitute a valuable strategy for the release of genetic material into bone tissue cells as non viral vectors. Liposomes have been prepared with 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) - 2-Dioleoyl-sn-Glycero-3-Phosphatidylethanolamine (DOPE) and DOTAP-Cholesterol, and characterised according to their size, zeta potential, DNA protection capacity and citotoxicity. Transfection studies have also been carried out using pCMVβgal plasmid in two osteoblastic cell lines (MG63 and MC3T3-E1) and in the 294T line, varying the charge ratio and the DNA applied dose. Inclusion of trasferrin to increase the expression was also tested.

Results suggest that there is a great dependency between the transfection activity and the lipidic formulation, charge ratios of the complexes, applied DNA dose and cell type. There were even some differences concerning both osteoblastic lines under study. The cells of the MC3T3-E1 line present greater expression levels than the cells of the MG-63 line. The conjugation of the transferrin with the complexes contributes for the increase of the transfection levels, possibly due to an increase of internalisation of complexes being therefore a good strategy to induce the expression of specific gene in osteoblastic-like cells

1. Introduction

The molecular mechanism underlying bone formation and repair is a complex and highly coordinated process that is not completely known. Despite that, bone is one the few organs that retains the potencial for regeneration into adult life. Fracture repair and segmental bone defects healing are commonly attempted procedures in orthopaedic surgery. Aged induced bone illnesses like osteoporosis, may produce bone defects whose dimensions limit self-repairing mechanisms [1,2]. There are many clinical options to treat bone defects including bone allograft, bone autograft, biomaterials implants and amputation. However, there are many problems associated with these procedures: lack of sufficient material to bone autografts; risk of pathologies transmission and impaired immune responses in bone allograft and xenographs, biomaterials failure and biological incompatibility [3-5]. These difficulties have resulted in the search for other methods to repair skeletal defects, namely gene therapy. Although the molecular mechanism underlying bone formation remain to be defined, it is known that bone morphogenic proteins (BMP's) are essential to osteoinduction, which is the activation of various growth factors to attract osteoblasts to the repair site and induce them to produce bone [6]. In such cases, BMPs have been reported to be effective in enhancing bone deposition. However, several problems are associated with it, in particular: requirement of large doses; short half-life and thus short-term bioavailability and lack of a practical method for sustained delivery of these exogenous proteins [5,7]. Gene therapy could provide an alternative method for the delivery of BMP protein into tissues thus stimulating osteogenesis.

Gene therapy is defined as the introduction of exogenous genetic material into cells or tissues in order to cure a disease or to avoid associated symptoms [8]. Research in osteogenic factor delivery for bone repair has elucidated endogenous BMP production by transfected cells at the fracture site to be more efficient than exogenous delivery of recombinant proteins, as indicated by the smaller amount of BMP required to stimulate healing [9,10]. Both non-viral and viral vectors have been used to mediate the transfer of genes encoding bone morphogenic proteins into target cells, aiming at promoting bone regeneration. Among other, crucial requirements for gene delivery vectors include the ability to accommodate an ilimited size of DNA; to be available in a concentrate form, to be easy to scale up; to be targeted to specific cells or tissues; to ensure high levels and long term gene expression, to be non-toxic and non-immunogenic. A number of viral delivery systems are available for gene delivery, in particular retroviral and adenoviral systems [11-14]. The usefulness of viral vectors is limited, however, by host immune and inflammatory reactions (in the case of adenovirus), difficulty of large-scale

production, size limit of the exogenous DNA (in the case of adeno-associated virus), random integration into the host genome (in the case of retroviruses), and the risks of inducing tumorigenic mutations and generating active viral particles through recombination [15,16]. In contrast to the viral-based vectors, liposome–DNA complexes and direct administration of pure DNA complexes can transfer expression cassettes nearly without size limitations. Additionally, these systems offer advantages such as proven stability under a variety of conditions, utilization in a number of delivery systems, and are less immunogenic than the viral vectors [17]. The major drawback of these systems is that gene transfer is very inefficient, requiring large quantities of materials, sustained site or repetitive administration to achieve clinical success [13].

Transfection efficiency is determined by the limiting barriers involved in this process, such as the entry of lipoplexes into the cell, their escape from the endosome, dissociation of the plasmid from the lipid, translocation into the nucleus and finally transcription of the transgene [18].

The *in vitro* transfection ability of lipoplexes depends on many parameters, such as their physico-chemical characteristics (size and zeta potential), lipid/DNA charge ratio, type of cells, incubation conditions and mode of lipoplex preparation [19]. Recently it has been demonstrated that the association of transferrin (Tf) with cationic liposomes followed by complexation with DNA promotes a significant enhancement of transfection as compared to conventional lipoplexes [20,21]. In these studies Tf-lipoplexes encapsulation efficiency, size and zeta potential were characterized.

In this work, the potential of cationic liposomes-DNA complexes associated or not to transferrin to mediate gene transfer into osteoblastic-like cells (MG-63 and MC3T3-E1 cells lines) was evaluated using 293T cells as positive control. Transfection activity, cytotoxicity and dependence of DNA dose of non-viral plasmid gene transfection were evaluated using two different lipoplex formulations: DOTAP-DOPE and DOTAP-CHOL. Lipoplexes were produced and characterized in terms of size, charge ratio (lipid/DNA) and DNA protection capacity.

2. Materials and methods

2.1. Preparation of cationic liposomes and their complexes with DNA

Small unilamellar cationic liposomes (SUV) were prepared from a 1:1 (mol ratio) mixture of 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) and 2-Dioleoyl-sn-Glycero-3-Phosphatidylethanolamine (DOPE) or cholesterol (Chol), by extrusion of multilamellar liposomes

(MLV). Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) dissolved in $CHCl_3$ were mixed at the desired molar ratio and dried under nitrogen. Remaining solvent was removed under vacuum for 8 h. The dried lipid films were hydrated with de-ionized water to a final lipid concentration of 4 µM. The tube was vortexed for 10 minutes and the resulting MLV were then sonicated, for 3 min, and extruded, 21 times, through two polycarbonate filters of 50 nm pore diameter using a mini-extruder (Avanitilipids). The resulting liposomes (SUV) were then diluted five times with deionized water. The cationic phospholipid concentration was determined by Fiske-Subbarow Method and Infinity Cholesterol Liquid Stable Reagent (Thermo Electron Corporation). Liposomes were stored at 4ºC under nitrogen and were used within 1 month after preparation.

Complexes were prepared by sequentially mixing 100 µl of a Hepes-buffered saline solution (HBS) (100 mM NaCl, 20 mM HEPES, pH 7.4), with liposomes (volume was dependent on the (+/-) charge ratio) and with 100 µl of HBS solution containing 1 µg of pCMVlacZ plasmid. The mixture was further incubated for 15 min at room temperature. The Tf-lipoplexes were obtained by gently mixing 100 µl of HBS with liposomes, with 100 µl of human transferrin solution (320 µg/ml of HBS) (Holo-transferrin Human, Sigma-Aldrich), 15 min prior to the addition of 100 µl DNA solution, and the resulting mixture was further incubated for 15 min. Lipoplexes were prepared immediately before experiments. Complexes prepared from the commercially available formulations (Lipofectamin® - Invitrogen) were obtained in a similar manner to that described above.

2.2. Physico-chemical characterization of complexes

Complexes were characterized with respect to their size and zeta potential using a Coulter N4 Plus and a Coulter DELSA 440 (Coulter Corporation, Miami, FL), respectively. The Coulter N4 Plus is a PCS (photon correlation spectroscopy) based technique, which uses autocorrelation spectroscopy of scattered laser light to determine its time dependent fluctuations resulting from the Brownian motion of the particles and hence their size. The detection angle is fixed at 90º. The DELSA 440 is a laser-based multiangle particle electrophoresis analyser that measures the electrophoretic mobility and zeta potential distribution simultaneously with the hydrodynamic size of particles in suspension. Cationic liposome/DNA complexes were prepared immediately before analysis. Samples of the prepared complexes were placed in the measuring cell, whose position was adjusted to cover a previously determined stationary layer, and an electric current of 4.0 mA was applied. Measurements were recorded and the zeta potential was calculated for each scattering angle (8.6º, 17.1º, 25.6º and 34.2º).

