Elsevier Editorial System(tm) for Biomaterials
Manuscript Draft

Manuscript Number:

Title: Mechanistic study of transfection of chitosan/DNA complexes coated by anionic poly(γ-glutamic acid)

Article Type: FLA Original Research

Section/Category: Biomaterials for the Delivery of Drugs, Genes, Vaccines and Active Biomolecules (BDGV)

Keywords: Keywords: gene transfection; γ-glutamyl transpeptidase; receptor-mediated endocytosis; γ-glutamyl unit; poly(γ-glutamic acid)

Corresponding Author: Dr. Hsing-Wen Sung,

Corresponding Author's Institution: National Tsing Hua University

First Author: Zi-Xian Liao

Order of Authors: Zi-Xian Liao; Shu-Fen Peng; Yi-Cheng Ho; Fwu-Long Mi; Barnali Maiti; Hsing-Wen Sung

Abstract: Abstract
Chitosan (CS) has been investigated as a non-viral carrier for gene delivery, but resulting in a relatively low transfection. To address this concern, we developed a ternary system comprised the core of CS/DNA complex and the outer coating of an anionic polymer, poly(γ-glutamic acid) (γ-PGA). In molecular dynamic (MD) simulations, we found that γ-PGA was entangle tightly with the excess CS emanating from the surface of test complexes, thus making them more compact. With γ-PGA coating, the extent of test complexes internalized and their transfection efficiency were evidently enhanced. Trypsin treatment induced a concentration-dependent decrease in internalization of the γ-PGA-coated complexes, suggesting a specific protein-mediated endocytosis. The endocytosis inhibition study indicates that the γ-glutamyl transpeptidase (GGT) present on cell membranes was responsible for the uptake of test complexes. The amine group in the N-terminal γ-glutamyl unit on γ-PGA played an essential role in the interaction with GGT. When entangled with CS, the free N-terminal γ-glutamyl unit of γ-PGA on test complexes was exposed and might thus be accommodated within the γ-glutamyl binding pocket of the membrane GGT. Above results suggest that the γ-PGA coating on CS/DNA complexes can significantly enhance their cellular uptake via a specific GGT-mediated pathway. Knowledge of the uptake mechanism is crucial for the development of an efficient vector for gene transfection.

Department of Chemical Engineering
National Tsing Hua University
101, Section 2, Kuang-Fu Road, Taiwan, 30013, R.O.C.
Tel：886-3-5719036 Fax：886-3-5715408

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

October 31, 2011

Dear Professor Williams:

Attached please find a manuscript entitled"Mechanistic study of transfection of chitosan/DNA complexes coated by anionic 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 journal.

Chitosan (CS) has been investigated as a non-viral carrier for gene delivery, but resulting in a relatively low transfection. To address this concern, we developed a ternary system comprised the core of CS/DNA complex and the outer coating of an anionic polymer, poly(γ-glutamic acid) (γ-PGA). Interestingly, we found that the cellular uptake of the CS/DNA complex was evidently enhanced by the outer γ-PGA coating, thus improving its gene expression level significantly. This study was designed to explore the role that γ-PGA coating may play in enhancing the cellular uptake of CS/DNA complexes. Knowledge of the uptake mechanism is crucial for the development of an efficient vector for gene 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,

Hsing-Wen Sung, Ph.D.
Tsing Hua Chair Professor
Department of Chemical Engineering
National Tsing Hua University
Hsinchu, Taiwan 30013
Phone: +886-3-574-2504
Fax: +886-3-572-6832
Email: hwsung@che.nthu.edu.tw

*Mandatory Author Declaration

AUTHOR DECLARATION

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work. [OR]

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from biomaterials@online.be.

Signed by all authors as follows:

[LIST AUTHORS AND DATED SIGNATURES ALONGSIDE]

Zi-Xian Liao Zi Xian Liao

Shu-Fen Peng Shu-Fen Peng

Yi-Cheng Ho Yi-Cheng Ho

Fwu-Long Mi Fwu-Long Mi

Barnali Maiti Barnali Maiti

Hsing-Wen Sung Hsing Wen Sung 11/1/2011

*Title Page

Mechanistic study of transfection of chitosan/DNA complexes coated by anionic poly(γ-glutamic acid)

Zi-Xian Liao[1†], Shu-Fen Peng[2,3†], Yi-Cheng Ho[4],
Fwu-Long Mi[4], Barnali Maiti[1], Hsing-Wen Sung[1*]

[1] Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan, ROC
[2] Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, ROC
[3] Department of Medical Research, China Medical University Hospital, Taichung, Taiwan, ROC
[4] Department of Biotechnology, Vanung University, Chungli, Taoyuan, Taiwan, ROC

*Correspondence to:
Hsing-Wen Sung, PhD
Tsing Hua Chair Professor
Department of Chemical Engineering
National Tsing Hua University
Hsinchu, Taiwan 30013
Phone: +886-3-574-2504
Fax: +886-3-572-6832
E-mail: hwsung@che.nthu.edu.tw

† The first two authors (Z.X. Laio and S.F. Peng) contributed equally to this work.

Abstract

Chitosan (CS) has been investigated as a non-viral carrier for gene delivery, but resulting in a relatively low transfection. To address this concern, we developed a ternary system comprised the core of CS/DNA complex and the outer coating of an anionic polymer, poly(γ-glutamic acid) (γ-PGA). In molecular dynamic (MD) simulations, we found that γ-PGA was entangle tightly with the excess CS emanating from the surface of test complexes, thus making them more compact. With γ-PGA coating, the extent of test complexes internalized and their transfection efficiency were evidently enhanced. Trypsin treatment induced a concentration-dependent decrease in internalization of the γ-PGA-coated complexes, suggesting a specific protein-mediated endocytosis. The endocytosis inhibition study indicates that the γ-glutamyl transpeptidase (GGT) present on cell membranes was responsible for the uptake of test complexes. The amine group in the *N*-terminal γ-glutamyl unit on γ-PGA played an essential role in the interaction with GGT. When entangled with CS, the free *N*-terminal γ-glutamyl unit of γ-PGA on test complexes was exposed and might thus be accommodated within the γ-glutamyl binding pocket of the membrane GGT. Above results suggest that the γ-PGA coating on CS/DNA complexes can significantly enhance their cellular uptake via a specific GGT-mediated pathway. Knowledge of the uptake mechanism is crucial for the development of an efficient vector for gene transfection.

Keywords: gene transfection; γ-glutamyl transpeptidase; receptor-mediated endocytosis; γ-glutamyl unit; poly(γ-glutamic acid)

1. Introduction

Chitosan (CS), a linear cationic amine-containing polysaccharide, has the potential to condense negatively-charged DNA into a compact structure via electrostatic interactions [1,2]. It has been shown that CS can protect DNA from nuclease degradation and transfect DNA into different cell types [3]. Although advantageous for DNA packing and protection, the transfection efficiency of such binary CS/DNA complex is relatively low [4]. To enhance its gene expression level, an approach through the modification of its internal structure by incorporating an anionic polymer, poly(γ-glutamic acid) (γ-PGA), inside the CS/DNA complex was previously reported by our group [5]. γ-PGA, a naturally occurring peptide that consists of D- and L-glutamic acids polymerized through γ-glutamyl bonds, is produced by certain *Bacillus* strains as capsular or extracellular viscous materials [6]; it is water-soluble, biodegradable and non-toxic. γ-PGA has been used as an adjuvant for oral delivery of insulin [7,8] and been employed to deliver protein vaccines [9].

Our previous results obtained in molecular dynamic (MD) simulations suggested that after forming a ternary complex, γ-PGA may be recognized by an intrinsic membrane protein, γ-glutamyl transpeptidase (GGT), resulting in a significant increase in its cellular uptake [10]. After internalization, a less percentage of co-localization of the ternary CS/DNA/γ-PGA complex with lysosomes was observed when compared to its binary CS/DNA counterpart. A greater cellular uptake together with a less entry into lysosomes thus enhanced the transfection efficiency of the CS/DNA/γ-PGA complex significantly. According to these results, γ-PGA plays an important role in augmenting the cellular uptake and transfection efficiency of CS-based gene carriers.

To take full advantage of this unique feature in the study, we first prepared the cationic CS/DNA complex; anionic γ-PGA was then electrostatically coated onto its surface (Figure 1). Membrane-active polyanions have been proposed as adjuvants for transfection of cationic DNA complexes [11-13]. It has been reported that polyanionic coatings on polycation/DNA complexes could efficiently reduce their non-specific interaction with blood components, which inhibit transfection in the serum-containing medium, and especially to the *in vivo* gene therapy [14,15].

In this work, the prepared CS/DNA complexes coated with different amounts of γ-PGA [(CS/DNA)/γ-PGA complexes] were characterized by means of the MD simulations and the gel retardation and dynamic light scattering (DLS) assays. Their cytotoxicity was evaluated via the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, while the internalization and transfection efficiency were examined using a confocal laser scanning microscope (CLSM) and a flow cytometer, respectively. Additionally, the interaction between γ-PGA and GGT present in the cell membranes was investigated through the MD simulations.

