Elsevier Editorial System(tm) for Biomaterials
Manuscript Draft

Manuscript Number: jbmt14650

Title: Enhancement of efficiencies of the cellular uptake and gene silencing of chitosan/siRNA complexes via the inclusion of a negatively charged poly(γ-glutamic acid)

Article Type: FLA Original Research

Section/Category: Biomaterials & Gene Transfer

Keywords: chitosan; small interfering RNA; poly(γ-glutamic acid); transfection; gene silencing

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Abstract: Although advantageous for siRNA packing and protection, chitosan (CS)-based complexes may lead to difficulties in siRNA release once they arrive at the site of action, due to their electrostatic interactions. To assist the intracellular release of siRNA and thus enhance its effectiveness in gene silencing, we incorporated a negatively charged poly(γ-glutamic acid) (γ-PGA) into CS/siRNA complexes. The inclusion of γ-PGA did not alter the complex-formation ability between CS and siRNA; additionally, their cellular uptake was significantly enhanced. The results obtained in our molecular dynamic simulations indicate that the binding between CS and siRNA remained stable in the cytosol environment. In contrast, the compact structure of the ternary CS/siRNA/γ-PGA complexes was unpacked; such a structural unpackage may facilitate the intracellular release of siRNA. In the gene silencing study, we found that the inclusion of γ-PGA into complexes could significantly expedite the onset of gene knockdown, enhance their inhibition efficiency and prolong the duration of gene silencing. These findings may be attributed to the fact that there were significantly more CS/siRNA/γ-PGA complexes internalized into the cells in company with their more rapid intracellular unpackage and release of siRNA when compared with their binary counterparts in the absence of γ-PGA. The aforementioned results suggest that CS/siRNA/γ-PGA complexes can be an efficient vector for siRNA transfection.

AUTHOR DECLARATION

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July 12, 2010

Professor D. F. Williams
Editor-in-Chief, Biomaterials

Dear Professor Williams:

Attached please find a manuscript entitled *"Enhancement of efficiencies of the cellular uptake and gene silencing of chitosan/siRNA complexes via the inclusion of a negatively charged poly(γ-glutamic acid)"*. The manuscript is intended to be published in *Biomaterials*. It has been solely submitted to *Biomaterials* and that it is not concurrently under consideration for publication in any other journals.

In this study, a chitosan (CS)-based carrier system with the inclusion of poly(γ-glutamic acid) (γ-PGA) was developed for siRNA delivery. The results obtained demonstrate that γ-PGA played an important role in improving the cellular uptake of CS complexes, expediting their intracellular unpackage and the release of siRNA, thus significantly enhancing the efficiency of gene silencing and prolonging the duration of its action. Taken together, CS/siRNA/γ-PGA complexes show much potential as an efficient vector for siRNA transfection. We believe these results are of very broad significance and should be of interests to the *Biomaterials* readers.

We value you and the reviewers' suggestions and comments. Thank you in advance for arranging the review process for our manuscript.

Sincerely yours,

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Enhancement of efficiencies of the cellular uptake and gene silencing of chitosan/siRNA complexes via the inclusion of a negatively charged poly(γ-glutamic acid)

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Abstract

Although advantageous for siRNA packing and protection, chitosan (CS)-based complexes may lead to difficulties in siRNA release once they arrive at the site of action, due to their electrostatic interactions. To assist the intracellular release of siRNA and thus enhance its effectiveness in gene silencing, we incorporated a negatively charged poly(γ-glutamic acid) (γ-PGA) into CS/siRNA complexes. The inclusion of γ-PGA did not alter the complex-formation ability between CS and siRNA; additionally, their cellular uptake was significantly enhanced. The results obtained in our molecular dynamic simulations indicate that the binding between CS and siRNA remained stable in the cytosol environment. In contrast, the compact structure of the ternary CS/siRNA/γ-PGA complexes was unpacked; such a structural unpackage may facilitate the intracellular release of siRNA. In the gene silencing study, we found that the inclusion of γ-PGA into complexes could significantly expedite the onset of gene knockdown, enhance their inhibition efficiency and prolong the duration of gene silencing. These findings may be attributed to the fact that there were significantly more CS/siRNA/γ-PGA complexes internalized into the cells in company with their more rapid intracellular unpackage and release of siRNA when compared with their binary counterparts in the absence of γ-PGA. The aforementioned results suggest that CS/siRNA/γ-PGA complexes can be an efficient vector for siRNA transfection.

Keywords: chitosan; small interfering RNA; poly(γ-glutamic acid); transfection; gene silencing

1. Introduction

Small interfering RNAs (siRNAs), known to silence target genes with high specificity, offers a potentially new therapeutic strategy that reduces undesirable gene expression [1–3]. Gene silencing using siRNAs has prospective applications in the treatment of a variety of diseases including cancer, viral infection and genetic disorders [4–6]. However, a major limitation in the therapeutic use of siRNAs is their rapid degradation in the plasma and intracellular cytosol, resulting in a short half life [7]. Additionally, naked siRNAs cannot be internalized into cells effectively. Therefore, a carrier system is needed for the protection and delivery of siRNAs [8]. Viral vectors enable an efficient inhibition of gene expression [9]; but the clinical application of viral vectors is associated with concerns of toxicity and immunogenicity [10]. Polycations have been considered as siRNA delivery carriers; although less toxic than viral vectors, there are still problems of low efficiency [11,12].

Chitosan (CS), a cationic polysaccharide, is biodegradable, non-toxic and tissue compatible and thus has been considered for the delivery of siRNAs [13,14]. It has the potential to condense anionic nucleic acids such as siRNAs into a compact structure through electrostatic interactions and provide an effective protection against enzymatic degradations [13]. Nevertheless, the transfection efficiency of CS/siRNA complexes is relatively low and their onset of gene silencing is comparatively slow [15]. We speculate that the strength of electrostatic interactions between CS and siRNA prevents their dissociation within the cells, thus precluding RNA interference (RNAi) and resulting in slow/low gene silencing. The challenge of the CS carrier is, therefore, to overcome this intracellular barrier to achieve an effective delivery of siRNA.

In this study, an approach for the enhancement of efficiencies of cellular uptake and gene silencing through the inclusion of a negatively charged poly(γ-glutamic acid) (γ-PGA) into the formulation of CS/siRNA complexes is reported. γ-PGA, a naturally occurring peptide, is water-soluble, biodegradable and non-toxic. γ-PGA-based complexes have been used as a carrier for the oral delivery of insulin [16,17] and been employed to deliver protein vaccines and appeared to hold a great potential as an adjuvant

[18].

The prepared CS/siRNA/γ-PGA complexes were characterized via dynamic light scattering (DLS). Their cytotoxicity, cellular uptake and gene silencing were evaluated by the MTS (methoxyphenyl-tetrazolium salt) assay, flow cytometery and confocal laser scanning microscopy (CLSM), respectively. In addition, the unpackage of CS/siRNA/γ-PGA complexes intracellularly was modeling using molecular dynamic (MD) simulations. CS/siRNA complexes were used as a control.

2. Materials and Methods

2.1. Materials

CS (80 kDa) with a degree of deacetylation of approximately 85% and γ-PGA (20 kDa) were purchased from Challenge Bioproducts and Vedan (Taichung, Taiwan), respectively. Alexa Fluor 488-siRNA and siRNA duplexes targeting luciferase (Luciferase GL3 siRNA, LUC-siRNA) or EGFP (GFP-22 siRNA, EGFP-siRNA) were acquired from Qiagen (Deutschland, Germany). The Cell Titer 96AQueous One Solution Cell Proliferation Assay reagent, passive lysis buffer and luciferase assay reagent were obtained from Promega (Madison, Wisconsin, USA).

2.2. Preparation and characterization of test complexes

The charge ratio (N/P/C) of test complexes was expressed as the ratio of moles of the amino groups (N) on CS to the phosphate groups (P) on siRNA and the carboxyl groups (C) on γ-PGA. Test complexes at various known N/P/C molar ratios (100/1/0, 100/1/10, 10/1/20, 100/1/30 and 100/1/50) were prepared by an ionic-gelation method in deionized (DI) water (pH 6.0) [19]. Briefly, an aqueous Alexa Fluor 488-siRNA was mixed with an aqueous γ-PGA at different molar ratios with a final volume of 100 μl. Test complexes were obtained upon addition of the mixed solution, using a pipette, into an aqueous CS (0.2 μg/μl, 100 μl) and then thoroughly mixed for 30–60 s by vortex and left for at least 1 h at room temperature.