2.3. Cells culture and maintenance

MG-63 and MC3T3-E1 were grown in Minimal Essential Medium – alpha medium (α-MEM) with 10% (v/v) foetal bovine serum (FBS), fungizone (1%), penicillin (100 U/ml), streptomycin (100 µg/ ml) and gentamicine (50 µg/ml). The 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) FBS heat-inactivated, penicillin (100 U/ml) and streptomycin (100 µg/ ml). The cells were maintained in a humidified atmosphere, with 5% CO_2, at 37 ºC.

2.4. Transfection studies

The cells were seeded 24h prior to transfection on 48-well culture plates at 50–60% confluence in 1 ml of their usual medium. On the day of transfection, cells were rinsed twice with serum-free medium and then covered with 0.3 ml of medium, without serum, before lipid/DNA complexes were added. Lipid/DNA complexes were added gently to cells in a volume of 0.2 ml per well. After 4 h incubation (in 5% CO2 at 37 ºC), the medium was replaced with 1 ml of complete medium, and the cells were further incubated for 48 h. The cells were then washed twice with phosphate-buffered saline solution (PBS) and 100 µl of lysis buffer (0,1%w/v Triton-X, 0.25 M Tris-base, pH = 8) were added to each well and incubated overnight at –80ºC. The cell lysate was centrifuged for 15 min at 14 000 rpm to pellet debris. In a 96 well culture plates was added sequentially 30 µl of PBS, 20 µl of the supernatant and 150 µl of ONPG solution (ONPG (1,5 mg/ml in 60 mM of di-basic phosphate buffer), 1 mM $MgCl_2$, 10 mM KCl, and 50 mM βmercaptoethanol, pH=8). Further incubation for 1 h at 37º C, the absorbance at 420 nm was measured (Sunrise, Bio-Rad). The data were expressed as µg of β-gal (based on standard curve for β-galactosidase activity) per mg of protein. The protein content of the lysates was measured by BCA protein assay Kit (Pierce).

Transfected cells that express β-galactosidase can be visualised on optic microscope. Cells were fixed and stained with X-gal substrate (5-bromo-4-chloro-3-indoyl-β-D-galoctoside) developing a blue colour when transfected.

2.5. Assessment of DNA Protection

The resistance of cationic liposome/DNA complexes to DNase I and the accessibility of ethidium bromide to the DNA associated with the complexes (Sigma) was monitored at 37 ºC, for 2 min, in a SPEX Fluorolog 2 fluorometer (SPEX Industries, Edison, NJ). The fluorescence was read at excitation

and emission wavelengths of 518 and 605 nm, respectively, using 1 mm excitation and 2 mm emission slits. The fluorescence scale was calibrated such that the initial fluorescence of EtBr (20 µl of a 2.5 mM solution added to a cuvette containing 2 ml HBS solution) was set at residual fluorescence.The value of fluorescence obtained upon addition of 1 µg DNA (control) was set as 100%. DNase I was maintained in a buffer solution (50 mM Tris–HCl (pH = 7.5); 10 mM $MnCl_2$; 50 µg/ml BSA). Cationic liposome/DNA complexes were submitted to DNase I action (5 units DNase I/µg of DNA), during 30 min to 37 ºC, followed by inactivation of the enzyme upon incubation with 0.5 M EDTA (1 µl/unit of DNase I). Parallel experiments were performed by incubating samples under the same experimental conditions, except that DNase I was previously inactivated. Following treatment of the complexes with DNase I (active or inactive) complexes were added to the cuvette containing 2 ml HBS solution followed by addition of 20 µl of EtBr. The amount of DNA available to interact with the probe was calculated by subtracting the values of residual fluorescence from those obtained for the samples and expressed as the percentage of the control. All experiments were carried out with and without Triton 10%.

The amount of DNA available to interact with the probe is calculated by subtracting the values of residual fluorescence from those obtained for the samples. Considering that the fluorescence emited by DNA, in the presence of triton and inactive Dnase I corresponds to total protection of DNA, the percentage of protected DNA was calculated in the presence of triton and active Dnase I.

2.6. Cytotoxicity assay

The percentage of viable cells was determined by the MTT test, 48 hours after the process of transfection, in which the cells were in contact with the complexes during 4 hours. After these 48 hours, the medium was substituted by medium without phenol red containing 10% FBS and 20 µl of 0.5% (w/w) MTT in PBS solution and then incubated for 3 h to allow the production of formazan crystal. This was dissolved by adding 200 ml of dimethylsulfoxide (DMSO). After centrifugation at 13.000 rpm during 2 minutes, the supernatant was transferred to 96 well plates and the optical density was determined at 540 nm. The 100% value was obtained from the OD value measured in non-transfected cells.

2.7. Statistical analysis

All the data presented represents mean ± standard deviation, obtained from triplicates of three independent experiments, except for the amount of complexed DNA which represents mean ±S.D, obtained form triplicate wells of two independent experiments. The statistical analysis was performed using multiple parameters analysis ANOVA , with a significance level of P(0.05).

3. Results

3.1. Physico-chemical characterization of the complexes

Transfection efficacy mediated by lipoplexes delivered genetic material efficiency into target cells is strongly dependent on their physico-chemical properties.

Fig. 1 and 2 illustrates the particles average size and zeta potential of DOTAP-DOPE/DNA and DOTAP-Chol liposomes and respective complexes when prepared at different lipid/DNA (+/-) charge ratios and in the presence or absence of transferrin. As it may be observed, the zeta potential values of DOTAP-DOPE liposomes are lower than those of DOTAP-Chol. Addition of transferrin to the liposomes led to a decrease of the zeta potential, this effect being more pronounced upon further complexation with DNA. Increasing the amount of cationic liposomes with respect to a fixed amount of DNA resulted in an increase of the overall charge of the complexes as evaluated by zeta potential measurements. The highest values were obtained in lipoplexes prepared at 4/1 charge ratio. For the same charge ratio, lipoplexes prepared with transferrin are more negative than plain lipoplexes. Except for 1/2 charge ratio, all lipoplexes prepared with DOTAP-Cholesterol had zeta potential values higher than those prepared with DOTAP-DOPE.

Free liposomes, liposomes with transferrin and complexes prepared at 1/2, 3/2, 2/1 e 4/1 charge ratios (+/-) exhibited mean diameters ranging from 180 to 300 nm, while lipoplexes prepared at 1/1, 1/1 Tf, 3/2 Tf e 2/1 Tf exhibited larger mean diameters, between 650 and 1150 nm.

3.2. Assessment of DNA Protection

DNA protection was estimated from the access of the ethidium bromide to the DNA of the complexes, after incubation with 10 unities of DNase I per microgram of DNA. The results presented in Figure 3 show that the resistance of cationic liposome/DNA complexes to DNase I is proportional to the increase of lipid/DNA charge ratio of the complexes. For the same charge ratio, the complexes prepared with transferrin presented lower ability to protect DNA. The highest levels of protection were

reached for the complexes prepared at 4/1 (+/-) charge ratios. Complexes prepared from DOTAP-DOPE presented higher levels of protection than those prepared from DOTAP-Cholesterol.

3.4. Cytotoxicity assay

The toxicity of the complexes was assessed by the MTT assay. For this purpose, the cells were incubated with the complexes for 4 hours and cell viability evaluated after 48 hours. Since toxicity of the lipoplexes is considered a crucial limiting factor for their use in gene therapy [22] cell viability was analyzed in three cell lines (MG-63, MC3T3-E1 and 293T) using different lipid/DNA charge ratios prepared in the presence or absence of transferrin (1/2, 1/1, 3/2, 2/1, 4/1, 3/2 Tf and 2/1 Tf). No significant differences occurred in terms of cytotoxicity independently of the lipid composition tested (DOTAP-DOPE or DOTAP-Cholesterol). Cell viability was higher than 80% for all charge ratios tested for the three cell lines, except for complexes prepared at 4/1 (+/-) charge ratios (Data not shown).