2. Materials and Methods

2.1. Materials

CS (15 kDa) with a degree of deacetylation of approximately 85% and γ-PGA (30 kDa) were purchased from Challenge Bioproducts and Vedan (Taichung, Taiwan), respectively. The plasmid DNA used in the study was pEGFP-N2 (4.7 kb, coding an enhanced GFP reporter gene, Clontech, Palo Alto, CA, USA). pEGFP-N2 was amplified using DH5α and purified by a Qiagen Plasmid Mega Kit (Valencia, CA, USA) according to the manufacturer's instructions. Plasmid purity was analyzed by gel electrophoresis (1% agarose), while its concentration was measured by ultraviolet-visible (UV) absorption at 260 nm (Jasco, Tokyo, Japan).

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 DNA and the carboxyl groups (C) on γ-PGA. Test complexes, (CS/DNA)/γ-PGA, at various known (N/P)/C ratios [(10/1)/0, (10/1)/4, (10/1)/12, (10/1)/20 and (10/1)/40] were prepared in deionized (DI) water (pH 6.0). CS/DNA complexes were first made via an ionic-gelation method by blending an aqueous DNA (pEGFP-N2, 33 μg in 200 μl) with an aqueous CS (40 μg in 200 μl) and then thoroughly mixed for 30–60 s and left for at least 1 h at room temperature. Subsequently, an aqueous γ-PGA (0.2 μg/μl) at various known volumes (0, 1.5, 4.5, 7.5 or 15.0 μl) was gently added into the mixed solution and left for another 1 h to form test complexes.

The hydrodynamic sizes and surface charges of test complexes were measured using DLS (Zetasizer Nano ZS, 3000HS, Malvern Instruments Ltd., Worcestershire, UK). The binding efficiency between DNA and carriers was evaluated by a gel retardation assay [16]. Electrophoresis was carried out onto a 1% agarose gel with a current of 100 V for 30 min in a 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. In vitro transfection

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 on 12-well plates at 2×10^5 cells/well and transfected the next day at 50–80% confluency. 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 or naked DNA at a concentration of 2 μg DNA/well.

At 2 h post transfection, the transfection media containing test complexes were removed; the cells were then rinsed twice with transfection media and refilled with FBS-containing media until analysis at 24 h after transfection. The transfected cells were observed under a fluorescence microscope (Carl Zeiss Optical, Chester, VA, USA) to monitor any morphological changes and to obtain an estimate of the transfection efficiency. Cells transfected with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) were used as a positive control, and those without any treatment were used as a negative control. Transfection efficiencies were presented by two numeric indicators: percentages of cellular uptake and cell transfected [17].

2.4. Preparation of fluorescence complexes

Fluorescence complexes were prepared to track their internalization by CLSM and quantify their cellular uptake by flow cytometry. FITC-labeled CS (FITC-CS), Cy5-labeled CS (Cy5-CS) and fluoresceinamine-labeled γ-PGA (FA-γ-PGA) were synthesized as per the methods described in the literature [18,19]. To remove the unconjugated FITC, Cy5 and FA,

the synthesized FITC-CS, Cy5-CS and FA-γ-PGA were dialyzed individually in the dark against DI water and replaced on a daily basis until no fluorescence was detected in the supernatant. The resultant samples were lyophilized in a freeze dryer and then used to prepare fluorescence complexes as described in Section 2.2.

2.5. CLSM visualization and flow-cytometry analysis

To track the internalization of test complexes, cells were seeded on 12-well plates with a sterile glass coverslip at 2×10^5 cells/well and incubated overnight. Subsequently, cells were rinsed twice with transfection media and transfected with (Cy5-CS/DNA)/FA-γ-PGA complexes. After incubation for 2 h, cells were washed twice with pre-warmed phosphate-buffered saline (PBS) before they were fixed in 4% paraformaldehyde. Finally, the fixed cells were examined under a CLSM (TCS SL, Leica, Germany).

To quantify the cellular uptake of test complexes, cells were plated on 12-well plates and transfected with (FITC-CS/DNA)/γ-PGA complexes at a concentration of 2 μg DNA/well for 2 h. Following transfection, cells were detached by 0.025% trypsin-EDTA and transferred to microtubes. Subsequently, cells were resuspended in PBS containing 1*mM* EDTA and fixed in 4% paraformaldehyde. Finally, the cells were analyzed by flow cytometry (Beckman Coulter, Fullerton, CA, USA) equipped with a 488 nm argon laser for excitation.

For each sample, 10,000 events were collected and fluorescence was detected. Signals were amplified in logarithmic mode for fluorescence to determine the EGFP-positive events by a standard gating technique. The percentage of positive events was calculated as the events within the gate divided by the total number of events, excluding cell debris.

2.6. MD simulations

MD simulations of the configurations of (CS/DNA)/γ-PGA complexes and the interaction between γ-PGA and GGT were performed by a MD method [20]. MD simulations were accomplished with the program NAMD [21] using parameters adapted from the CHARMM 27 force field [22]. 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 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 with hydrogen bondings were generated using VMD [23] and UCSF Chimera [24].

2.7. Endocytosis inhibition

To study whether the cell-surface proteins were involved in the uptake of test complexes, cells were incubated with distinct concentrations of trypsin (0.01%, 0.025% and 0.05% by w/v in Hanks' salt solution) for 5 min prior to transfection [25]. Cells were then treated with FITC-CS/DNA complexes with or without the γ-PGA coating for 2 h. Subsequently, cells were washed three times with PBS, collected according to the methods described above and analyzed by flow cytometry. The groups in the presence of test complexes but without trypsin treatment were used as controls, and their fluorescence intensities were expressed as 100%.

To study the effect of a highly selective GGT inhibitor (GGs) on the uptake of test complexes, cells were pre-incubated with GGs at varying known concentrations (0, 50 and 100*nM*) which were not toxic to the cells [26]. Following pre-incubation for 1 h, the inhibitor solutions were removed, and the freshly prepared FITC-labeled complexes in media containing GGs at the same concentrations were added and further incubated for 2 h. Subsequently, cells were washed three times with PBS, collected according to the methods described above and analyzed by flow cytometry.

2.8. MTT assay

The cytotoxicity of test complexes was evaluated *in vitro* using the MTT assay [27]. HT1080 cells were seeded on 24-well plates at 5×10^4 cells/well, allowed to adhere overnight and transfected by test complexes containing 1 μg DNA. After 2 h, test samples were

aspirated, and cells were incubated for another 22 h. Subsequently, cells were incubated in a growth medium containing 1 mg/ml MTT reagent for an additional 4 h; a 500 µl of dimethyl sulfoxide (DMSO) was added to each well to ensure the solubilization of formazan crystals. Finally, the optical density readings were performed using a multiwell scanning spectrophotometer (Dynatech Laboratories, Chantilly, VA, USA) at a wavelength of 570 nm.

2.9. Modification of the terminal amine group on γ-PGA

To evaluate its effect on cellular uptake, the terminal amine group on γ-PGA was modified as per a method reported in the literature [28]. In brief, 6 mg of sodium dodecyl sulfate (SDS) and 5 ml of acetic anhydride were added into an aqueous γ-PGA (0.2 µg/µl, 2 ml); the mixture was emulsified under magnetic stirring for 24 h to block the terminal amine group on γ-PGA. Upon completion of the reaction, 100 ml of acetone was added to the reaction flask to precipitate the acetylated γ-PGA. The precipitated γ-PGA derivative was washed five times with acetone to remove the unreacted acetic anhydride. The acetylated γ-PGA was then transferred to a dialysis tube (MWCO: 6000–8000) and dialyzed against DI water for 3 days. The final product was lyophilized and then stored at –20°C until used. The purified acetylated γ-PGA was analyzed by ^{1}H NMR (Varian Unityionva 500 NMR Spectrometer, MO, USA). The degree of acetylation on the terminal amine group of γ-PGA was estimated using the ninhydrin assay [29].

2.10. 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

CS has been investigated as a non-viral carrier for gene delivery, but resulting in a relatively low transfection [4]. To address this concern, we developed a ternary gene-transfection system, which comprised the core of CS/DNA complex and the outer coating of an anionic polymer, γ-PGA. Interestingly, we found that the cellular uptake of the

CS/DNA complex was evidently enhanced by the outer γ-PGA coating, thus improving its gene expression level significantly. Normally, anionic complexes are not taken up well by cells, as they have a tendency to be electrostatically repelled by the negatively-charged cell membranes. Therefore, the ternary complex containing γ-PGA developed in the study must have a different mechanism in cellular uptake. This study was designed to explore the role that γ-PGA coating may play in enhancing the cellular uptake of CS/DNA complexes.