The hydrodynamic sizes of test complexes were measured using a Zetasizer Nano ZS (3000HS, Malvern Instruments Ltd., Worcestershire, UK). The binding efficiency

between siRNA and CS was evaluated by a gel retardation assay [20]. Electrophoresis was carried out onto a 2% agarose gel with a current of 100 V for 30 min in TAE buffer solution (40*mM* Tris-HCl, 1% v/v acetic acid and 1*mM* EDTA). The retardation of test complexes was visualized by staining with ethidium bromide.

2.3. Cellular uptake

Prior to CS-mediated delivering siRNAs for targeted gene silencing, we optimized the transfection conditions using Alexa Fluor 488-siRNA as the reporter. HT1080 (human fibrosarcoma) cells were cultured in DMEM media supplemented with 2.2 g/l sodium bicarbonate and 10% fetal bovine serum (FBS). Cells were subcultured according to the ATCC recommendations without using any antibiotics. For transfection, cells were seeded in 24-well plates at 1×10^5 cells/well and transfected the next day. Prior to transfection, the media were removed and cells were rinsed twice with transfection media (DMEM without FBS, pH 6.0). Cells were replenished with 0.3 ml transfection media containing test complexes at a concentration of 0.25 μg siRNA/well. At 1 h post transfection, the transfection media containing test complexes were removed, cells rinsed twice with transfection media and refilled with FBS-containing media until analysis at 24 h after transfection.

The percentage of cellular uptake was quantitatively assessed by flow cytometry. Cells were detached by 0.025% trypisin. Suspensions of cells were transferred to microtubes, fixed by 4% paraformaldehyde and determined the transfection efficiency using a flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an argon laser at 488 nm excitation and a band-pass filter at 505–530 nm to detect Alexa Fluor 488. Test cells were appropriately gated by forward and side scatters and 10,000 events per sample were collected. The untreated cells were used as the control.

2.4. Silencing EGFP and luciferase expression

In the study, recombinant HT1080 cells with the constitutive EGFP and luciferase expression were prepared as described in the literature [20]. In the EGFP silencing study, recombinant HT-1080 cells were cultured in 35-mm dishes with glass cover-slip bottoms and incubated overnight. Subsequently, cells were rinsed twice with

transfection media and treated with test complexes containing EGFP-siRNA. At 1 h after incubation, test samples were aspirated; cells were then washed twice with the pre-warmed phosphate buffered saline (PBS) before they were fixed in 4% paraformaldehyde. The fixed cells were then counterstained to visualize nuclei by propidium iodide (PI, Sigma-Aldrich) and examined under a CLSM (Zeiss LSM510, Carl Zeiss, Jena GmbH, Germany). The untreated recombinant HT1080 cells were used as a control.

For silencing the luciferase expression, cells were seeded in 24-well plates at 1×10^5 cells/well overnight and then treated with test complexes containing LUC-siRNA. At different time points, test cells were lysed using a 100 µl of passive lysis buffer. The cell lysate was transferred into a microcentrifuge tube and the cell debris was separated by centrifugation (12,000 rpm, 5 min). Subsequently, a 100 µl of the luciferase assay reagent was added into a 20 µl of the supernatant; the relative luminescence of the sample was determined by a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized to the total cell protein concentration by the Bradford method. The cells transfected with Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) were used as a positive control and those without any treatment were used as a negative control.

2.5. MTS assay

The cytotoxicity of test complexes was evaluated *in vitro* using the MTS assay [20]. HT1080 cells were seeded in 24-well plates at 1×10^5 cells/well, allowed to adhere overnight and transfected by test complexes containing 0.25 µg siRNA. After 1 h, test samples were aspirated and cells were incubated for another 23 h. Subsequently, cells were incubated in a growth medium with 1 mg/ml MTS reagent for 15 min. Subsequently, the optical density readings were performed using a multiwall scanning spectrophotometer (Dynatech Laboratories, Chantilly, VA, USA) at a wavelength of 490 nm. The untreated HT1080 cells were used as a control.

2.6. MD simulations

MD simulations of the self-assembly of test complexes (CS/siRNA and

CS/siRNA/γ-PGA) prepared in DI water and their unpackage in the intracellular environment (pH 7.0) with time were performed by a MD method [21]. MD simulations were accomplished with the program NAMD [22] using parameters adapted from the CHARMM 27 force field [23]. The models were minimized to remove unfavorable contacts, brought to 310 K by velocity rescaling and equilibrated for 1 ns. Before any MD trajectory was run, 40 ps of energy minimization were performed to relax the conformational and structural tensions. This minimum structure was the starting point for the MD simulations. For this purpose, the molecule was embedded into a cubic simulation box of 120 Å. A cutoff distance of 12 Å was employed for the nonbonded and electrostatic interactions. The heating process was performed from 0 to 310 K through Langevin damping with a coefficient of 10 ps^{-1}. A time step of 2 fs was employed for rescaling the temperature. After 20 ps heating to 310 K, equilibration trajectories of 1 ns were recorded, which provided the data for the structural and thermodynamic evaluations. The equations of motion were integrated with the Shake algorithm with a time step of 1 fs. Figures displaying atomistic pictures of molecules were generated using VMD [24].

2.7. Statistical analysis

Comparison between groups was analyzed by the one-tailed Student's *t*-test (SPSS, Chicago, Ill). All data are presented as a mean value with its standard deviation indicated (mean ± SD). Differences were considered to be statistically significant when the *P* values were less than 0.05.

3. Results and Discussion

A major challenge for therapeutic applications of siRNA is the design of a suitable vector for their delivery. CS/siRNA complexes have been considered as a candidate for gene silencing; they generally transfect cells more efficiently than naked siRNA [13,14]. Although advantageous for siRNA packing and protection, CS-based complexes may lead to difficulties in siRNA release once they arrive at the site of action, due to their electrostatic interactions. To assist the intracellular release of siRNA and thus enhance its effectiveness in gene silencing, we incorporated a negatively charged γ-PGA into CS/siRNA complexes.

3.1. MD simulations of the self-assembly of CS/siRNA and CS/siRNA/γ-PGA complexes

MD simulations were performed in a full-atom model to gain insight into the self-assembly behavior of CS/siRNA and CS/siRNA/γ-PGA complexes at the molecular level. Each atom was treated as a bead and the beads interacted with each other via the Charmm potentials. Both CS and γ-PGA molecules considered in the MD simulation contained 10 monomer units and siRNA was a short double-stranded RNA with 22 mer on each strand (Fig. 1). The present atomistic simulation would allow the capture of the aggregation of CS, siRNA and γ-PGA in complexes. The methodology adopted has recently been applied to enable simulations of the self-assembly of protein and detergent into mixed micelles [25,26].

The p*K*a values of CS and γ-PGA are 6.5 and 2.9, respectively [27]. When prepared in DI water (pH 6.0), CS, siRNA and γ-PGA are ionized. These ionized polyelectrolytes can form complexes by electrostatic interactions between the positively charged amino groups ($-NH_3^+$) on CS and the negatively charged phosphate groups ($-PO_4^-$) on siRNA and the carboxyl groups ($-COO^-$) on γ-PGA. Fig. 2 displays the snapshots of the complex formation of CS/siRNA and CS/siRNA/γ-PGA complexes in DI water, starting from the initial condition, obtained by the MD simulations. The N/P/C ratios set for the binary (CS/siRNA) and ternary (CS/siRNA/γ-PGA) complexes were 100/1/0 and 100/1/50, respectively. Because CS molecules were in large excess of siRNA in terms of the prescribed N/P ratio of 100/1, only a small fraction of CS molecules (in blue) were able to bind with siRNA (in yellow) to form a small primary aggregate for the binary complex.

The complex thus formed was overcharged (i.e., it carried an overall positive charge) such that the excess CS molecules were expelled out of the complex due to the electrostatic repulsion and remained well dispersed in the bulk solution (Supplementary Video 1).