3.5. Transfection studies

Gene expression was evaluated in terms of transfection activity using lipoplexes prepared with DOTAP-DOPE (1:1) and DOTAP-Cholesterol (1:1). Each formulation was tested at 1/2, 1/1, 3/2, 2/1 e 4/1 lipid/DNA charge ratio. In order to optimize the system, the transfection activity was determined with ternary complexes (Tf-lipoplexes), upon association of 32 μg of transferin/ μg of DNA to cationic liposome/DNA complexes (binary complexes or lipoplexes). MG-63 and MC3T3-E1 cells are osteoblastic-like cells, originated from human osteosarcoma and from calvary of small rats, respectively. MG63 is a human osteosarcoma derived cell line, which has lost many features of osteoblasts, although it can be used in screening tests as an osteoblastic-like cell line. The 293T cells, originated from embryonic cells of human kidney, were used as positive control, since these cells are well known as easily transfectable.

Fig. 4, 5 and 6 show that transfection activity was affected by lipid composition and net charge of the complexes, presence of transferrin and cell type. The levels of transfection are higher for 293-T cells (positive control), obtaining an expression 100 times higher than on osteoblastic cells.

The levels of transfection in 293-T cells mediated by DOTAP-Cholesterol is slightly higher than that observed for DOTAP-DOPE-containing lipoplexes for all the charge ratios tested (except ½, 2/1, 4/1 and Tf-2/1). In contrast, for MG-63 cells higher levels of tansfection activity were observed when cells were transfected with DOTAP-DOPE-containing lipoplexes, whereas no significant differences between the two formulations were observed for MC3T3-E1 cells. . Transfection activity of MC3T3-1

is significantly higher than that observed for MG-63 cells, this effect being particularly relevant for the 1/1 lipid/DNA charge ratio and for all ternary complexes. Independently of the cell line, an enhancement of transfection was observed upon association of transferrin to lipoplexes, this effect this effect being more pronounced for the 2/1 and 3/2 lipid/DNA charge ratio.

The activity of complexes (associated or not to transferrin) prepared from cationic liposomes produced in our laboratory was also compared with Lipofectamine. The levels of transfection were in the same order of magnitude of plain lipoplexes, therefore significantly less active than ternary complexes.

DNA dosage is a parameter that affects transfection efficiency. In order to study the influence of the amount of DNA complexed with the liposomes in osteoblastic cells transfection, MG-63 and MC3T3-E1 cells were transfected with complexes containing 1, 2 and 4 μg of DNA. As illustrated in Fig 7, for both, MC3T3-E1 and MG63 cells a dose effect was observed for up to 2 μg of DNA, the highest levels of gene expression being observed upon transfection of the cells with ternary complexes prepared at a 2/1 lipid/DNA charge ratio.. Further increase on the amount of DNA does not seem to induce to a consistent enhancement of transfection.

To assess the percentages of cells transfected by the different liposome mixtures the βgal staining results of the transfected 293T cells are shown in Fig 8 as an example. Images concerning MG63 and MC3T3-E1 cells showed a much lower number of transfected cells.

4. Discussion

4.1 Zeta Potential and complexes size

Electrostatic interactions between cationic liposomes and DNA have a significant impact on the physico-chemical properties and biological activities of complexes [23-25]. According to the described results, complexes prepared with a ½ (+/-) charge ratio presented negative zeta potential, indicating that an excess of non-complexed exposed DNA is present, leading to a negative surface charge. The increase of the lipid content gave rise to an increase in the zeta potential value, the highest values being observed for the complexes prepared at a 4/1 (+/-) charge ratio where almost all negative charges have been neutralized by the excess of cationic lipid. For these complexes the zeta potential is very similar to that obtained for free cationic liposomes. Transferrin has a net negative global charge and therefore for the same lipid/DNA charge ratio, ternary complexes are more negative than the binary complexes. The electrostatic interaction between DNA and liposomes is triggered by negatively charged DNA

phosphate groups and positive charges of the cationic lipid DOTAP, the co-lipids used in this work (DOPE and Cholesterol) exhibiting a net neutral charge. However, according to Hirsch-Lerner [26] the presence of DOPE can cause a decrease in the positive charge. This effect is probably caused by the partial neutralization of quaternary ions of DOTAP ammonia by the negative charge of DOPE. The amine group of DOPE can also interact with the DNA phosphate groups. On the other hand although Cholesterol does not have ionogenic groups, the OH-β in C3 only exhibiting a reduced polarity. Inclusion of Cholesterol in the formulation does not contribute to the net surface charge of the resulting liposome, it cause significant de-hidratation of cationic lipid bilayer, exposing the cationic charge [26]. The results presented in this work are in accordance with this theory, since DOTAP-Cholesterol complexes presented higher net zeta potential values then those evaluated for DOTAP-DOPE containing complexes. Exception occurred in complexes with negative zeta potential (1/2 charge ratio), being DOTAP-Cholesterol more negative, probably due to an inefficient complexes formation.

A significant increase of particle size is observed upon complexation of DNA to cationic liposomes, being neutral complexes (1/1 charge ratio) significantly bigger than those that exhibiting a positive or negative net charge. This phenomenon is explained by the absence of electrostatic repulsive forces present in 1/1 complexes, that favors aggregation. Results obtained of poly-dispersion index (results not shown) also support this theory, indicating that complex population prepared in the 1/1 charge ratio is heterogeneous.

Transferrin (Tf) association significantly increased the complex size, as Tf negative charge contributes to a lower DNA condensation.

4.2. DNA protection

It is known that an increase in DNA protection results in an increase of transfection efficiency, due to the lower extent of DNA degradation by DNases. According to the results for the ½ charge ratio, 80% of DNA is non-protected, being this result also confirmed by zeta potential results that revealed negative values for these complexes. As it was expected for the other charge ratios, an increase in the lipid/DNA charge ratio leads to a higher extent of DNA protection. However, for all the lipid/DNA charge ratios tested, the protection of DNA is relatively low, being the maximum obtained for complexes prepared at the 4/1 (+/-) charge ratio. Nevertheless, it is important to refer that the highest value of DNA protection does not correlate to the higher values of transfection activity. These apparently unexpected results are most likely due to the difficulty of the complexes to undergo

dissociation, thus hampering the DNA release inside the cell. No relevant impact of the liposome composition on the extent of DNA protection was observed.

In contrast to what have been reported by other authors, that reported that association of transferrin to the lipoplexes to an increase in DNA protection by the complexes [20], our results clearly indicate that the presence of the protein render the resulting ternary complexes les efficient to protect DNA.

4.3. Citotoxicity evaluation

High doses of cationic liposomes can raise transfection efficiency, but can also lead to higher levels of cytotoxic effects. Therefore, to better evaluate this effect, 3 different cell lines (MG-63, MC3T3-E1 and 293T cells) were used to assess the potential cytotoxic effect mediated by the different formulations. In this regard, it should be emphasized that independently of the liposome composition, lipid/DNA charge ratio and of the presence or absence of transferrin, no significant effects on cell viability were observed.

4.4. Transfection activity

Transfection activity of cationic liposomes-DNA was evaluated in bone derived cell lines (MG63 and MC3T3) and a human kidney derived cell line (293T) as a positive control. MG63 cells are derived from a human osteosarcoma, widely used in biocompatibility studies *in vitro*. In these cells, a wide range of cytokines, growth factors and receptors are very similar to those usually found in normal human osteoblasts. However, the altered gene expression responsible for the tumoral phenotype can attenuate or enhance some cell phenomena, therefore not reflecting the behaviour of primary cultured osteoblasts. [27]. This is why the same transfection studies were carried out on a further cell line, MC3T3-E1, deriving from mice and typically used in *in vitro* tests as models for osteoblastic cells.

Tranfection with the developed liposomes was observed for all the three studied cell lines, once that significantly different results were obtained when compared to controls, where cells were transfected with free DNA. However it was observed that the cell type is a factor to be taken into account when using liposomes as vectors. Positive control was done using 293T cells thus showing the cationic liposomes produced in this work are internalised by the cells and generate βgalactosidase expression.

Besides that, for all the charge ratios significantly higher values were obtained than when free DNA or Lipofectamine® were used. MG-63 e MC3T3-E1 cells did not present similar transfection activities, instead these were considerably lower.