3.1. Characterization of test complexes

The binding capacity of DNA with CS was previously examined by our group using the gel retardation assay [5]; the obtained data showed that as its N/P ratio increased to 10/1, the migration of DNA was retarded efficiently. Therefore, preparation of the CS/DNA complex was carried out at an N/P ratio of 10/1 in the study. No significant release of DNA from test complexes containing γ-PGA at varying (N/P)/C ratios was observed, an indication of their DNA binding stability (Figure 2a).

The particle size and zeta potential of test complexes in aqueous media were investigated by DLS. According to Figure 2b, test complexes coated with γ-PGA had a smaller particle size than their counterpart in the absence of γ-PGA coating [the CS/DNA complex with an (N/P)/C ratio of (10/1)/0, $P < 0.05$]; additionally, γ-PGA coating significantly reduced their surface charge ($P < 0.05$, Figure 2c). With increasing the coating of γ-PGA, the zeta potential of test complexes decreased appreciably and reached its minimum value (approximately –27 mV) at an (N/P)/C ratio of (10/1)/12 ($P < 0.05$); further increasing the amount of γ-PGA used [(N/P)/C ratios of (10/1)/20 and (10/1)/40] did not significantly alter their surface charge ($P > 0.05$).

3.2. MD simulations of configurations of test complexes

MD simulations were performed in a full-atom model to model the configurations of (CS/DNA)/γ-PGA complexes formed at distinct (N/P)/C ratios. Both CS and γ-PGA molecules considered in MD simulations contained 10 monomer units, and DNA was a double helix with 20 mer on each strand. Figure 3 displays the conformations of (CS/DNA)/γ-PGA complexes self-assembled in DI water. In the CS/DNA complex [(10/1)/0], as CS molecules were in excess of DNA in terms of the prescribed N/P ratio of 10/1, only a

fraction of CS molecules (in blue) were able to bind with DNA (in yellow) to form a primary aggregate for the binary complex. The complex thus formed was overcharged due to the excess CS used; namely, the CS/DNA complex carried an overall positive charge.

γ-PGA can be in different conformational states, including α-helix, β-sheet, random coil, helix-coil transition and enveloped aggregation, depending on its exposed environmental pH [30]. In DI water (pH 6.0), γ-PGA molecules are mainly in the form of linear random-coil conformation and show a polyanionic characteristic [30], allowing themselves to entangle tightly with the excess CS emanating from the surface of the complex, thus making the complex more compact. The amount of γ-PGA entangled with CS on the complex was saturated when the (N/P)/C ratio was increased to (10/1)/12. With further increasing the (N/P)/C ratio, there were excess γ-PGA molecules suspending in the aqueous solution in the vicinity of the (CS/DNA)/γ-PGA complex.

3.3. Cellular uptake and transfection efficiency

The cellular uptake and transfection efficiency were used as the criteria to optimize the (N/P)/C ratio when preparing (CS/DNA)/γ-PGA complexes. To visualize the cellular uptake under CLSM, CS and γ-PGA were fluorescently labeled with Cy5 and FA, respectively; the percentage of cells that internalized test complexes (labeled by FITC) was quantified by flow cytometry at 2 h after transfection. As shown in Figure 4a, the free form γ-PGA (in green) was not seen inside the cells. As is well known, polyanions cannot interact effectively with the cell membranes due to the electrostatic repulsion and consequently hardly be internalized [31].

Accumulation of the CS/DNA complex [(10/1)/0] was observed intracellularly. With the γ-PGA coating, the extent of test complexes internalized (Figure 4a) and the percentage of fluorescent cells (Figure 4b) were evidently increased ($P < 0.05$), indicative of a greater cellular uptake. With increasing the coating of γ-PGA, the fluorescence intensity observed in cells increased notably. The test complex with an N/P/C ratio of (10/1)/12 had the maximum

cellular uptake ($P < 0.05$). Further increasing γ-PGA coating on test complexes reduced their cellular uptake appreciably. Of note is that the fluorescence intensity of γ-PGA coating (in green) observed intracellularly was not as distinct as that of CS complexes (in red, Figure 4c).

To define the percentage of cells that actually expressed the transgene, we counted the number of EGFP-positive cells using flow cytometry at 24 h post transfection. When transfected with the CS/DNA complex, approximately 25% of the cells produced EGFP (Figure 5a). With the γ-PGA coating on test complexes, both the percentage of cells expressed EGFP (Figure 5a, $P < 0.05$) and their fluorescence intensity (Figure 5b) increased considerably. Among all studied groups, the cells treated with test complexes prepared at an (N/P)/C ratio of (10/1)/12 had the most evident expression of EFGP (85%, $P < 0.05$). Beyond this (N/P)/C ratio, the transfection efficiency of test complexes decreased appreciably.

3.4. MTT assay

Figure 5C shows the viability of cells cultured in the media treated with distinct test complexes. As compared to the negative control (NC, the group without any treatment), the cytotoxicity of naked DNA (NK), γ-PGA and CS was minimal. The viability of cells treated with the γ-PGA-coated complexes decreased relatively, due to their high expression of EGFP. It is well recognized that EGFP protein is toxic to the cells [32].

3.5. Receptor-mediated endocytosis

The aforementioned results indicate that with γ-PGA coating, the cellular uptake of CS/DNA complexes was significantly enhanced, suggesting that there might be a γ-PGA-specific receptor-mediated pathway involved in internalization of test complexes [33]. To distinguish differences in their uptake mechanism, the interaction of test complexes, in the absence/presence of γ-PGA coating, with cell membranes was investigated by treating cells with trypsin at different concentrations prior to transfection. Trypsinization can cause a significant reduction in surface-bound proteins on plasma membranes [34]. As shown in Figure 6a, trypsinization resulted in a substantial decrease in internalization of test complexes with or without the γ-PGA coating ($P < 0.05$). However, trypsin treatment induced a concentration-independent effect on the uptake of test complexes in the absence of γ-PGA

coating [(10/1)/0, $P > 0.05$], while producing a concentration-dependent decrease in internalization of the γ-PGA-coated complexes [(10/1)/12, $P < 0.05$]. These results imply that with γ-PGA coating, CS-based complexes can be internalized by cells via a specific protein-mediated endocytosis.

GGT, a highly glycosylated heterodimeric enzyme, is bound to the external surface of plasma membrane and is expressed in high concentration in mammalian cells [10]. GGT catalyzes the cleavage of extracellular glutathione and other γ-glutamyl-containing compounds such as γ-PGA to water (hydrolysis) or to amino acids and peptides (transpeptidation) [35,36]. To study the effect of GGT present in the cell membranes on the uptake of test complexes (labeled by FITC), cells were treated with varying concentrations of GGs, an inhibitor specific to GGT, prior to transfection. Their counterparts in the absence of inhibitors were used as controls. Compared with the control, the fluorescence intensity observed by flow cytometry in cells treated with the CS/DNA complex [(10/1)/0] was GGs-concentration independent ($P > 0.05$, Figure 6b), indicating that the GGT-mediated pathway was not involved in uptake of the CS/DNA complex. In contrast, that treated with the γ-PGA-coated complexes was dependent upon the GGs concentration used ($P < 0.05$), suggesting that γ-PGA coating substantially enhanced their internalization via the GGT-mediated pathway.

3.6. MD simulations of the interaction between γ-PGA and GGT

The role that γ-PGA may play in enhancing the cellular uptake of CS-based complexes was explored using MD simulations. In the free form γ-PGA, its terminal amine group ($-NH_2$) may form intramolecular hydrogen bonds with the carbonyl groups (−C=O) on its neighboring units and thus is hidden (Figure 7a). The hidden *N*-terminal γ-glutamyl unit makes the free form γ-PGA difficult to interact with the γ-glutamyl binding pocket of the membrane GGT (Figure 7b).

In contrast, in the entanglements of γ-PGA/CS on test complexes, the amine groups of CS may form intermolecular hydrogen bonds with the carbonyl groups on γ-PGA; the amine group in the *N*-terminal γ-glutamyl unit on γ-PGA is therefore freed and exposed (Figure 7a). The exposure of the free *N*-terminal γ-glutamyl unit of γ-PGA on test complexes may thus be

accommodated within the γ-glutamyl binding pocket of GGT on cell membranes (Figure 7b). It has been reported that GGT may exert the specificity to those substrates containing free γ-glutamyl units in their *N*-terminal ends [37].

For test complexes containing γ-PGA up to an (N/P)/C ratio of (10/1)/12, γ-PGA was tightly bound on their surfaces (Figure 3) and consequently might effectively entangle with CS and expose their free *N*-terminal γ-glutamyl units to interact with the membrane GGT, resulting in a significant increase in their cellular uptake (Figures 4a and 4b). Beyond this (N/P)/C ratio [(10/1)/20 or (10/1)/40], the hidden *N*-terminal γ-glutamyl units of the excess (or pendant) γ-PGA molecules surrounding test complexes may not interact with GGT competently, thus reducing their cellular uptake.