The inclusion of γ-PGA into the system introduced more negative charges; in this case, siRNA and γ-PGA were able to attract most of the CS molecules to form larger primary aggregates for the ternary complex (Supplementary Video 2). As the amount of cationic CS molecules in the complex was still greater than that required to neutralize all negative charges, the complex still carried an overall positive charge. In the real system, the molecular weights of CS and γ-PGA were much larger than those considered in the MD simulation; a given polyelectrolyte chain may participate in the complexation in a number of primary complex aggregates. Therefore, the primary aggregates were "bridged" to yield much larger aggregates or particles that were observed by the experiment. The sizes of CS/siRNA and CS/siRNA/γ-PGA complexes were 207 ± 31.1 nm and 216.6 ± 13.0 nm, respectively, as measured by DLS (n = 6 batches).

3.2. Characteristics of CS/siRNA complexes

The binding capacity of siRNA with CS prepared at various N/P ratios was evaluated using the gel retardation assay; siRNA only was included as a control. Compared to control siRNA (Fig. 3a), no migrated bands were seen for complexes with N/P ratios ranging from 50/1 to 200/1, suggesting that siRNA was fully complexed or CS was able to effectively combine siRNA into complexes. The transfection efficiency and cytotoxicity were used as the criteria to optimize the N/P ratio in the preparation of CS/siRNA complexes. After transfection for 1 h, the percentage of cells that internalized test complexes containing Alexa Fluor 488-siRNA and their fluorescence intensity were quantified by flow cytometry. In general, with increasing the N/P ratio in test complexes, the percentage of fluorescent cells (Fig. 3b) and their fluorescence intensity (Fig. 3c) increased relatively. No significant cytotoxicity was observed for CS/siRNA complexes prepared at an NP ratio of 50/1 or 100/1 ($P > 0.05$, Fig. 3d), while the viability of cells decreased significantly when the N/P ratio was increased up to 150/1 ($P < 0.05$). Therefore, preparation of CS/siRNA complexes was carried out using an N/P ratio of 100/1 in the subsequent experiments.

3.3. Characteristics of CS/siRNA/γ-PGA complexes

A gel retardation study was conducted to confirm the formation of complexes among CS, siRNA and γ-PGA. As shown in Fig. 4a, the inclusion of γ-PGA in the preparation of complexes in an N/P/C ratio of 100/1/0 to 100/1/50 did not alter the complex-formation ability between CS and siRNA. However, at a higher ratio of γ-PGA (N/P/C ratios greater than 100/1/50), a significant aggregation of the prepared complexes was observed. As shown in Fig. 4b and 4c, following an increase in the ratio of γ-PGA, the percentage of cells that internalized CS/siRNA/γ-PGA complexes and their fluorescence intensity increased significantly ($P < 0.05$). As an example, the percentage of cellular uptake for the group treated with CS/siRNA/γ-PGA complexes in an N/P/C ratio of 100/1/50 was 96% while that treated with CS/siRNA complexes (N/P/C ratio of 100/1/0) was 82%. Little toxicity was observed after the inclusion of γ-PGA into CS/siRNA complexes (Fig. 4d, $P > 0.05$); a required characteristic of a siRNA delivery system is that it is not cytotoxic [8]. The aforementioned results indicate that CS/siRNA/γ-PGA complexes with an N/P/C ratio of 100/1/50 had the highest cellular uptake among all studied groups and therefore were chosen for the rest of study. Their counterparts prepared in the absence of γ-PGA (CS/siRNA complexes with an N/P/C ratio of 100/1/0) were used as a control.

3.4. MD simulations of the unpackage of test complexes in the cytosol environment

Non-viral vectors functionalized with polycationic polymers can deliver drugs and/or DNA/siRNA into the cytosol [11,12]. It has been reported that initial interaction between the cationic macromolecules and the negatively charged cell membranes is mediated by electrostatic interactions [28]; the vectors are then likely taken up by the cells through endocytosis. Having been taken up by the cells, vectors must escape from the endosome to reach the cytosol. A proposed mechanism for this is the "proton sponge effect"; this is thought to cause osmotic swelling and physical rupture of the endosome, resulting in the escape of the vector from the degradative lysosomal trafficking pathway [29]. After the endosomal escape, the vector must unpackage and release siRNA to the RNAi machinery. RNAi is induced by siRNAs, which become incorporated into the RNA-induced silencing complex (RISC) and serve as a guide for endonucleolytic cleavage of the complementary

target mRNA (Fig. 1).

MD simulations were also performed at pH 7.0 (simulating the pH environment in the cytosol) to gain insight into the mechanism of intracellular unpackage of test complexes [30]. Under this condition, most amino groups on CS were in the form of $–NH_2$ due to deprotonation and the polymer became hydrophobic. Interestingly, instead of disruption of the compact structure formed at pH 6.0, the binding between CS and siRNA became even tighter for CS/siRNA complexes at pH 7.0 (Fig. 5, Supplementary Video 3). Moreover, some CS chains were found to embed within the groove regions of siRNA double helices to reduce the contact with water. As the electrostatic interaction was no longer significant, the tight CS/siRNA binding should be driven by another favorable interaction, which was most likely to be the hydrogen bonding between the $–NH_2$ groups on CS and the phosphate groups of siRNA. Because CS/siRNA complexes were not disrupted under the physiological condition, the observed intracellular release of siRNA might be accomplished by the enzymatic degradation of CS. It is known that CS can be readily degraded either by lysozyme, chitinase or other enzymes in the physiological environment [31,32].

In contrast to the binary complex, the originally formed compact structure of CS/siRNA/γ-PGA complexes disintegrated at pH 7.0 (Fig. 5). In this case, a portion of CS remained bound with siRNA due to the amine-phosphate hydrogen bonding while the other were associated with γ-PGA via the hydrogen bonding between the amine groups (CS) and the carbonyl groups (γ-PGA). As both CS/siRNA and CS/γ-PGA aggregates carried negative charges, the electrostatic repulsion between the two led to disintegration of the compact structure (Supplementary Video 4). Such a structure disruption may facilitate the intracellular release of siRNA, as observed experimentally.

3.5. Cellular uptake and gene silencing

Effects of the inclusion of γ-PGA into test complexes containing Alexa Flour 488-siRNA on their cellular uptake was further investigated by CLSM. Results of the fluorescence images of cells after exposure to CS/siRNA or CS/siRNA/γ-PGA complexes are shown in Fig. 6. At 24 h after incubation of test complexes with cells, a greater accumulation of fluorescence signals was observed in the cytosol for the group treated with

CS/siRNA/γ-PGA complexes than that treated with CS/siRNA complexes. Interestingly, these results, consistent with those measured by flow cytometry (Fig. 4b and 4c), demonstrate that the cellular uptake of the CS-mediated siRNA transfection was significantly enhanced through the inclusion of γ-PGA into complexes. Generally, anionic complexes are not taken up well by cells because of the electrostatic repulsion induced by the negatively charged cell membranes. However, Kurosaki *et al.* reported that cationic complexes coated with the negatively charged γ-PGA not only reduced their cytotoxicity but also significantly enhanced their cellular uptake, suggesting that there might be a γ-PGA-specific receptor-mediated pathway involved in the internalization of these complexes [33].

The stably expressing EGFP cell line (recombinant HT1080 cells) was used to investigate the gene silencing efficacy of CS/EGFP-siRNA and CS/EGFP-siRNA/γ-PGA complexes. CLSM was used to show the decrease in fluorescence of cells after receiving test complexes containing EGFP-siRNA. The fluorescence of cells treated with free EGFP-siRNA was unchanged (data not shown), indicating no inhibition by naked EGFP-siRNA due to a poor transfection into the cells. It is known that the physicochemical properties of siRNA (relative large size, negative charge and hydrophilicity) make it difficult for siRNA to cross cellular membranes and to reach the cytosol [34]. Consequently, a variety of carrier systems have been developed in order to deliver siRNA into the cells [8,11–14,20].

As compared to the untreated control, the intensity of the cell fluorescence dramatically diminished with prolonged exposure to CS/EGFP-siRNA or CS/EGFP-siRNA/γ-PGA complexes (at 48 h after treatment), suggesting a successful reduction of the EGFP expression (Fig. 7). It appears that the reduction of fluorescent cells for the group transfected with CS/EGFP-siRNA/γ-PGA complexes was more prominent than its counterpart treated with CS/EGFP-siRNA complexes.