This might be the result of deficient internalisation of complexes by the cells, or it may not be expressed, in particular due to its degradation by endonucleases.

Studies that have used chitosan nanoparticles as vectors for gene therapy in MG-63 cells have shown that the particles remain at the cells surface at least for a week after the incubation period [28]. The composition of the cell membrane changes according to the various cell types, being able to enhance or damage binding to complexes at the surface or their internalization It is well known that a deficient synthesis of proteoglicans may negatively affect the transfection mediated by liposomes [29].

MC3T3-E1 cell lines present higher transfection activity than MG-63 in all tested charge ratios and for both lipidic formulations. These results were not expected considering that tumour cells divide faster and typically have a higher number of transferrin receptors at their membranes.

In MC3T3-E1 cells, the association of transferrin to complexes produced a considerable increase in transfection activity and efficiency, possibly due to a better capability of complexes internalization, due to the ligants capacity to promote the endocitosis process[25,30,31]. In the case of MG-63 The effect of tranferrin was only noticed for a 2/1 charge ratio (DOTAP-DOPE formulation), and with complexes prepared from DOTAP-Cholesterol. However, in this case, the tranfection values for the binary complexes were very low. The transferrin receptors in MG-63 cells may be somehow altered therefore not promoting the complexes internalization.

The increase of complexes transfection associated to transferrin is observed both for the DOTAP-DOPE formulation and for DOTAP-Cholesterol, indicating that this is not a specific effect for a specific lipidic formulation.

As previously stated the association of transferrin to complexes produces an increase in particles size. Many authors admit that the smaller complexes are responsible for enhanced internalization efficiency. However it is recognised by other authors that larger complexes are more effective as they deposit easier over the cells surface [32,33]. In that case, either the size increase is not relevant or the effect of conjugation of a specific ligant will be the main aspect to consider, as for binary complexes the size increase is not translated into a transfection activity increase.

It is important to realize that the transfection activity changes with the cell type, the complexes charge ratio, the lipidic formulation and with the interactions of these three factors. Thus there is no ideal charge ratio or lipidic formulation for osteoblasts.

Indeed, higher values of MG63 cells transfection with DOTAP-DOPE formulation, are associated to transferrin and to a 2/1 ratio, while in the case of MC3T3-E1 the highest transfection value was obtained for a 3/2 ratio in complexes associated to transferrin, prepared from DOTAP-Cholesterol.

Therefore the ideal charge ratio depends on the lipidic formulation and the cell type.

In MG-63 cells, for the ratios where the transfection activity was higher there were no significant differences between DOTAP-DOPE and DOTAP-Cholesterol, showing that there was no effect of lipids in the transfection of these cells. For MG-63 cells, DOTAP-DOPE formulation clearly dominates positively except for (1/1 Tf) charge ratio. The differences in activity cannot be explained through citotoxicity differences, as there were no differences in the toxicity in cells, except for 4/1 ratio. Increasing the DNA dosage from 2 e 4 μg, a two-fold (and some times three-fold) increase in the transfection activity was found

This activity increase may be explained due to an increased access of DNA to the cells and its consequent expression. In the case of MC3T3-E1, these results are not confirmed, as the highest transfection rates were found for 2 μg of DNA. There may be a saturation of the receptors or the effect of citotoxicity may be more clear, as during the complexes preparation the amount of DNA was increased and consequently also the amount of cationic liposomes to maintain the charge ratios. So, the complexes prepared with a 2/1 charge ratios, with 4 μg of DNA, have a liposome volume four times higher than those prepared with1 μg DNA.

5. Conclusion

Repairing fractures and bone defects or lesions is a target of therapies that have well known limitations. Gene therapy might become a good alternative for the release of ontogenesis stimulating factors. Cationic liposomes are good alternatives to viral vectors as they are safe, simple to prepare and use, may be modified to enhance the internalisation rate, and are associated to a transient expression adequate in the treatment of several bone pathologies and damage caused by fracture.

The results obtained in this work indicate that liposomes are an alternative to consider as non-viral vectors for gene therapy for osteoblastic-like cells. The association of transferrin introduced a remarkable improvement in the system. It would be interesting in the future to associate other ligants to the complexes, trying to further improve the system for osteoblastic transfection.

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Figure Captions

Fig. 1. Effect of charge ratio and liposome composition on lipoplex zeta potential. Liposomes composed of of DOTAP-DOPE (1:1) or DOTAP-Cholesterol (1:1) were complexed with 1μg DNA at different lipid/DNA charge ratios. Lipoplexes containing Tf were also prepared by associating 32 μg transferrin/μg DNA. Data represent the mean ±S.D. of two independent experiments.

Fig. 2. Distribution of the vesicles size (diameters) and poly-dispersion index of DOTAP-DOPE (1:1) and DOTAP-Cholesterol (1:1) liposomes using 1μg DNA at different lipid/DNA charge ratios. Tf – lipoplex with 32 μg transferrin/μg DNA. Data represent the mean ±S.D. of two independent experiments.

Fig.3. Extent of DNA protection, as a function of liposome composition, lipid/DNA charge ratio and presence or absence of Transferrin. DNA protection was calculated from the access of ethidium bromide to the DNA of the complexes prepared with DOTAP-DOPE (1:1) and DOTAP-Cholesterol (1:1), after incubation with DNase I (ten units/μg DNA). Tf – lipoplexes associated with 32 μg transferrin/μg DNA.

Fig. 4. Effect of lipoplex charge ratio (+/-) and lipid composition on βgalactosidase gene expression in 293-T cells, using DOTAP-DOPE (1:1) e DOTAP-Cholesterol (1:1). Tf – lipoplexes associated with 32 μg transferrin/μg DNA; C1 – cells only treated with 1μg DNA; Lp. – cells transfected using lipofectamine®. The data are expressed as micrograms of beta-galactosidase per milligram of total cell protein. The mean ± S.D., obtained from triplicates, are representative of three independent experiments.

Fig.5. Effect of lipoplex charge ratio (+/-) and lipid composition on βgalactosidase gene expression in MC3T3-E1 cells, using DOTAP-DOPE (1:1) e DOTAP-Cholesterol (1:1). Tf – lipoplex with 32 μg transferrin/μg DNA; C1 – cells only treated with 1μg DNA; Lp. – cells transfect using lipofectamine®. The data are expressed as micrograms of beta-galactosidase per milligram of total cell protein. The mean ±SD, obtained from triplicates of three independent experiments.

Fig. 6. Effect of lipoplex charge ratio (+/-) and lipid composition on βgalactosidase gene expression in MG-63 cells, using DOTAP-DOPE (1:1) and DOTAP-Cholesterol (1:1). Tf – lipoplex with 32 μg transferrin/μg DNA; C1 – cells only treated with 1μg DNA; Lp. – cells transfect using lipofectamine®.

The data are expressed as micrograms of beta-galactosidase per milligram of total cell protein. The mean ±SD, obtained from triplicates of three independent experiments.

Fig. 7. Effect of the amount of DNA complexed with DOTAP-DOPE (1:1) liposomes on β-galactosidase gene expression in MG-63 (A) and MC3T3-E1 (B) cells. Tf – lipoplex with 32 μg transferrin/μg DNA; C1 – cells treated with 1μg DNA. The mean ±S.D, obtained form triplicate wells are representative of two independent experiments.

Fig. 8. Transfection efficiency images of β-galactosidase gene in 293T cells using DOTAP-Cholesterol lipoplexes (1:1) associated with transferrin. Several charge ratio (+/-): 1/1 Tf (A) ; 3/2 Tf (B) and 2/1 Tf (C) are shown. The bar stands for 50μm.