In addition, the enzyme GGT may cleave (or hydrolyze) the γ-glutamyl bonds of γ-PGA to transfer the γ-glutamyl units to water before test complexes were internalized (Figure 7b). This fact may explain the reason why the fluorescence intensity of the γ-PGA coating seen intracellularly was not as evident as that of CS complexes (Figure 4c).

3.7. Effect of acetylated γ-PGA on cellular uptake

To further support the aforementioned proposed uptake mechanism, the amine group in the *N*-terminal γ-glutamyl unit on γ-PGA was protected via acetylation. Figure 8a shows the ^{1}H NMR spectrum of γ-PGA; the chemical shifts observed at δ 1.79 and 1.93 ppm corresponded to β-CH_2, whereas the peaks at δ 2.20 and 3.99 ppm represented the protons of γ-CH_2 and α-CH, respectively. After acetylation (Figure 8b), the characteristic peak of α-CH shifted from δ 3.99 to 5.15 ppm due to the electron withdrawing nature of the acetyl moiety attached to the primary amine of γ-PGA. Similarly, the chemical shift of NH proton shifted from 7.92 to 4.26 ppm. In addition, a new peak corresponded to the CH_3 protons of the acetyl group appeared at δ 2.0 ppm, indicating that the terminal amine group on γ-PGA was successfully acetylated. The degree of acetylation of γ-PGA, determined by the ninhydrin assay, was estimated to be 91%.

The acetylated γ-PGA was then used to coat CS/DNA complexes, and its transfection efficiency was examined by flow cytometry. At 2 h after transfection, the fluorescence intensity in cells treated with test complexes containing the acetylated γ-PGA decreased

significantly (approximately 60% reduction) when compared to the γ-PGA-coated counterpart, indicating that the terminal amine group on γ-PGA did play a crucial role on cellular uptake.

Above results suggest that the γ-PGA coating on CS/DNA complexes can significantly enhance their cellular uptake via a specific GGT-mediated pathway, using its exposed free *N*-terminal γ-glutamyl unit (Figure 1). After internalization, as both γ-PGA and DNA in test complexes carried negative charges, the electrostatic repulsion between the two might lead to the disintegration of test complexes. Such a structure disruption may facilitate the intracellular release of DNA, thus augmenting their transfection efficiency.

4. Conclusions

In the study, we found that γ-PGA coating can significantly enhance the cellular uptake of CS/DNA complexes, consequently augmenting their gene expressing level. The γ-PGA-coated complexes can be internalized by cells via a specific GGT-mediated pathway. The amine group in the *N*-terminal γ-glutamyl unit on γ-PGA played a significant role in uptake of test complexes. Once entangled with CS, the *N*-terminal γ-glutamyl unit on γ-PGA was exposed and might be effectively interact with the γ-glutamyl binding pocket of the membrane GGT. Detailed knowledge of the uptake mechanism is important for the development of more efficient carriers.

Acknowledgment

This work was supported by a grant from the National Science Council (NSC 100-2120-M-007-003), Taiwan, Republic of China.

Figure Captions

Figure 1. Schematic illustrations showing the self-assembly of chitosan (CS), DNA, poly(γ-glutamic acid) (γ-PGA) into test complexes (CS/DNA and CS/DNA coated with γ-PGA) and the mechanism of their cellular uptake and gene expression.

Figure 2. (a) Results of the gel retardation assay of (CS/DNA)/γ-PGA complexes prepared at different (N/P)/C ratios. Samples were run on a 1% agarose gel and subsequently stained by ethidium bromide; (b) Particle size and zeta potential of (CS/DNA)/γ-PGA complexes prepared at different (N/P)/C ratios.

Figure 3. Configurations of (CS/DNA)/γ-PGA complexes prepared at different (N/P)/C ratios in deionized water, obtained by molecular dynamic simulations. γ-PGA: poly(γ-glutamic acid).

Figure 4. (a) Confocal images of cellular uptake of (CS/DNA)/γ-PGA complexes prepared at different (N/P)/C ratios; (b) Percentages of cellular uptake of (CS/DNA)/γ-PGA complexes prepared at different (N/P)/C ratios, analyzed by flow cytometry (n = 6); (c) Confocal images of cellular uptake of (CS/DNA)/γ-PGA complexes prepared at an (N/P)/C ratio of (10/1)/12.

Figure 5. (a) Percentages of EGFP-expressing cells treated by (CS/DNA)/γ-PGA complexes prepared at different (N/P)/C ratios (n = 6) and (b) their intracellular EGFP intensities, analyzed by flow cytometry; (c) Results of the viability of cells treated by (CS/DNA)/γ-PGA complexes prepared at different (N/P)/C ratios, determined by the MTT assay (n = 6). NC: negative control (the group without any treatment); NK: naked DNA; LF: Lipofectamine^{TM} 2000.

Figure 6. (a) Percentages of cellular uptake of (CS/DNA)/γ-PGA complexes, determined by flow cytometry; cells were pretreated with different concentrations of trypsin (n = 6); (b) Relative fluorescence intensities observed intracellularly; cells were pretreated with varying concentrations of γ-glutamyl transpeptidase and then transfected with (CS/DNA)/γ-PGA complexes, determined by flow cytometry (n = 6). Control: the group treated with test complexes only.

Figure 7. Results obtained in molecular dynamic simulations showing (a) the role that the free form γ-PGA or γ-PGA/chitosan entanglement might play in enhancing the cellular uptake of (CS/DNA)/γ-PGA complexes and (b) the interaction between the hidden or exposed *N*-terminal γ-glutamly unit and the γ-glutamyl binding pocket of the membrane γ-glutamyl transpeptidase (GGT).

Figure 8. ^{1}H NMR spectra of (a) γ-PGA and (b) acetylated γ-PGA.

References

[1] Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit JP. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials. 2008;29:3477–96.

[2] Kim TH, Jiang HL, Jere D, Park IK, Cho MH, Nah JW, et al. Chemical modification of chitosan as a gene carrier in vitro and in vivo. Prog Polym Sci. 2007;32:726–53.

[3] Huang M, Fong CW, Khor E, Lim LY. Transfection efficiency of chitosan vectors: effect of polymer molecular weight and degree of deacetylation. J Control Release. 2005;106:391–406.

[4] Koping-Hoggard M, Varum KM, Issa M, Danielsen S, Christensen BE, Stokke BT, et al. Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. Gene Ther. 2004;11:1441–52.

[5] Peng SF, Yang MJ, Su CJ, Chen HL, Lee PW, Wei MC, et al. Effects of incorporation of poly(gamma-glutamic acid) in chitosan/DNA complex nanoparticles on cellular uptake and transfection efficiency. Biomaterials. 2009;30:1797–808.

[6] Chunhachart O, Itoh T, Sukchotiratana M, Tanimoto H, Tahara Y. Characterization of gamma-glutamyl hydrolase produced by Bacillus sp isolated from Thai Thua-nao. Biosci Biotech Bioch. 2006;70:2779–82.

[7] Lin YH, Mi FL, Chen CT, Chang WC, Peng SF, Liang HF, et al. Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery. Biomacromolecules. 2007;8:146–52.

[8] Mi FL, Wu YY, Lin YH, Sonaje K, Ho YC, Chen CT, et al. Oral delivery of peptide drugs using nanoparticles self-assembled by poly(gamma-glutamic acid) and a chitosan derivative functionalized by trimethylation. Bioconjugate Chem. 2008;19:1248–55.

[9] Wang X, Uto T, Akagi T, Akashi M, Baba M. Poly(gamma-glutamic acid) nanoparticles as an efficient antigen delivery and adjuvant system potential – for an AIDS vaccine. J Med Virol. 2008;80:11–9.

[10] Peng SF, Tseng MT, Ho YC, Wei MC, Liao ZX, Sung HW. Mechanisms of cellular uptake and intracellular trafficking with chitosan/DNA/poly(gamma-glutamic acid)

complexes as a gene delivery vector. Biomaterials. 2011;32:239–48.

[11] Trubetskoy VS, Wong SC, Subbotin V, Budker VG, Loomis A, Hagstrom JE, et al. Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery. Gene Ther. 2003;10:261–71.

[12] Cheung CY, Murthy N, Stayton PS, Hoffman AS. A pH-sensitive polymer that enhances cationic lipid-mediated gene transfer. Bioconjugate Chem. 2001;12:906–10.

[13] Kichler A, Zauner W, Ogris M, Wagner E. Influence of the DNA complexation medium on the transfection efficiency of lipospermine/DNA particles. Gene Ther. 1998;5:855–60.

[14] Maruyama K, Iwasaki F, Takizawa T, Yanagie H, Niidome T, Yamada E, et al. Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar-containing polyanion ternary complex. Biomaterials. 2004;25:3267–73.

[15] Oupicky D, Konak C, Dash PR, Seymour LW, Ulbrich K. Effect of albumin and polyanion on the structure of DNA complexes with polycation containing hydrophilic nonionic block. Bioconjugate Chem. 1999;10:764–72.