To further evaluate the knockdown efficiency of test complexes, we employed a luciferase reporter gene system in HT1080 cells. As expected, free LUC-siRNA did not alter the basal luciferase activity within the cells throughout the entire course of the study ($P > 0.05$). When compared to the untreated cells, the group treated with CS/LUC-siRNA

complexes showed low luciferase knockdown (5% reduction only) at 12 h after transfection, whereas the group treated with CS/LUC-siRNA/γ-PGA complexes demonstrated a significantly greater gene silencing efficiency (42% reduction, $P < 0.05$). CS-delivered LUC-siRNA reduced the luciferase level to approximately 50% within 24 h after treatment, and the reduction remained stable for up to 48 h. In comparison, at 24 h, CS/γ-PGA-delivered LUC-siRNA resulted in a 72% luciferase knockdown; the action of gene silencing continued with time and the maximum suppression of luciferase expression was found to be approximately 80% at 48 h. In contrast, the commercial Lipofectamine™ 2000 resulted in a maximum 70% luciferase knockdown. At 72 h post transfection, the reduction in luciferase expression for the cells received CS/LUC-siRNA/γ-PGA complexes (55%) was still statistically significant, an indication of a prolonged duration of action, as compared to their counterparts treated with CS/LUC-siRNA complexes (30%).

The aforementioned results indicate that the inclusion of γ-PGA into complexes could significantly expedite the onset of gene knockdown, enhance their inhibition efficiency and prolong the duration of gene silencing. These observations may be attributed to the fact that with the inclusion of γ-PGA, there were significantly more complexes internalized into the cells (Figs. 4b, 4c and 6) along with a more rapid structural unpackage after their endosomal escape into the cytosol when compared with those in the absence of γ-PGA (Fig. 5). A prolonged duration of gene silencing, being able to reduce the need of frequent administrations, is a key issue when considering therapeutic uses of siRNA [35].

4. Conclusions

A CS-based carrier system with the inclusion of γ-PGA was developed in the study for siRNA delivery. The results obtained demonstrate that γ-PGA played an important role in improving the cellular uptake of CS complexes, expediting their intracellular unpackage and the release of siRNA, thus significantly enhancing the efficiency of gene silencing and prolonging the duration of its action. Taken together, CS/siRNA/γ-PGA complexes show much potential as an efficient vector for siRNA transfection.

Acknowledgment

This work was supported by a grant from the National Science Council (NSC 98-2120-M-007-007), Taiwan, Republic of China.

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Figure Captions

Figure 1. Schematic illustrations showing the self-assembly of chitosan (CS), small interfering RNA (siRNA), poly(γ-glutamic acid) (γ-PGA) into test complexes (CS/siRNA and CS/siRNA/γ-PGA) and their intracellular release of siRNA, using the molecular dynamic (MD) simulations.

Figure 2. Snapshots of the complex formation of CS/siRNA and CS/siRNA/γ-PGA complexes in deionized water, starting from the initial condition, obtained by the molecular dynamic simulations. CS: chitosan; siRNA: small interfering RNA; γ-PGA: poly(γ-glutamic acid).

Figure 3. (a) Gel retardation analyses of CS/siRNA complexes prepared at different N/P ratios. Samples were run on a 2.0% agarose gel and subsequently stained using ethidium bromide; (b) Percentages of the cellular uptake of CS/Alexa Fluor 488-siRNA complexes prepared at different N/P ratios, analyzed by flow cytometry (n = 6); (c) Intracellular fluorescence intensities of CS/Alexa Fluor 488-siRNA complexes prepared at different N/P ratios determined by flow cytometry; (d) Results of the cell viability after being treated with CS/siRNA complexes prepared at different N/P ratios determined by the MTS assay (n = 6).

Figure 4. (a) Gel retardation analyses of CS/siRNA/γ-PGA complexes prepared at different N/P/C ratios. Samples were run on a 2.0% agarose gel and subsequently stained using ethidium bromide; (b) Percentages of the cellular uptake of CS/Alexa Fluor 488-siRNA/γ-PGA complexes prepared at different N/P/C ratios, analyzed by flow cytometry (n = 6); (c) Intracellular fluorescence intensities of CS/Alexa Fluor 488-siRNA/γ-PGA complexes prepared at different N/P/C ratios determined by flow cytometry; (d) Results of the cell viability after being treated with CS/siRNA/γ-PGA complexes prepared at different N/P/C ratios determined by the MTS assay (n = 6).

Figure 5. Snapshots of the intracellular release of siRNA from CS/siRNA and CS/siRNA/γ-PGA complexes in the cytosol obtained by the molecular dynamic simulations. CS: chitosan; siRNA: small interfering RNA; γ-PGA: poly(γ-glutamic acid).

Figure 6. Confocal images (scale bar, 50 μm) of the cellular uptake of CS/Alexa Fluor 488-siRNA (N/P/C ratio of 100/1/0) and CS/Alexa Fluor 488-siRNA/γ-PGA (N/P/C ratio of 100/1/50) complexes. Area defined by a square is shown at a higher magnification in the inset.

Figure 7. Confocal images (scale bar, 50 μm) of EGFP silencing of recombinant HT-1080 cells transfected with CS/EGFP-siRNA (N/P/C ratio of 100/1/0) or CS/EGFP-siRNA/γ-PGA (N/P/C ratio of 100/1/50)

complexes. The recombinant human HT-1080 fibroblast line stably expressed EGFP reporter. Control: the group without any treatment.

Figure 8. Suppression of luciferase gene expression after cells being treated with CS/Luciferase-siRNA (N/P/C ratio of 100/1/0) or CS/Luciferase-siRNA/γ-PGA (N/P/C ratio of 100/1/50) complexes, determined by a microplate luminometer (n = 6). The recombinant human HT-1080 fibroblast line stably expressed luciferase reporter. Control: the group without any treatment.

Data Supplement

Video 1: Formation of CS/siRNA complexes in deionized water, starting from the initial condition, obtained by the molecular dynamic simulations. Blue chain: chitosan; yellow chain: siRNA.

Video 2: Formation of CS/siRNA/γ-PGA complexes in deionized water obtained by the molecular dynamic simulations. Blue chain: chitosan; yellow chain: siRNA; red chain: γ-PGA.

Video 3: Structure of CS/siRNA complexes in the cytosol environment obtained by the molecular dynamic simulations. Blue chain: chitosan; yellow chain: siRNA.

Video 4: Unpackage of CS/siRNA/γ-PGA complexes in the cytosol environment obtained by the molecular dynamic simulations. Blue chain: chitosan; yellow chain: siRNA; red chain: γ-PGA.

Figure 1

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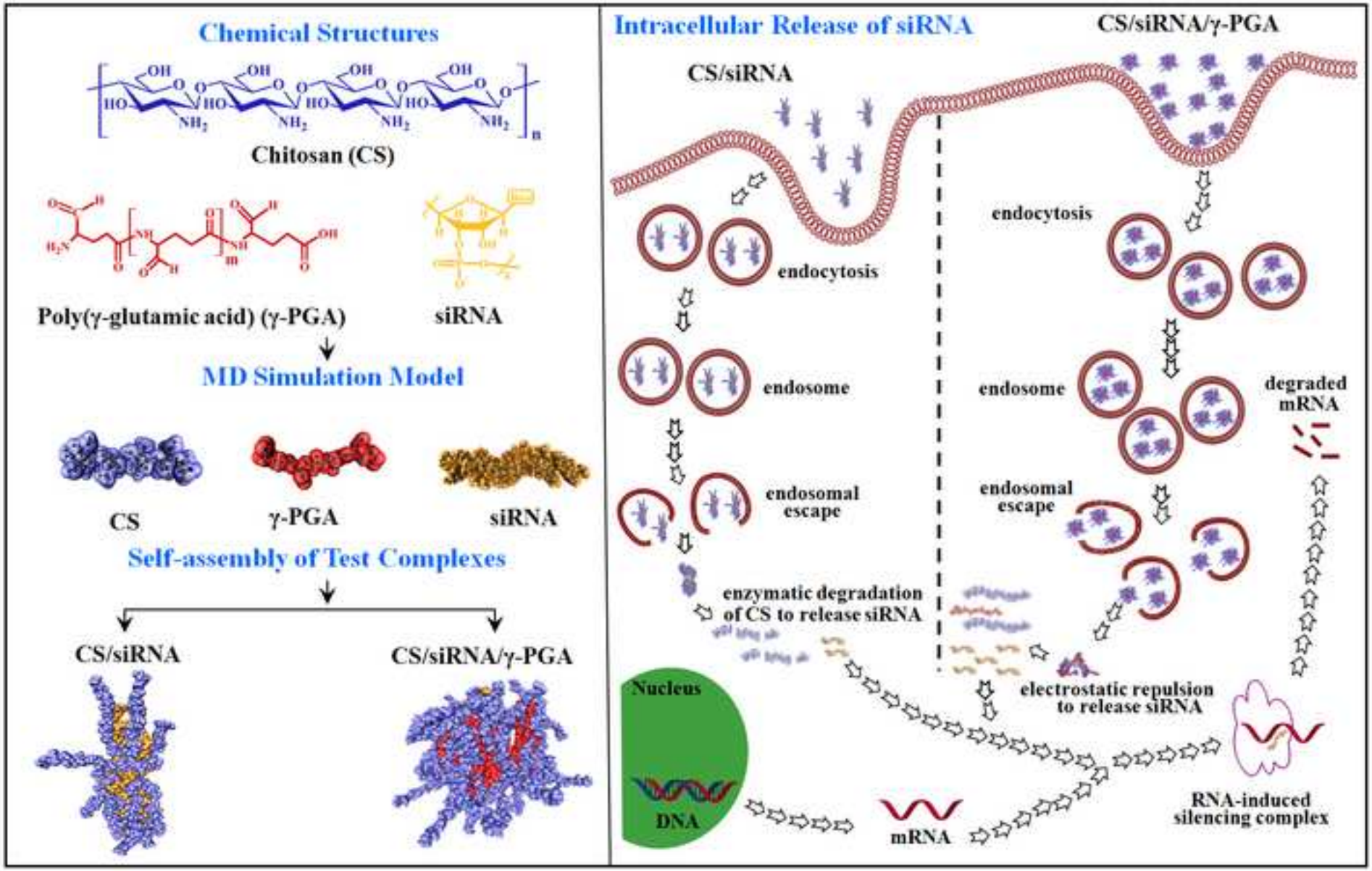