Figure 1

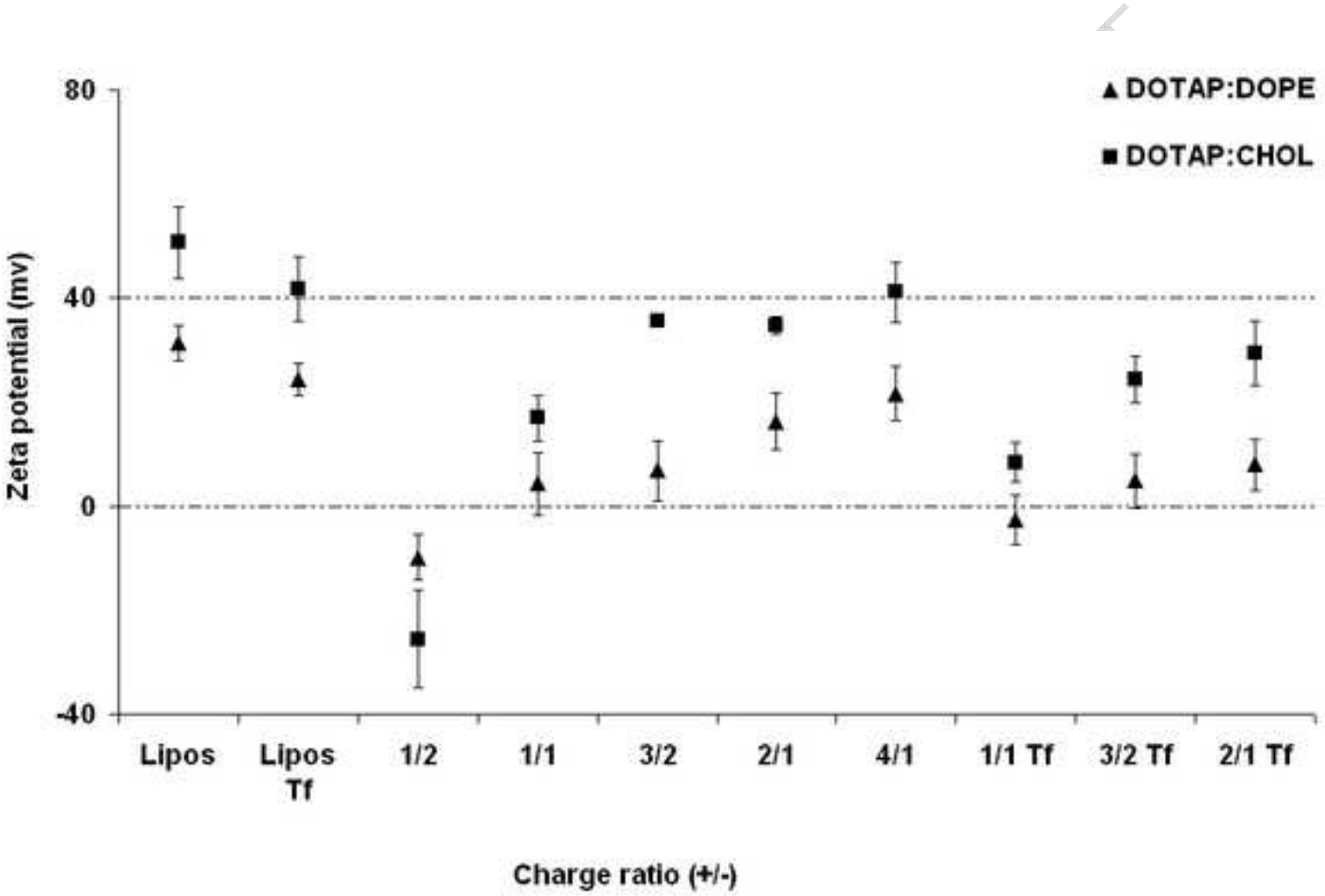
80
40
0
-40
Zeta potential (mv)
▲ DOTAP:DOPE
■ DOTAP:CHOL
Lipos
Lipos Tf
1/2
1/1
3/2
2/1
4/1
1/1 Tf
3/2 Tf
2/1 Tf
Charge ratio (+/-)

Figure 2

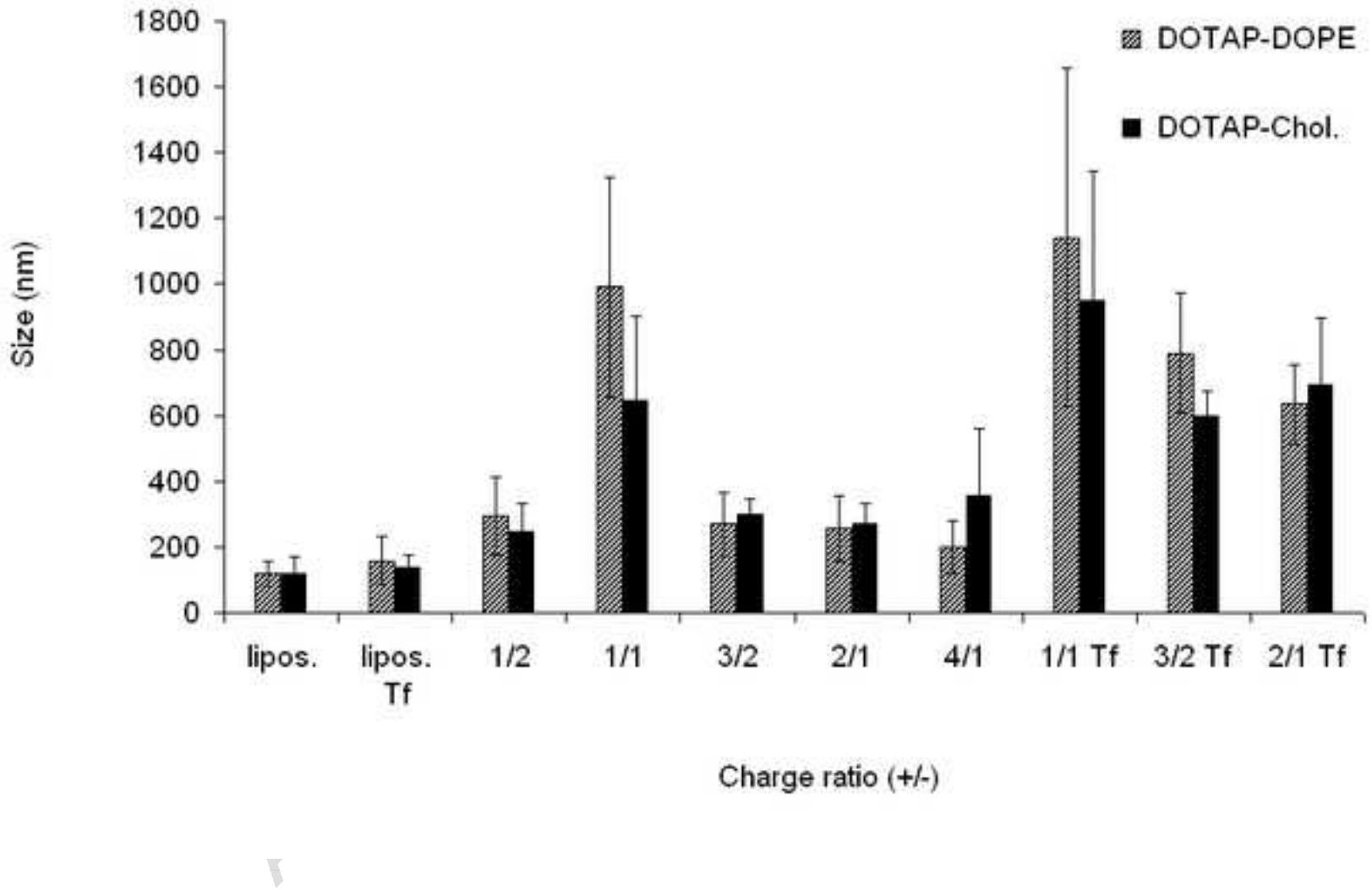
1800
1600
1400
1200
1000
800
600
400
200
0
Size (nm)
DOTAP-DOPE
DOTAP-Chol.
lipos.
lipos. Tf
1/2
1/1
3/2
2/1
4/1
1/1 Tf
3/2 Tf
2/1 Tf
Charge ratio (+/-)