[16] Liao ZX, Ho YC, Chen HL, Peng SF, Hsiao CW, Sung HW. 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). Biomaterials. 2010;33:8780–8.

[17] Ko IK, Ziady A, Lu SW, Kwon YJ. Acid-degradable cationic methacrylamide polymerized in the presence of plasmid DNA as tunable non-viral gene carrier. Biomaterials. 2008;29:3872–81.

[18] Qaqish RB, Amiji MM. Synthesis of a fluorescent chitosan derivative and its application for the study of chitosan-mucin interactions. Carbohyd Polym. 1999;38:99–107.

[19] Ho YP, Chen HH, Leong KW, Wang TH. Evaluating the intracellular stability and unpacking of DNA nanocomplexes by quantum dots-FRET. J Control Release. 2006;116:83–9.

[20] Marrink SJ, de Vries AH, Mark AE. Coarse grained model for semiquantitative lipid simulations. J Phys Chem B. 2004;108:750–60.

[21] Nelson MT, Humphrey W, Gursoy A, Dalke A, Kale LV, Skeel RD, et al. NAMD – a parallel, object oriented molecular dynamics program. Int J Supercomput Ap. 1996;10:251–68.

[22] Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M. Charmm – a program for macromolecular energy, minimization, and dynamics calculations. J Comput Chem. 1983;4:187–217.

[23] Humphrey W, Dalke A, Schulten K. VMD – visual molecular dynamics. J Mol Graphics. 1996;14:33–8.

[24] Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF chimera – a visualization system for exploratory research and analysis. J Comput Chem. 2004;25:1605–12.

[25] Szolnoky G, Bata-Csorgo Z, Kenderessy AS, Kiss M, Pivarcsi A, Novak Z, et al. A mannose-binding receptor is expressed on human keratinocytes and mediates killing of Candida albicans. J Invest Dermatol. 2001;117:205–13.

[26] Han L, Hiratake J, Kamiyama A, Sakata K. Design, synthesis, and evaluation of gamma-phosphono diester analogues of glutamate as highly potent inhibitors and active site probes of gamma-glutamyl transpeptidase. Biochemistry-Us. 2007;46:1432–47.

[27] von Gersdorff K, Sanders NN, Vandenbroucke R, De Smedt SC, Wagner E, Ogris M. The internalization route resulting in successful gene expression depends on polyethylenimine both cell line and polyplex type. Mol Ther. 2006;14:745–53.

[28] Naik S, Bhattacharjya G, Talukdar B, Patel BK. Chemoselective acylation of amines in aqueous media. Eur J Org Chem. 2004:1254–60.

[29] Starcher B. A ninhydrin-based assay to quantitate the total protein content of tissue samples. Anal Biochem. 2001;292:125–9.

[30] Ho GH, Ho TI, Hsieh KH, Su YC, Lin PY, Yang J, et al. Gamma-polyglutamic acid produced by Bacillus subtilis (natto) – structural characteristics, chemical properties and biological functionalities. J Chin Chem Soc-Taip. 2006;53:1363–84.

[31] Jo JI, Tabata Y. Non-viral gene transfection technologies for genetic engineering of stem cells. Eur J Pharm Biopharm. 2008;68:90–104.

[32] Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ. Is green fluorescent protein toxic to the living cells? Biochem Bioph Res Co. 1999;260:712–7.

[33] Kurosaki T, Kitahara T, Fumoto S, Nishida K, Nakamura J, Niidome T, et al. Ternary complexes of pDNA, polyethylenimine, and gamma-polyglutamic acid for gene delivery systems. Biomaterials. 2009;30:2846–53.

[34] Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med. 2004;10:310–5.

[35] Allison RD. Gamma-glutamyl-transferase transpeptidase – kinetics and mechanism. Methods in Enzymology. 1985;113:419–37.

[36] Keillor JW, Menard A, Castonguay R, Lherbet C, Rivard C. Pre-steady-state kinetic studies of rat kidney gamma-glutamyl transpeptidase confirm its ping-pong mechanism. J Phys Org Chem. 2004;17:529–36.

[37] Lieberman MW, Barrios R, Carter BZ, Habib GM, Lebovitz RM, Rajagopalan S, et al. Gamma-glutamyl-transpeptidase – what does the organization and expression of a multipromoter gene tell us about its functions. Am J Pathol. 1995;147:1175–85.