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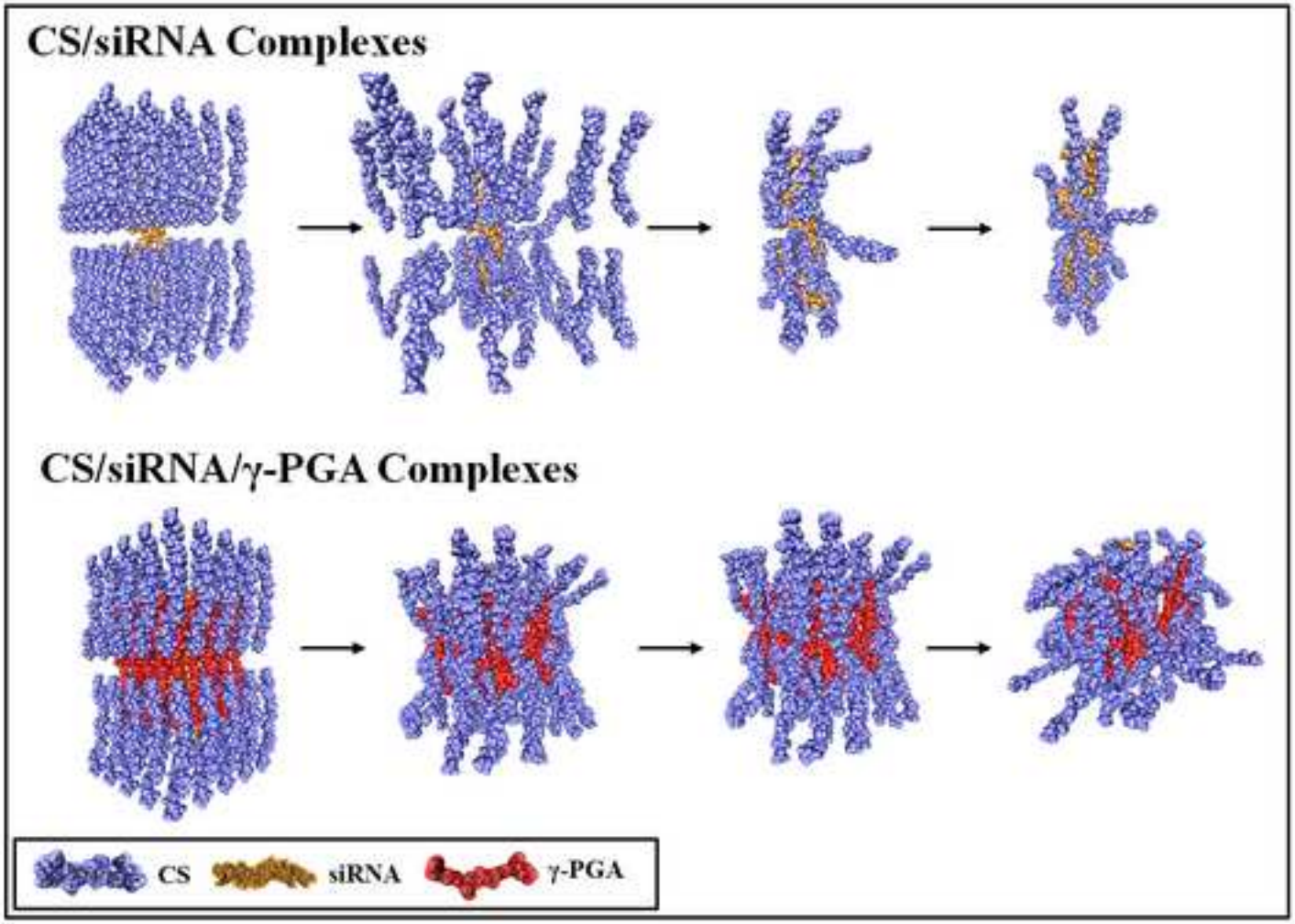

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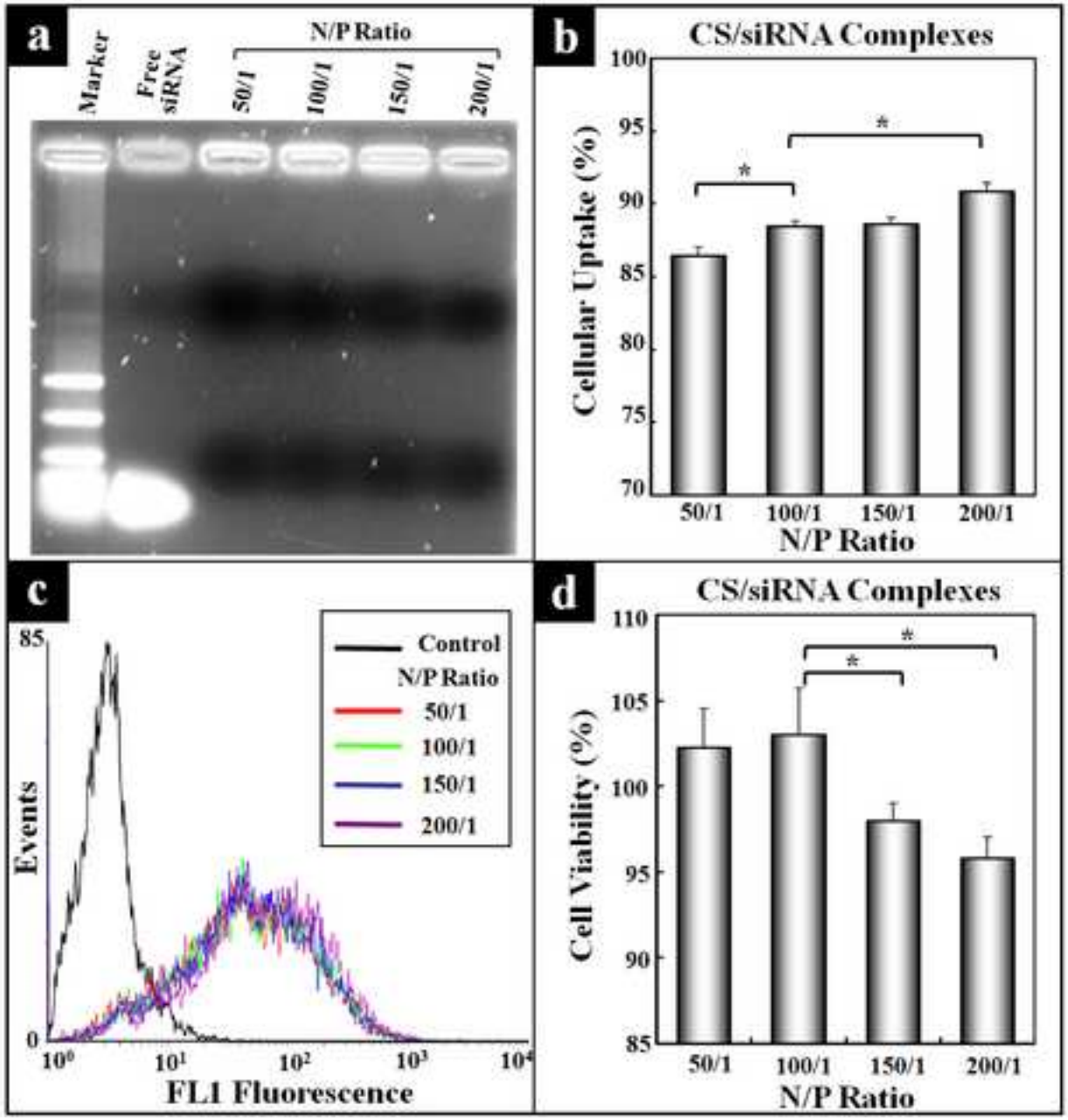