Figure 3

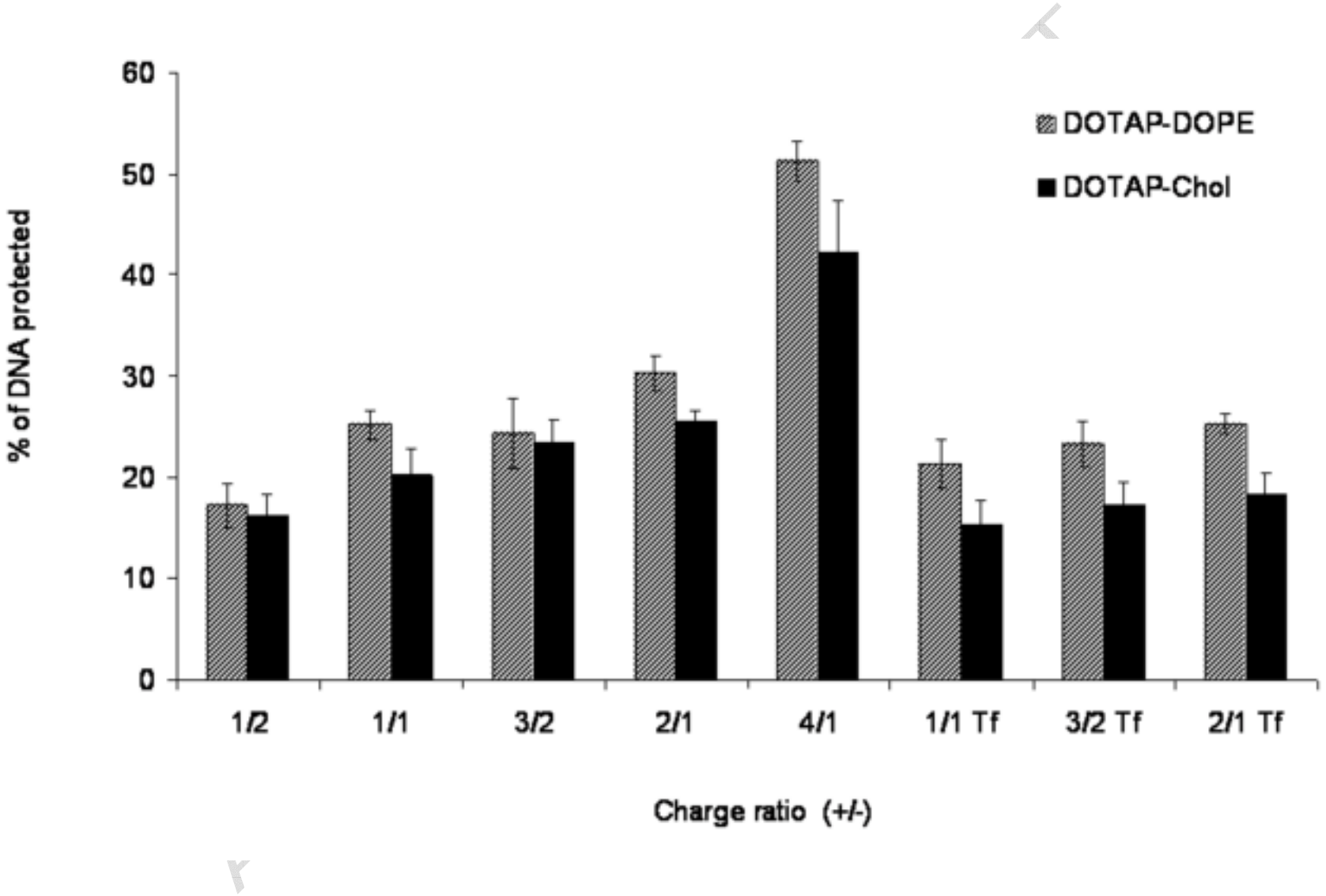
60
50
40
30
20
10
0
% of DNA protected
DOTAP-DOPE
DOTAP-Chol
1/2
1/1
3/2
2/1
4/1
1/1 Tf
3/2 Tf
2/1 Tf
Charge ratio (+/-)

Figure 4

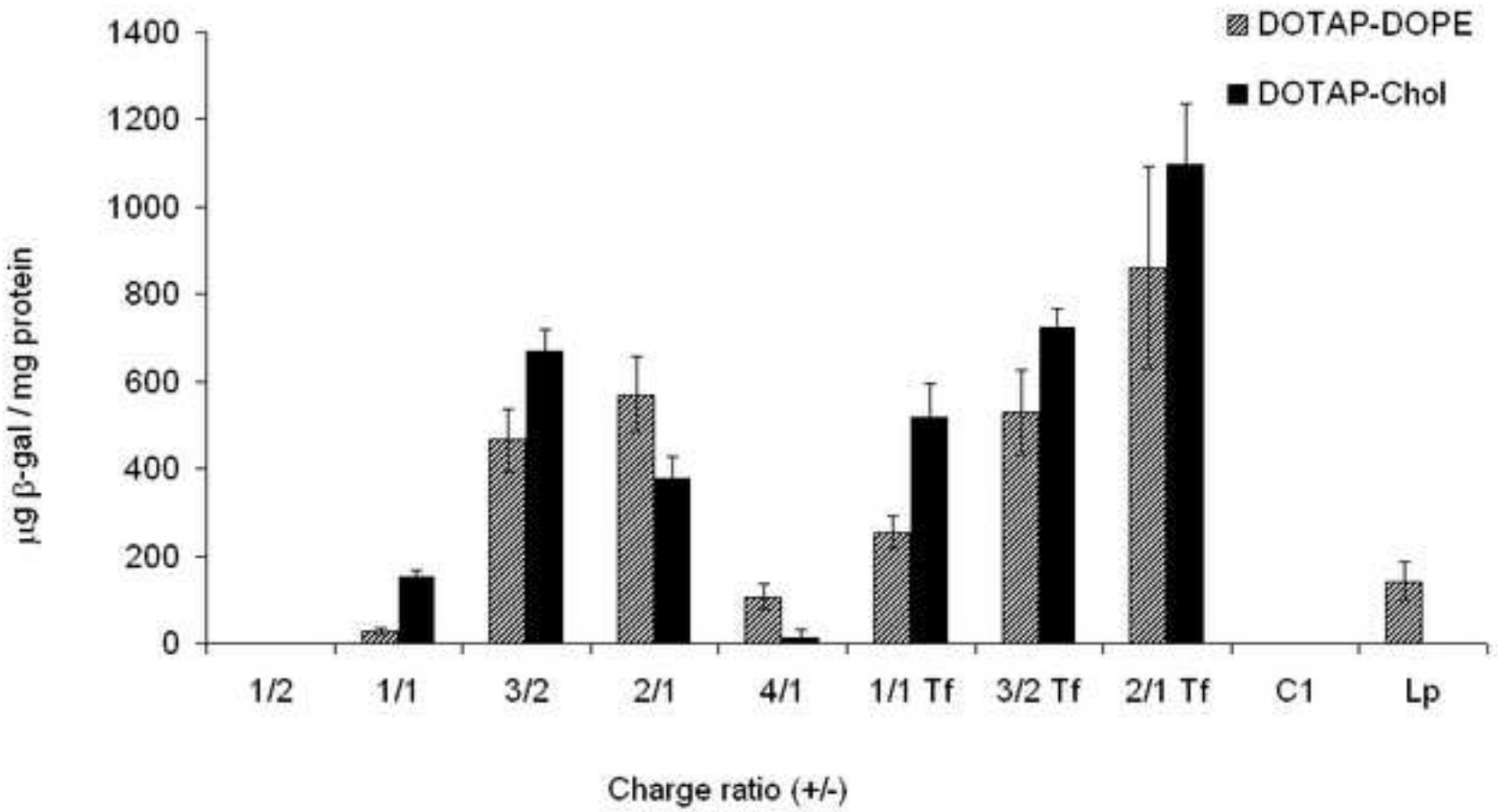
DOTAP-DOPE
DOTAP-Chol
µg β-gal / mg protein
1400
1200
1000
800
600
400
200
0
1/2
1/1
3/2
2/1
4/1
1/1 Tf
3/2 Tf
2/1 Tf
C1
Lp
Charge ratio (+/-)