Figure 1

Click here to download high resolution image

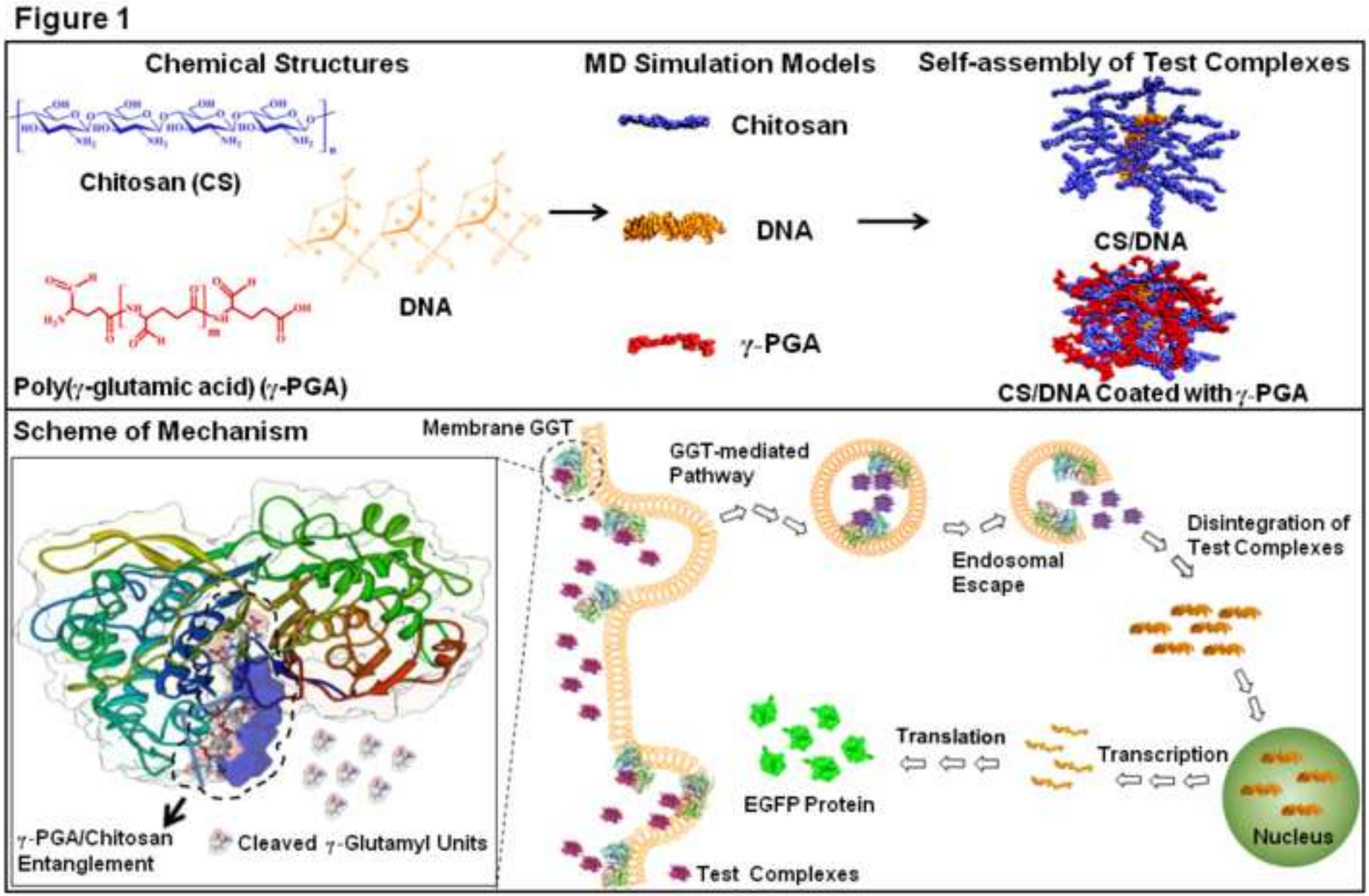

Figure 2

Click here to download high resolution image

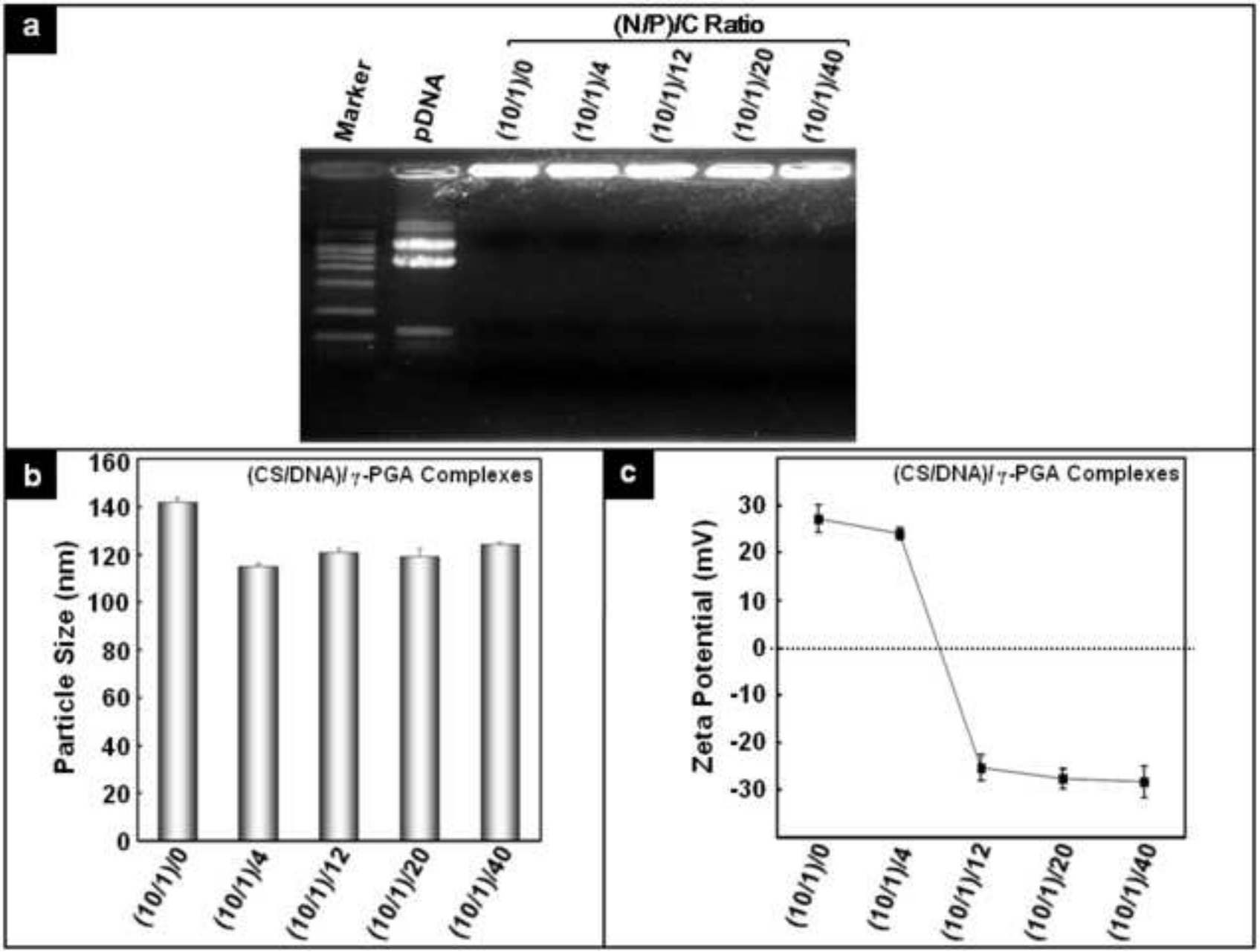

Figure 3
Click here to download high resolution image

Figure 3

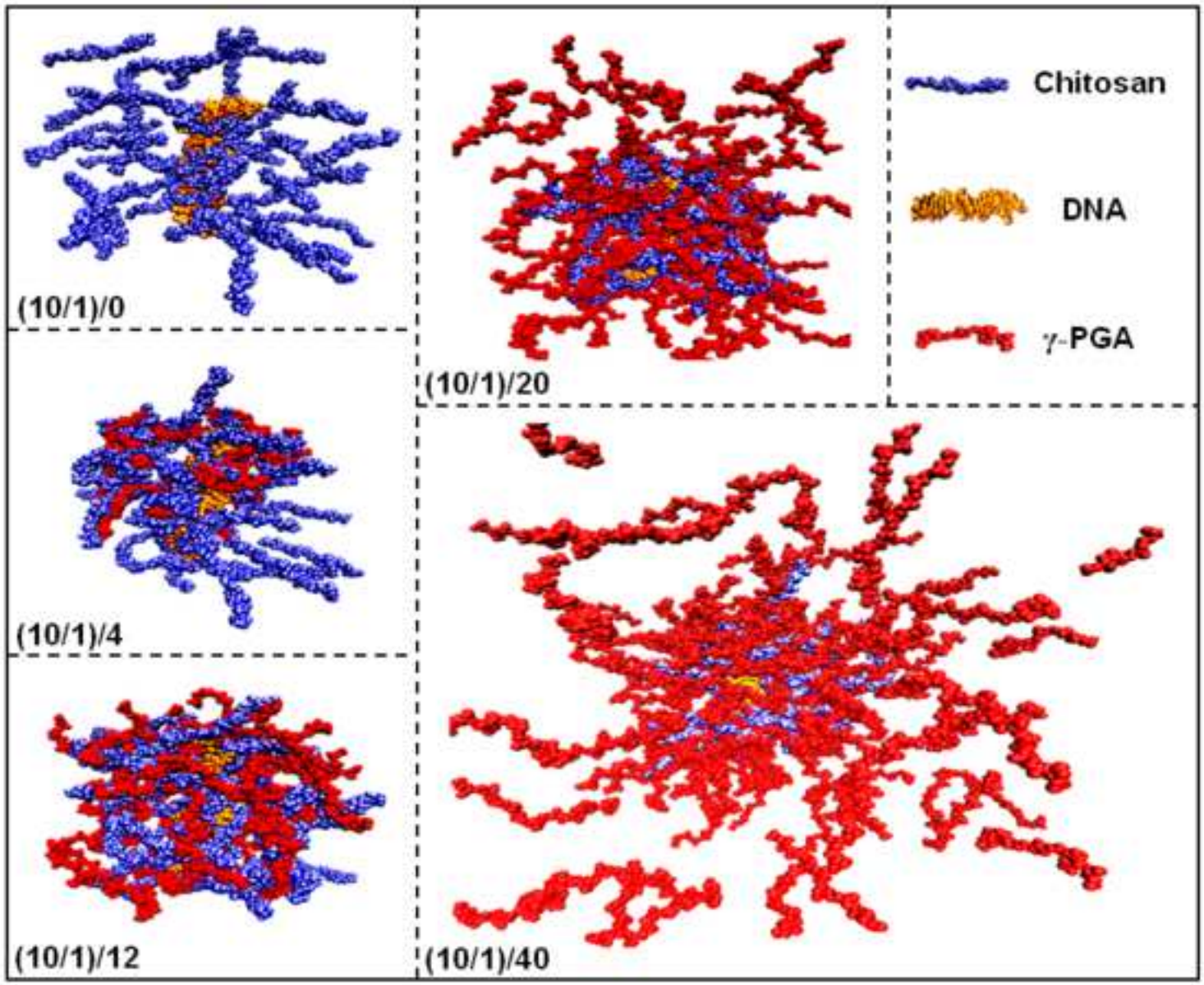