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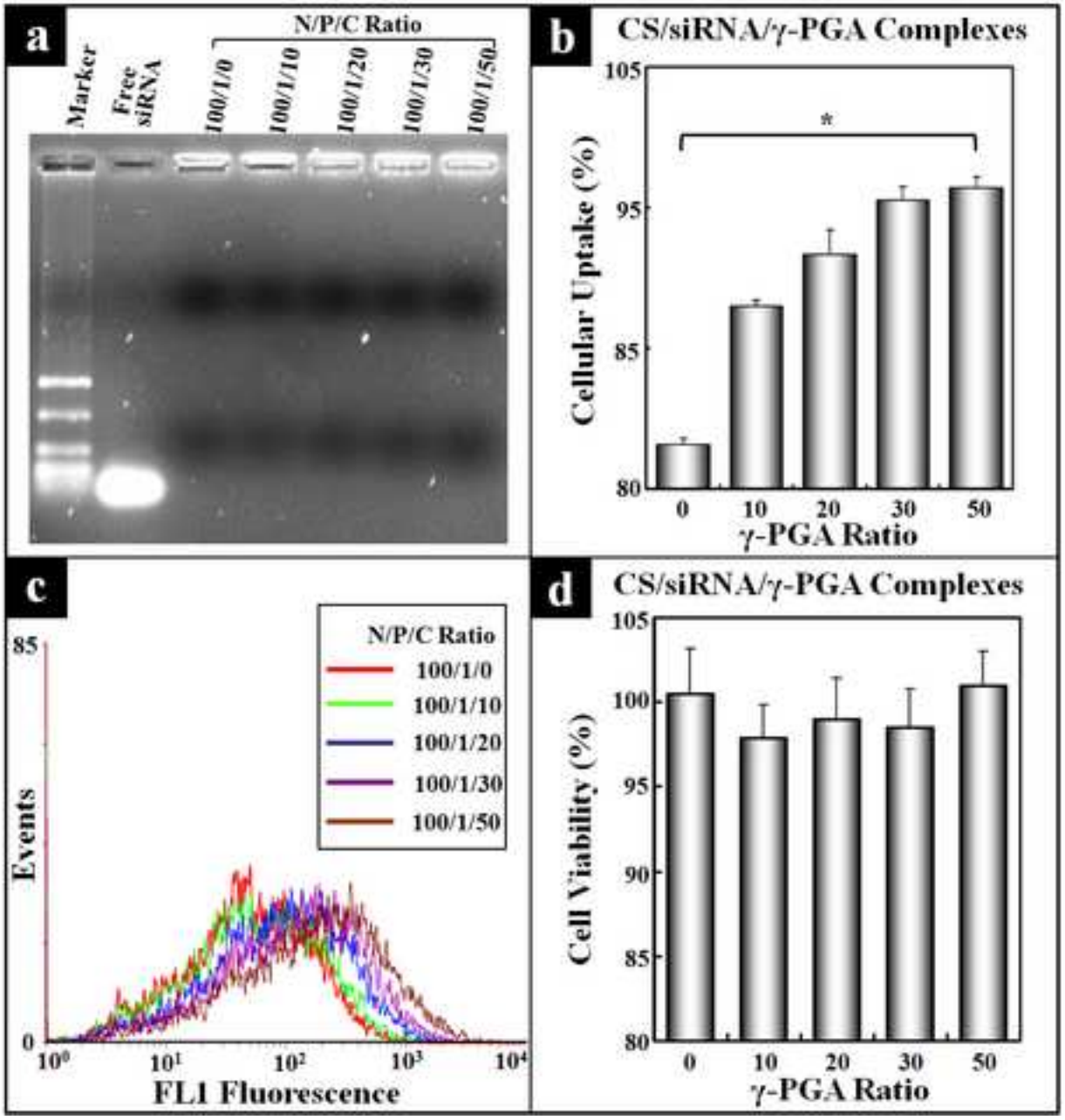

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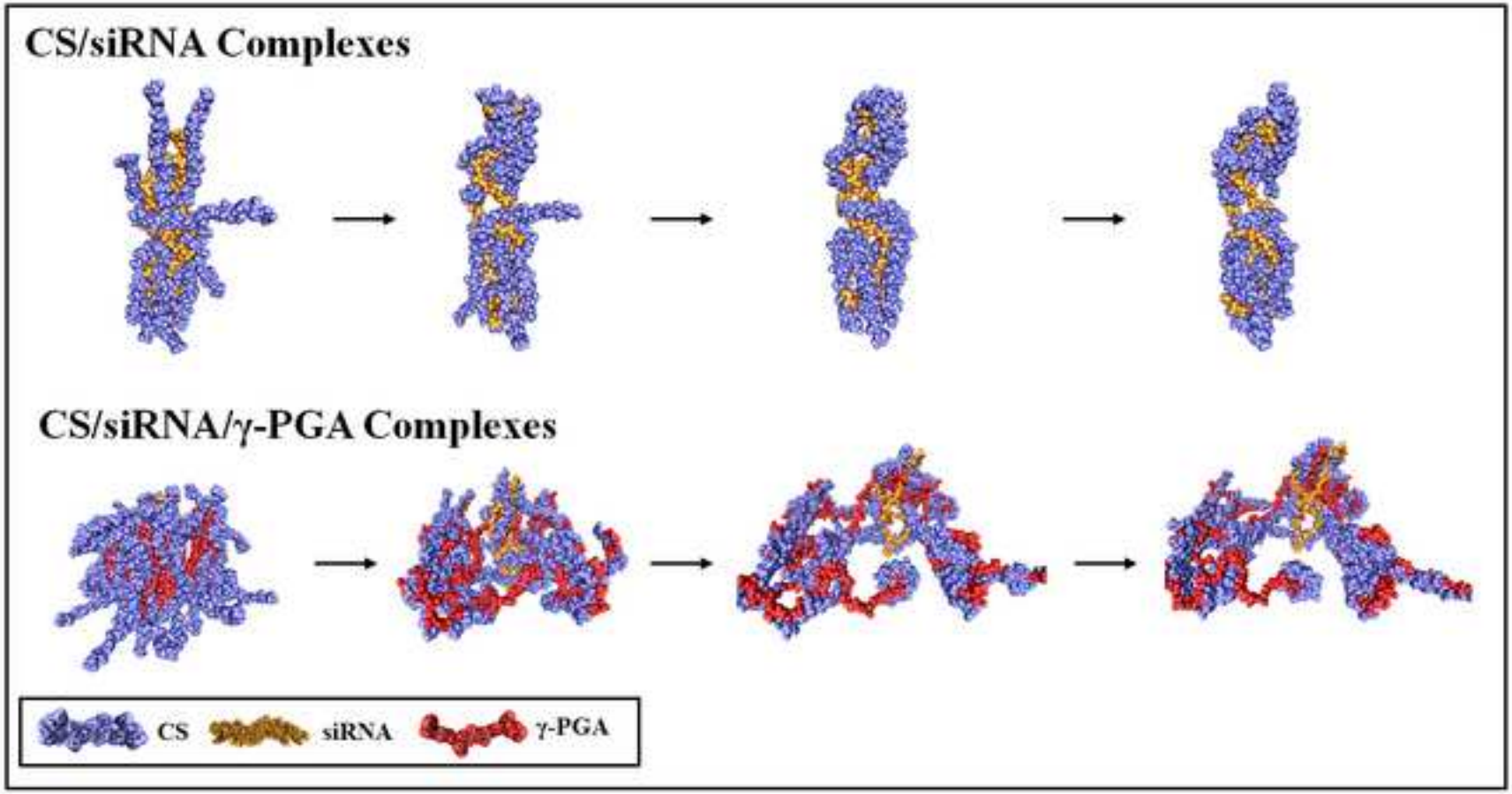