Figure 5

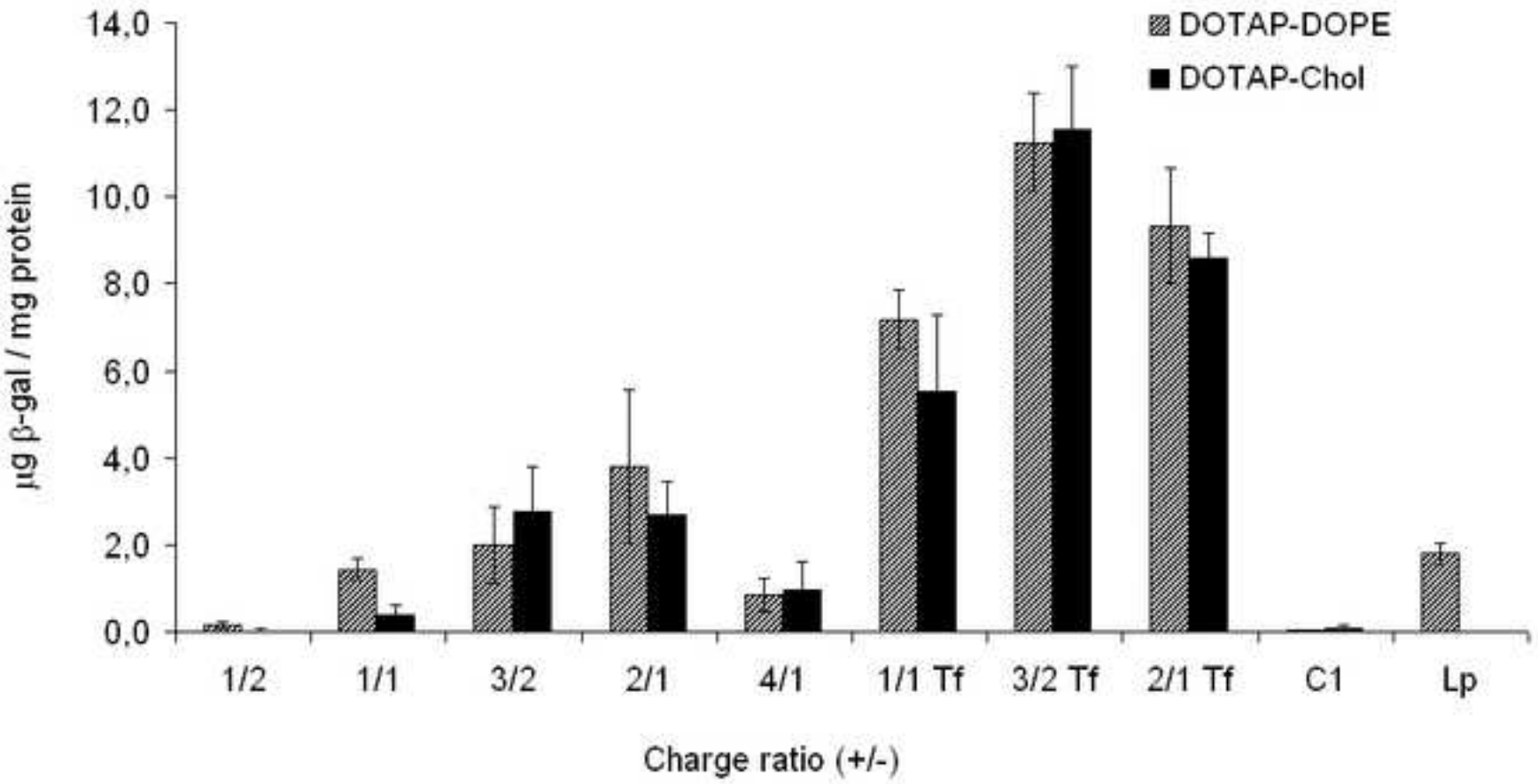
DOTAP-DOPE
DOTAP-Chol
µg β-gal / mg protein
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
1/2
1/1
3/2
2/1
4/1
1/1 Tf
3/2 Tf
2/1 Tf
C1
Lp
Charge ratio (+/-)

Figure 6

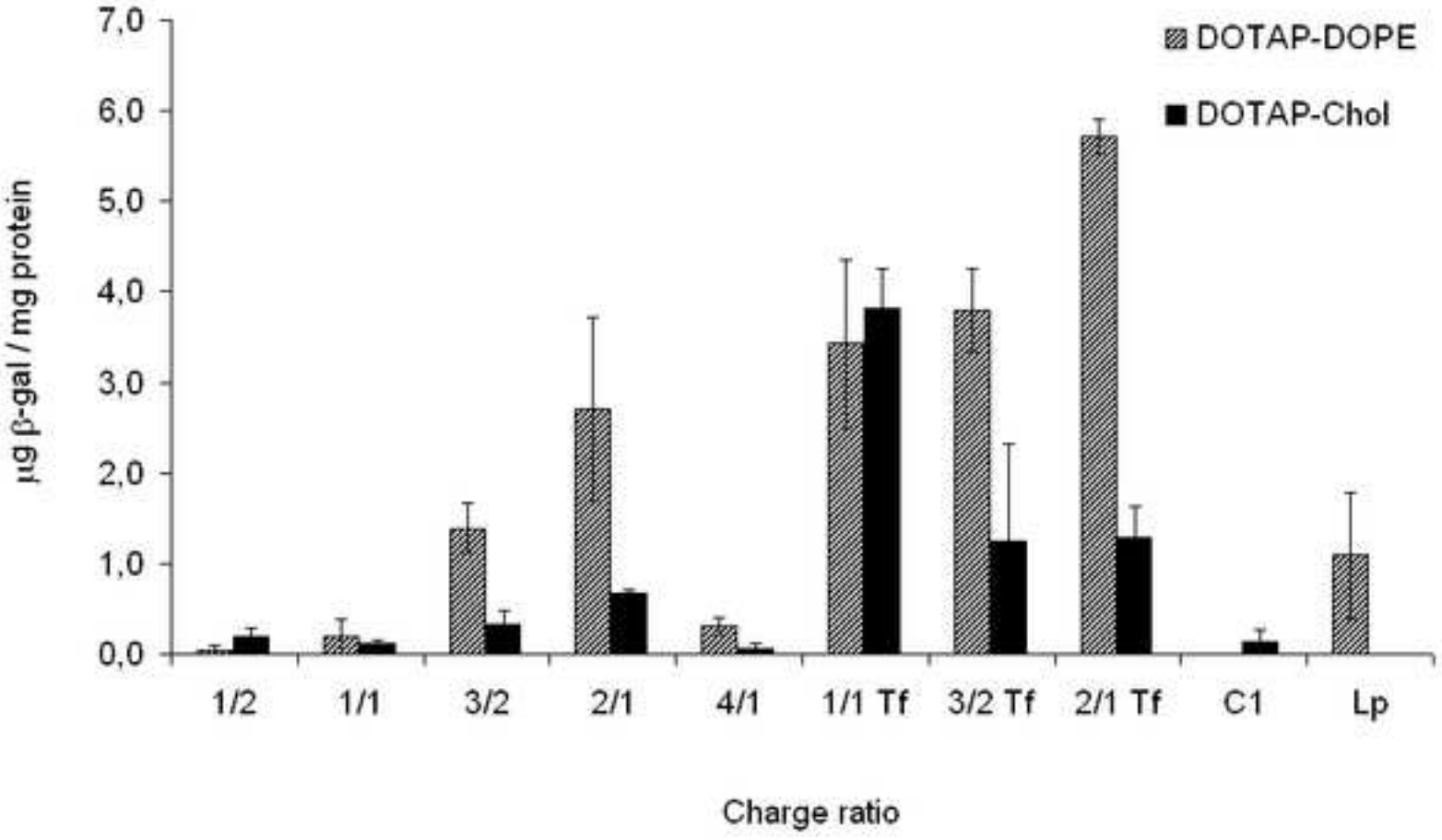
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
µg β-gal / mg protein
DOTAP-DOPE
DOTAP-Chol
1/2
1/1
3/2
2/1
4/1
1/1 Tf
3/2 Tf
2/1 Tf
C1
Lp
Charge ratio