Figure 4

Click here to download high resolution image

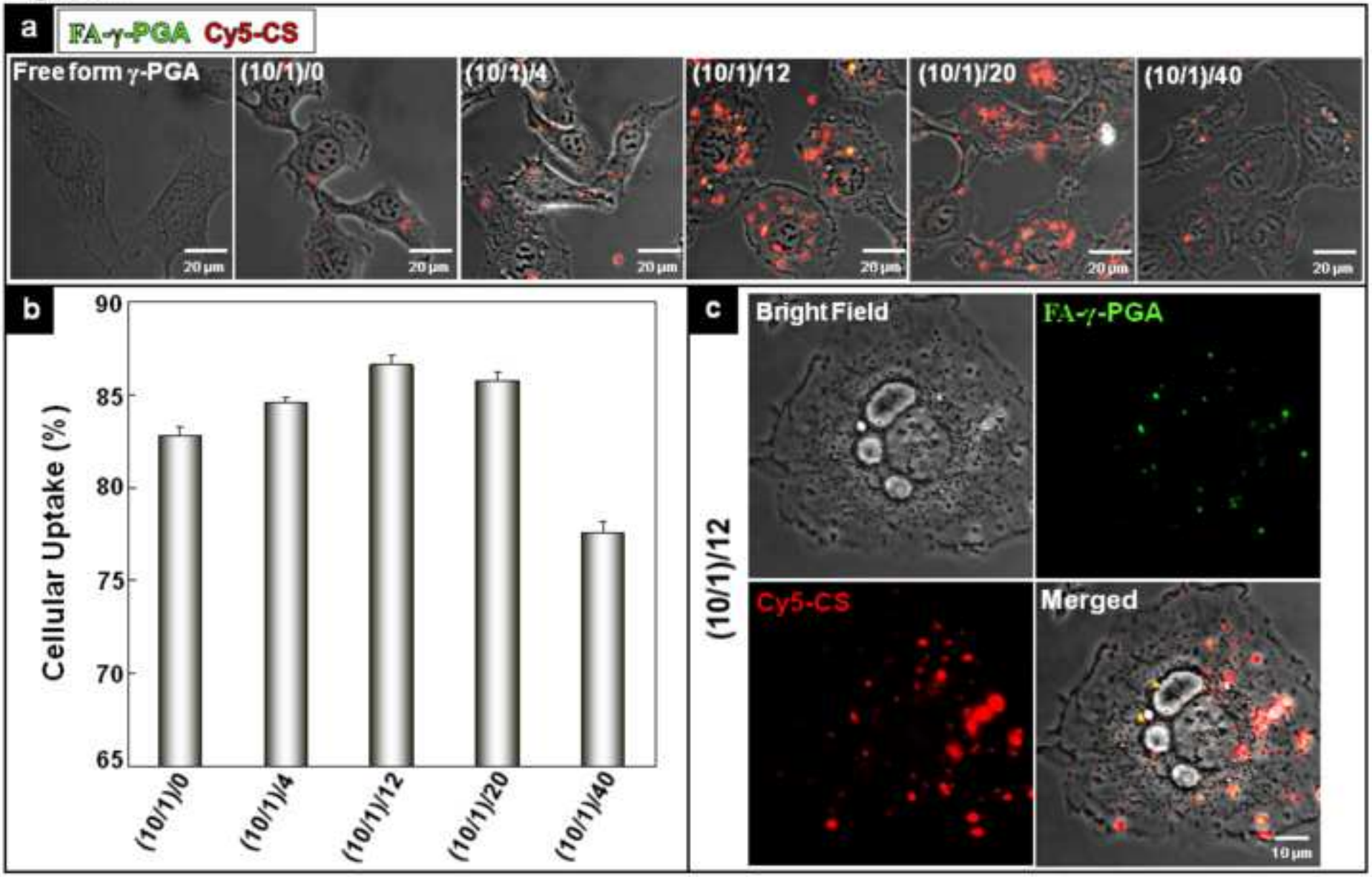

Figure 5

Click here to download high resolution image

Figure 5

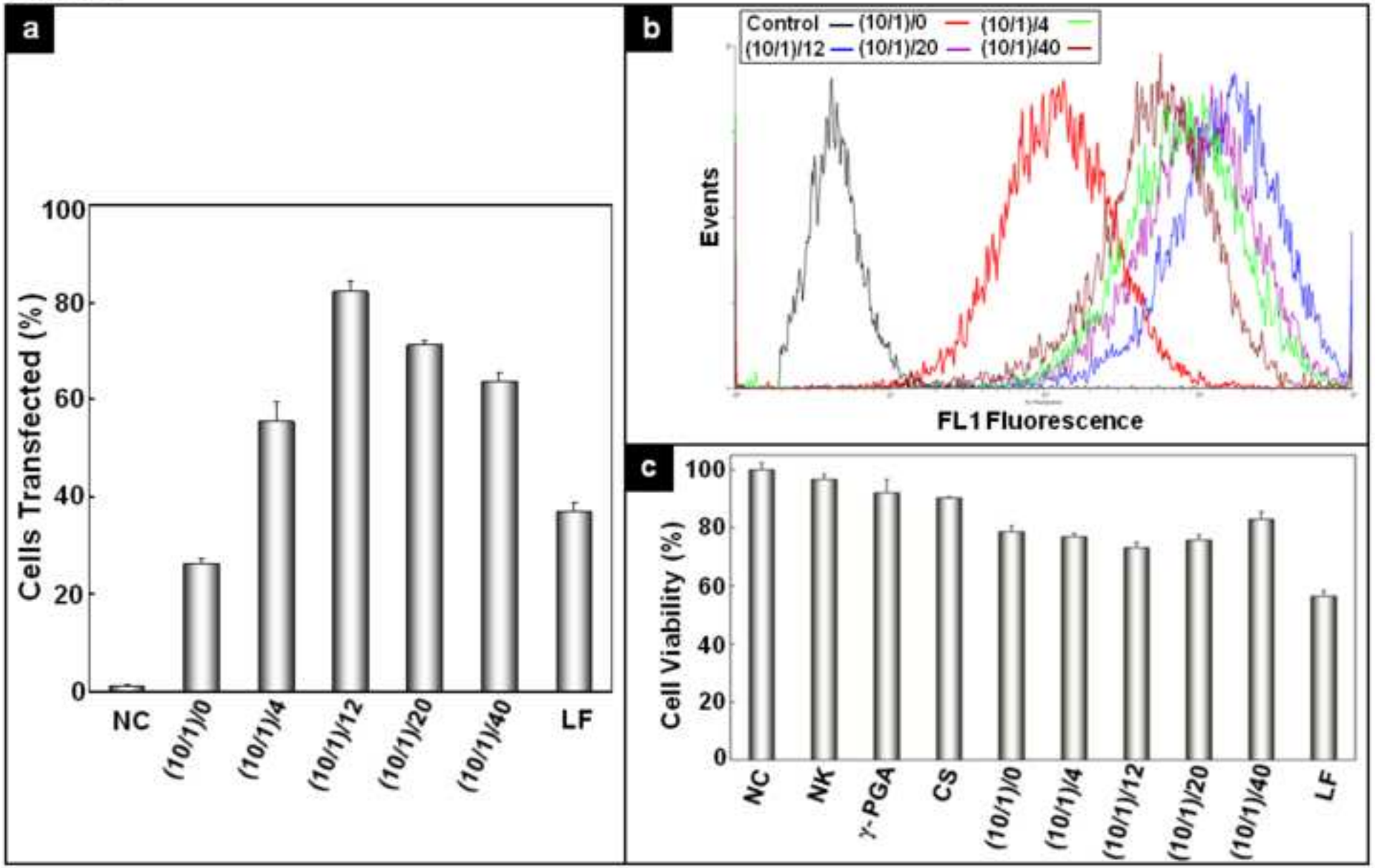

Figure 6

Click here to download high resolution image

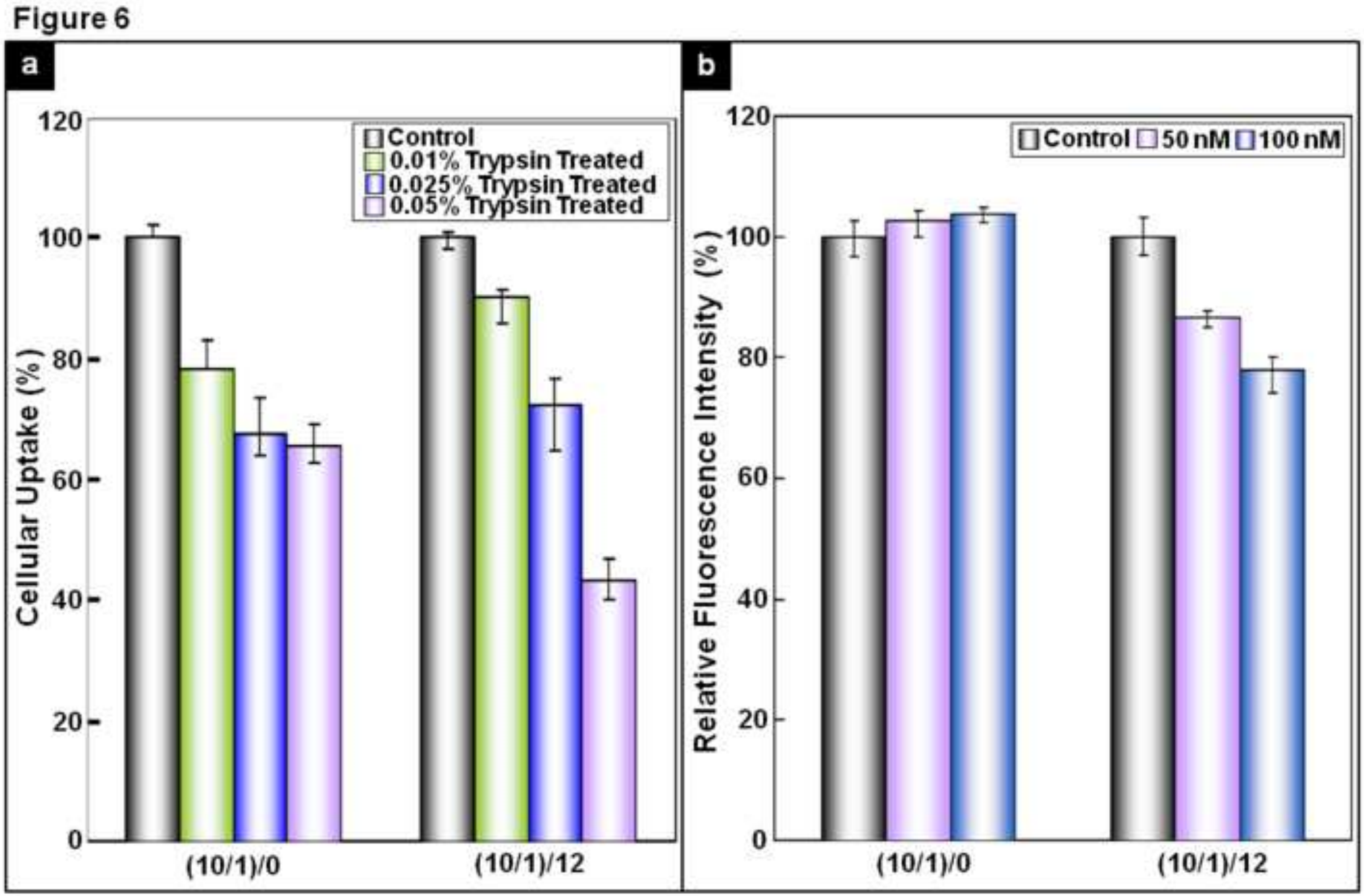