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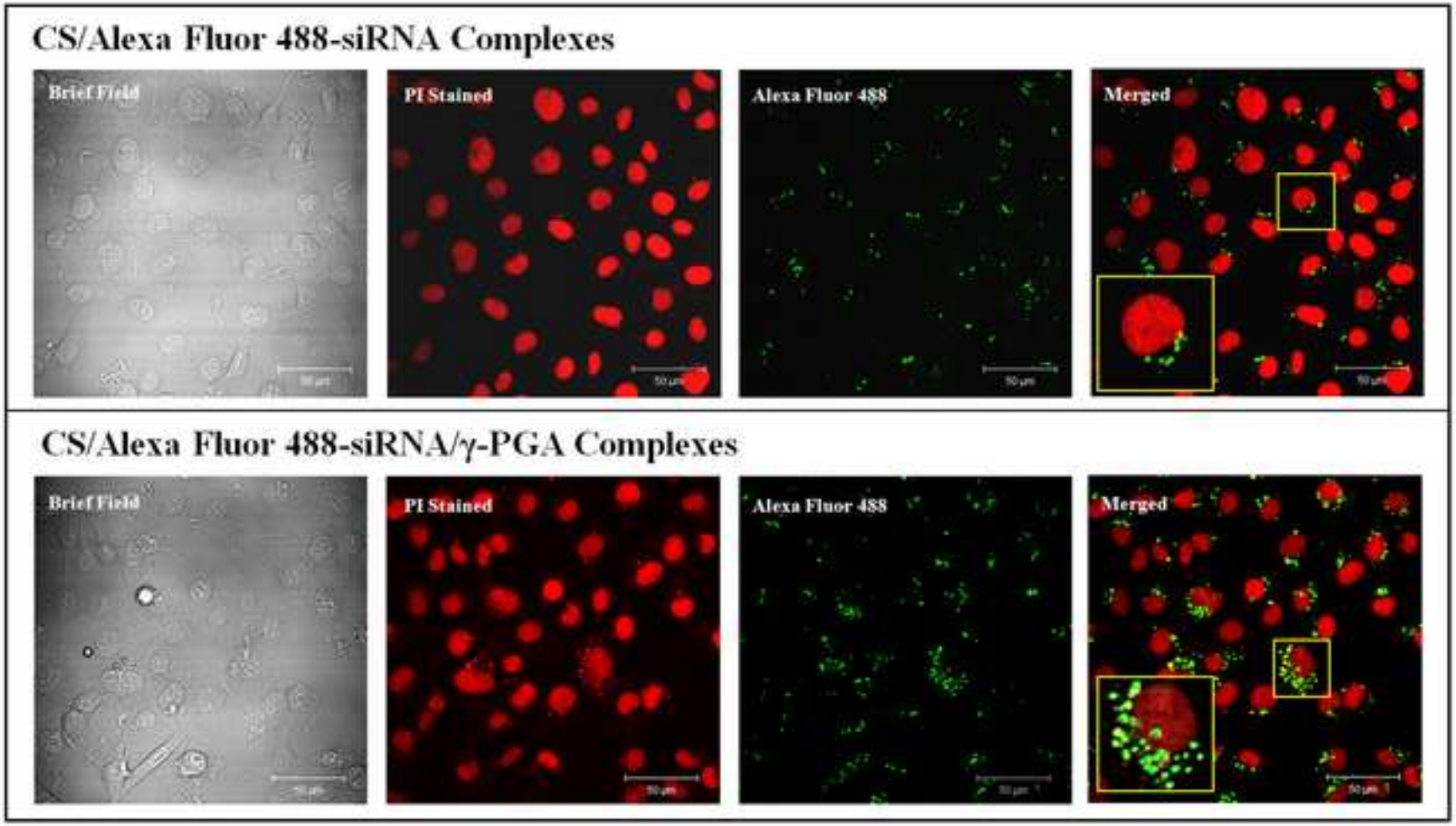

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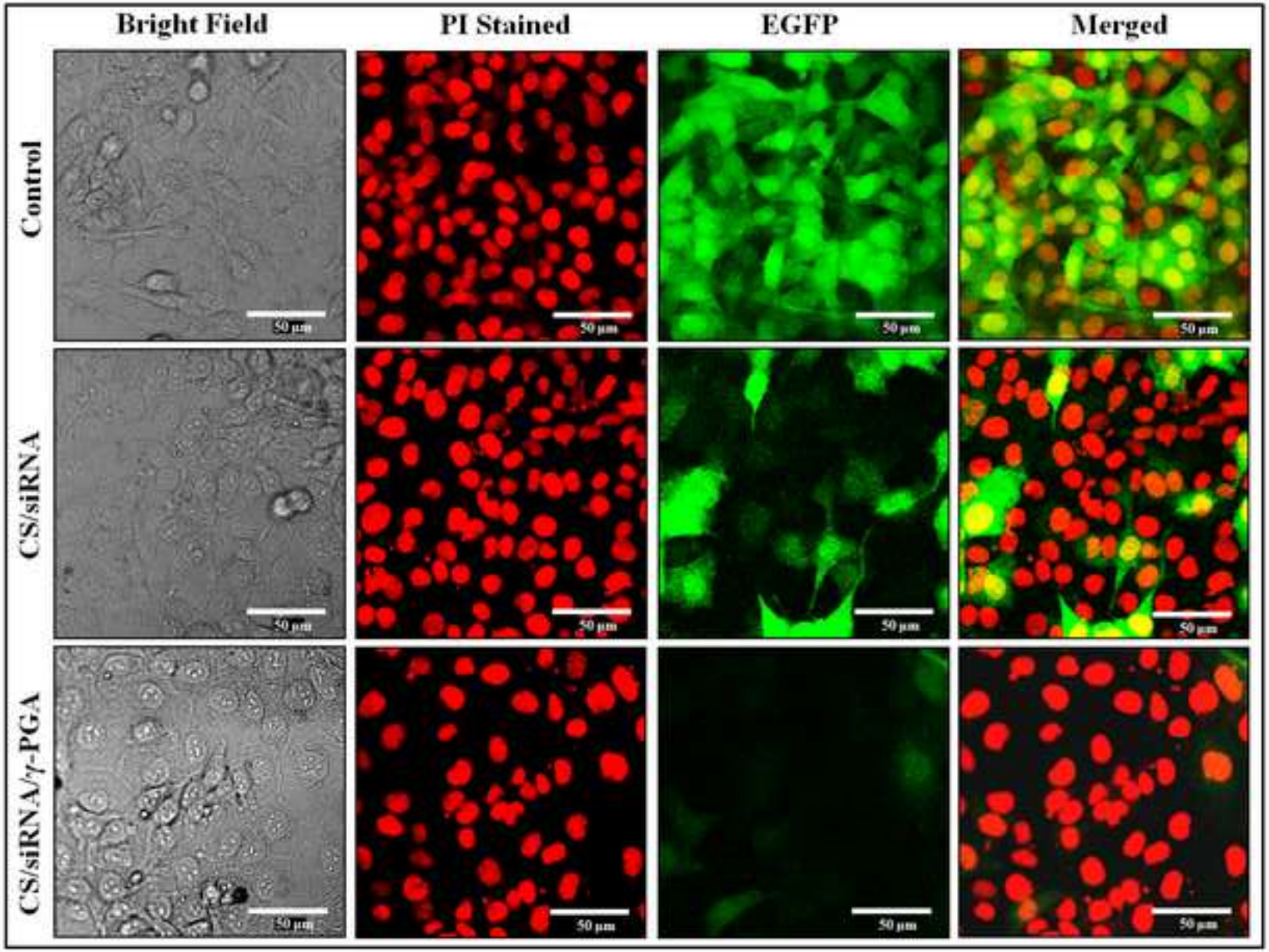