Figure 7A

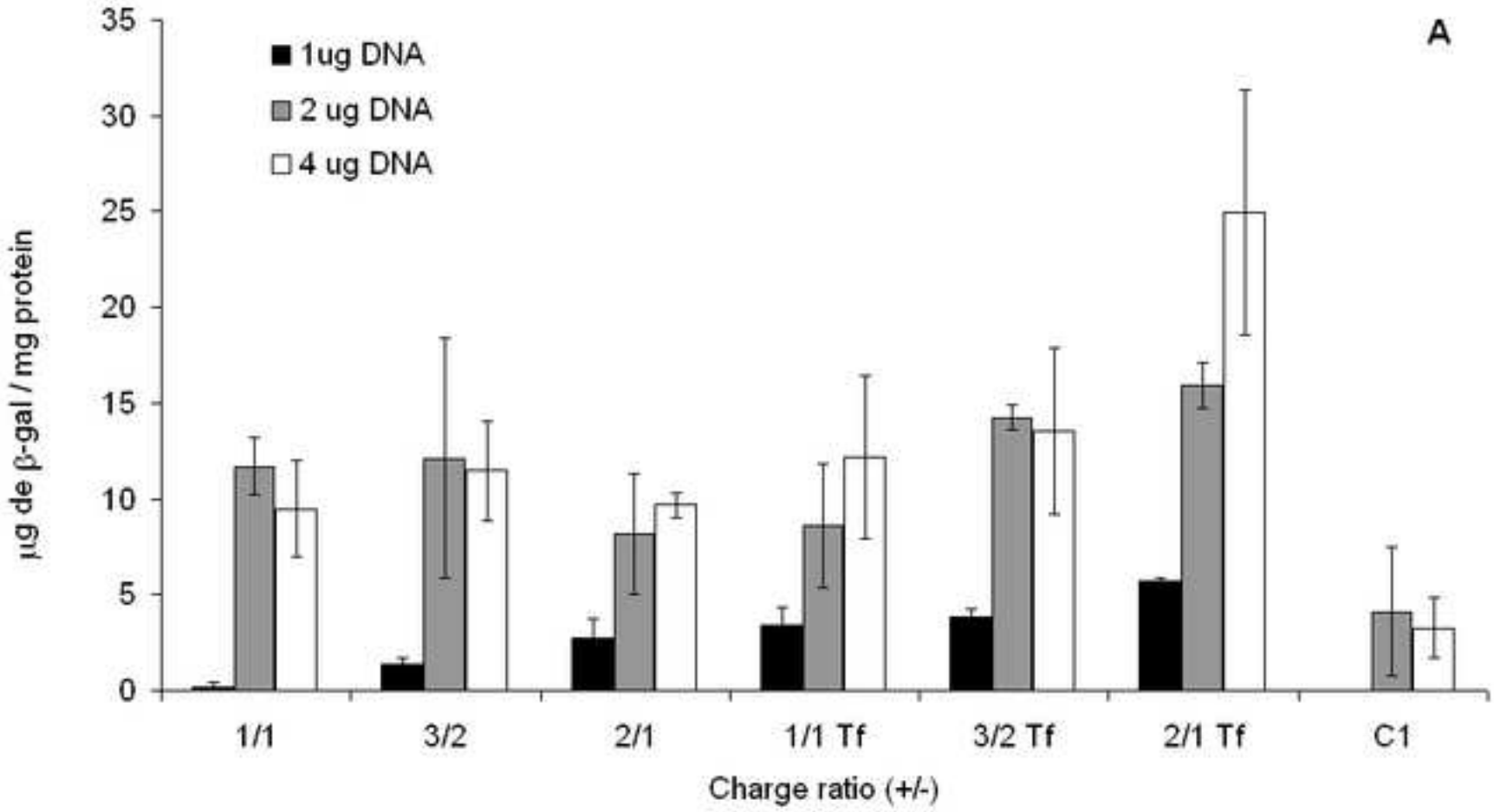
A
1ug DNA
2 ug DNA
4 ug DNA
µg de β-gal / mg protein
35
30
25
20
15
10
5
0
1/1
3/2
2/1
1/1 Tf
3/2 Tf
2/1 Tf
C1
Charge ratio (+/-)

Figure 7B

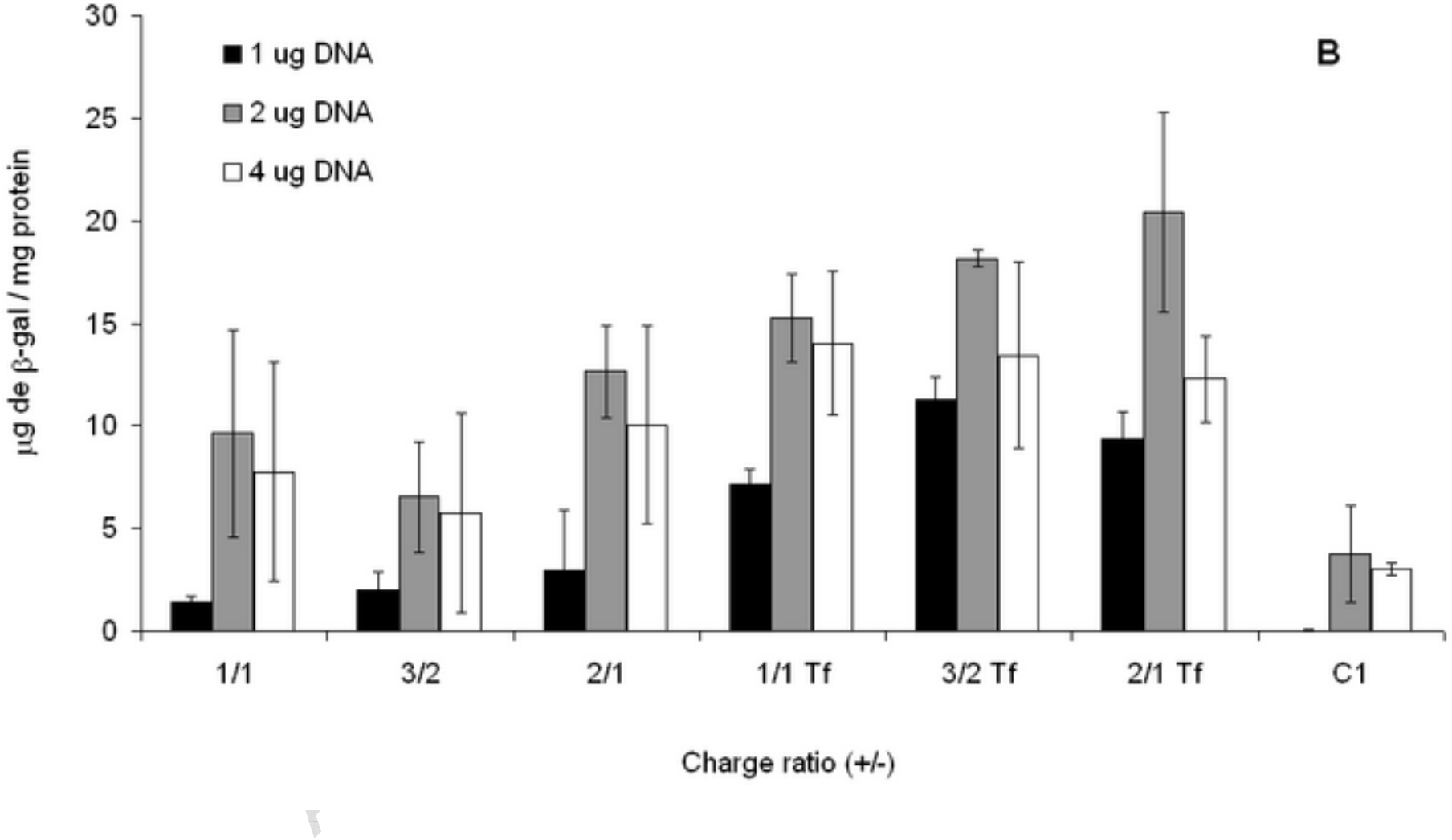
B
1 ug DNA
2 ug DNA
4 ug DNA
µg de β-gal / mg protein
30
25
20
15
10
5
0
1/1
3/2
2/1
1/1 Tf
3/2 Tf
2/1 Tf
C1
Charge ratio (+/-)

Figure 8