Figure 7a

Click here to download high resolution image

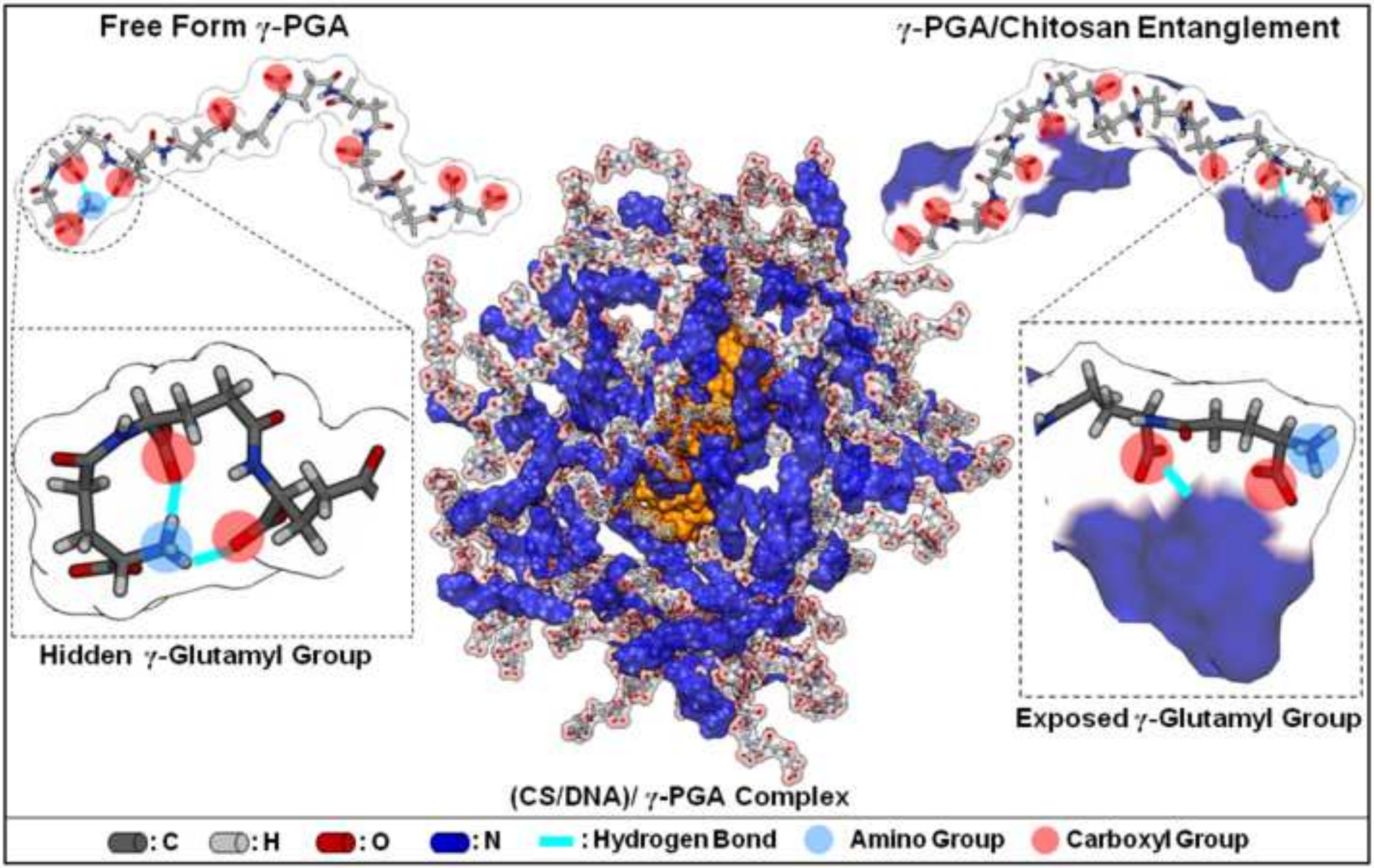

Figure 7b

Click here to download high resolution image

Figure 7b

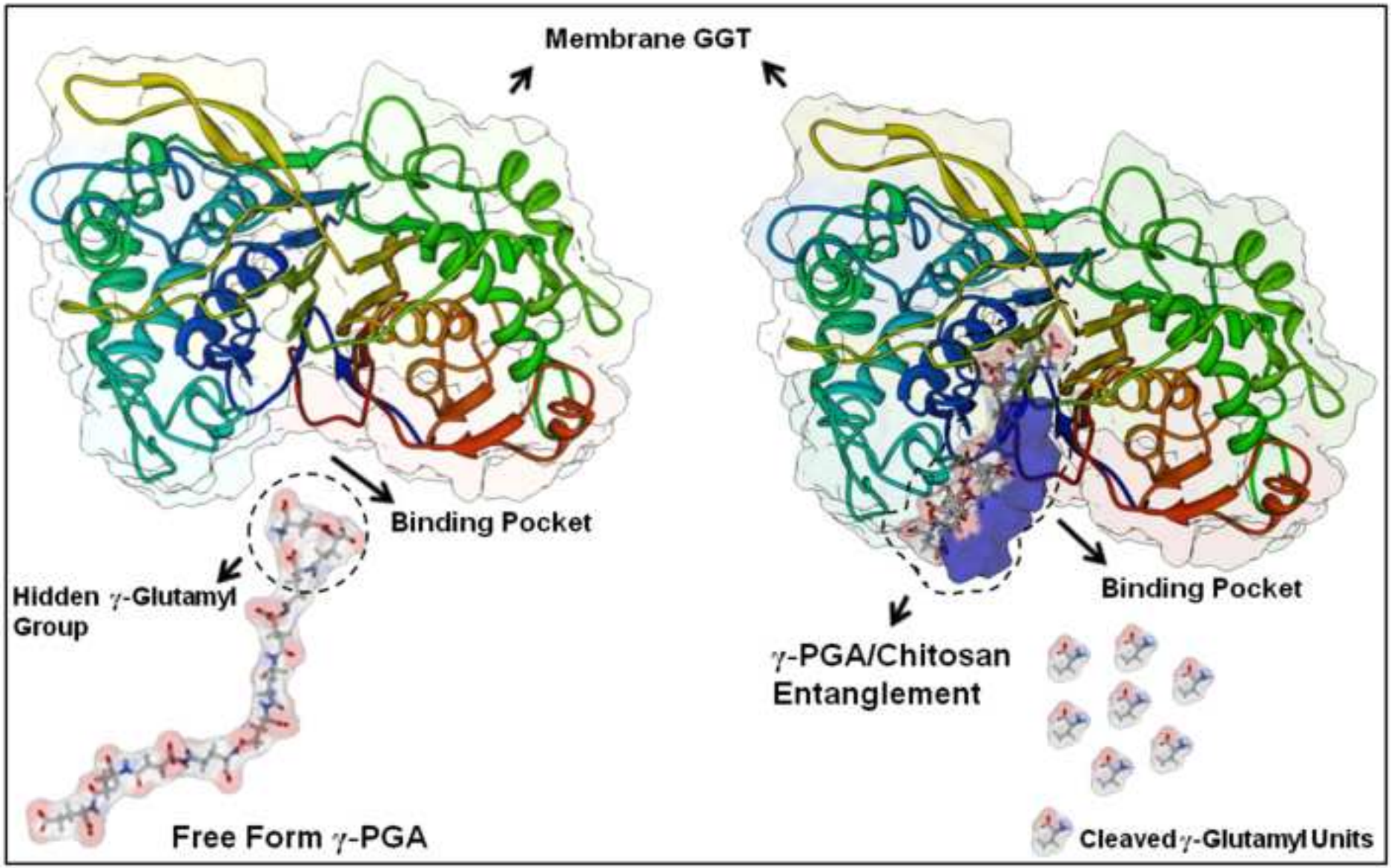

Figure 8

Click here to download high resolution image

Figure 8