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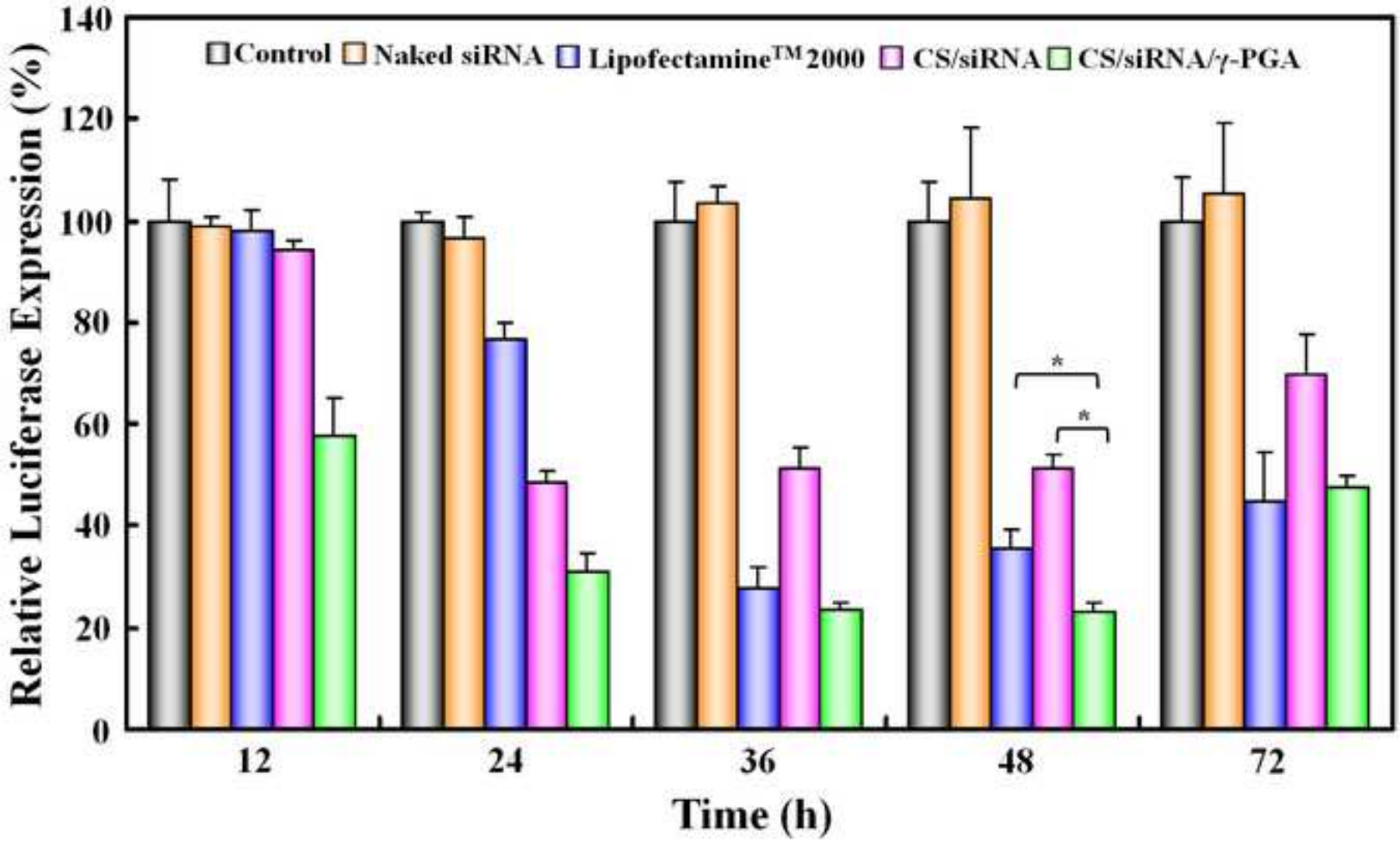

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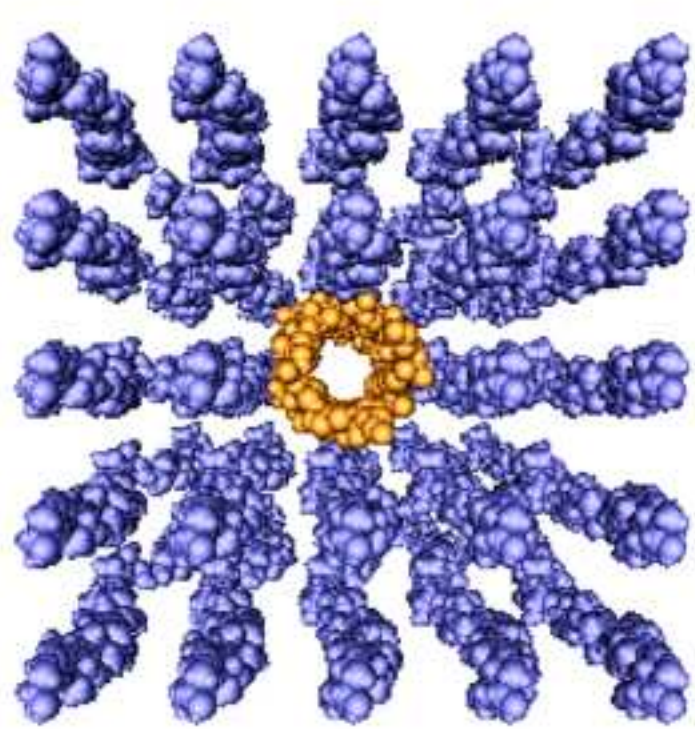

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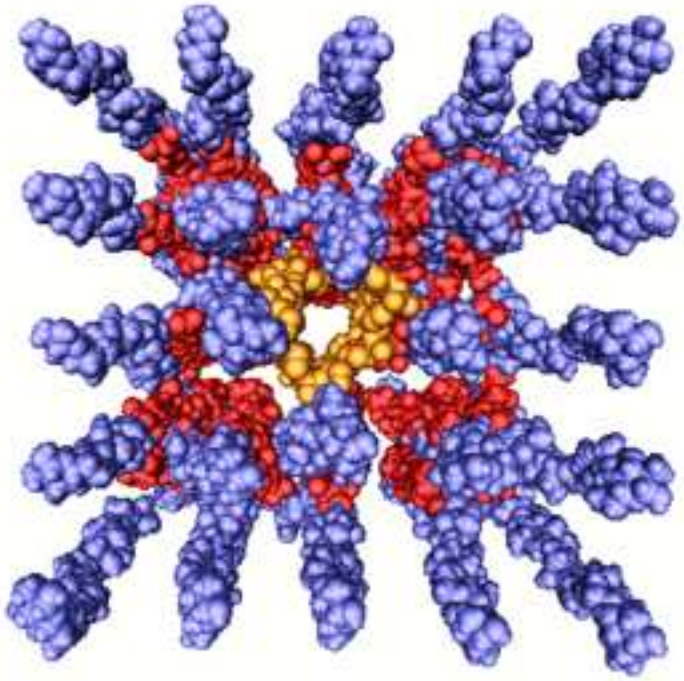

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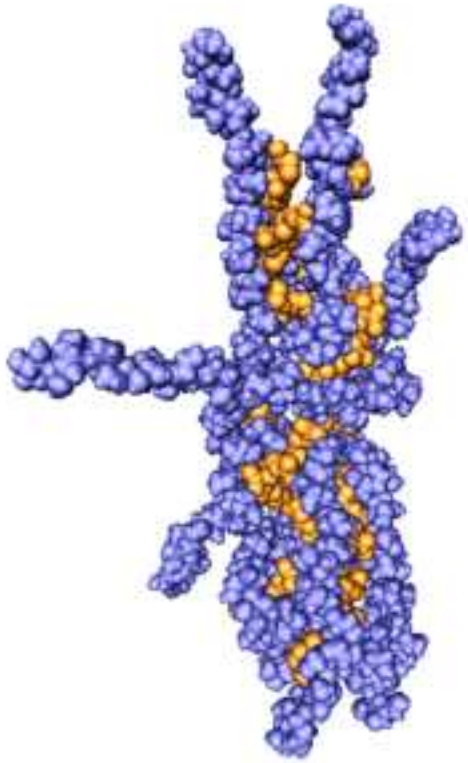

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