Formulation of nanoparticles from short chain chitosan as gene delivery system and transfection against T47D cell line

Formulasi nanopartikel menggunakan kitosan rantai pendek sebagai sistem penghantaran gen dan transfeksinya pada sel kanker T47D

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Abstract

Recently numerous prototype DNA-based biopharmaceuticals can be used to control disease progression by induction and inhibitin the overexpression of genes. Since there are poor cellular uptake and rapid in vivo degradation of DNA-based therapeutics therefore the use of delivery systems to facilitate cellular internalization and preserve their activity is necessary. Cationic polymers commonly used as carriers to delivery gene because of easy to form complexes and higher stability compared to that lipoplexs. Chitosan, a cationic, are polymer most widely used in gene delivery systems because of the low toxicity, and biocompatible. The aim of this study was to formulate nanoparticles of short chain chitosan-pEGFP-C1 and short chain chitosan/TPP-pEGFP-C1 by coaservation complex method. Stability test of the formula was performed by incubating the nanoparticles complex with DNase I and Artificial Intestinal Fluid. Cytotoxicity and transfection studies were evaluated against T47D cell line. The diameter of Chitosan-pEGFP-C1 and chitosan/TPP-pEGFP-C1 nanoparticles were on the range of 56–282.8 nm. The zeta potential was determined to be +14.03 - +16.6 mV. Stability studies showed that chitosan-pEGFP-C1 and chitosan/TPP-pEGFP-C1 nanoparticles were stable, undegradable by DNase I and artificial intestinal fluid. Cytotoxic Assay of Chitosan-pEGFP-C1 and chitosan/TPP-pEGFP-C1 nanoparticles (pH 4.0) showed that the viability of cell was > 90% for all formulas. EGFP-C1 plasmid gene delivered by chitosan nanoparticles can be expressed in T47D cell culture. According to these results chitosan and chitosan/TPP nanoparticles had potentially to be used as a non-viral vector system delivery for gene therapy.
Key words: Chitosan, Nanoparticles, Plasmid EGFP-C1, Cell culture T47D

Abstrak

Sejumlah prototipe DNA sekarang dapat mengendalikan perkembangan penyakit melalui induksi atau inhibisi gen, namun *cellular uptake* yang jelek dan degradasi yang cepat in vivo dari terapi berbasis DNA membutuhkan penggunaan sistem penghantaran yang dapat memfasilitasi internalisasi seluler dan mempertahankan aktivitasnya. Polimer kationik umum digunakan sebagai pembawa gen karena mudah membentuk kompleks dan stabilitas yang lebih tinggi dibandingkan dengan lipopleks. Kitosan suatu polimer kationik banyak digunakan dalam sistem penghantaran gen karena toksisitas rendah, dan biokompatibel. Tujuan dari penelitian ini adalah untuk memformulasi nanopartikel kitosan rantai pendek-pEGFP-C1 dan kitosan rantai pendek/TPP-pEGFP-C1 melalui metode komplek koaservasi. Uji stabilitas terhadap formula dilakukan melalui inkubasi komplek nanopartikel dengan DNase I dan *Artificial Intestinal Fluid*. Uji sitotoksik dan transfeksi dievaluasi terhadap sel kanker T47D.

Diameter nanopartikel kitosan-pEGFP-C1 dan nanopartikel kitosan/TPP-pEGFP-C1 berkisar antara 56–282.2 nm. Zeta potensial permukaan sebesar +14.03–+16.6 mV. Hasil uji stabilitas menunjukkan bahwa nanopartikel kitosan-pEGFP-C1 dan nanopartikel kitosan/TPP-pEGFP-C1 stabil dan tidak terdegradasi oleh DNase I maupun *Artificial Intestinal Fluid* pH 7. Uji sitotoksik nanopartikel kitosan-pEGFP-C1 dan nanopartikel kitosan/TPP-pEGFP-C1 yang dibuat pada pH 4 menunjukkan viabilitas sel > 90 % untuk semua formula. Plasmid EGFP-C1 yang dihantarkan dengan nanopartikel kitosan dapat diekspresikan pada kultur sel kanker T47D. Dari hasil penelitian ini nanopartikel kitosan dan nanopartikel kitosan/TPP potensial digunakan sebagai sistem penghantaran gen non viral untuk terapi gen.
Kata Kunci: Kitosan, nanopartikel, plasmid EGFP-C1, kultur sel kanker T47D

Introduction

The DNA molecule is one of the most vital sources for the development of a novel group of therapeutics model on its endogenous structure. DNA-based therapeutics includes covalently closed circular plasmids containing transgenes for gene therapy, ribozymes, DNAzymes and oligonucleotides for antisense and antigene applications (Patil, *et al.,* 2005). Gene therapy processes involve the introduction of one or more functional and specific genes into a human recipient to repair certain genetic defects and aberrations.

The fundamental principle for gene therapy is to deliver gene-based therapeutics to target cells for specific gene targeting. Succeed gene therapy relies on a suitable carrier system that can efficiently deliver specific genes to the desired cell with minimum cytotoxicity on target cells (Nguyen, *et al.,* 2007).

Chitosan is interesting to be used as carriers of gene delivery because it has a high positive charge and low toxicity to cells (Hirano, *et al.,* 1990). Chitosan is a biodegradable polysaccharide consist of two subunits of D-glucosamine and N-acetyl-D-glucosamine that is bound together by (1,4) glycosidic chain (LeHoux and Grondin, 1993). Amine group on the glucosamine unit of chitosan is an important part because it provides a high positive charge and highly reactive. The positive charge of chitosan can form complex with plasmids. DNA complex provides protection against enzyme degradation and promote internalization of plasmids that have been condensed.

To evaluate the potential of chitosan and chitosan/TPP (crosslinked chitosan) for gene delivery, there fore the aim of this study were to synthesize and characterize a nanoparticulate delivery vehicle by complex coacervation of pDNA with non cross-linked chitosan and cross-linked chitosan using Na-TPP as crosslinker and to evaluate its cell uptake. MTT assays were used to measure the in vitro cytotoxicity of these nanoparticles.

Methodology

Materials

Low viscous chitosan [2-amino-2-deoxy-(1→4)-*β*-Dglucopyranan] was obtained from Sigma, Germany. The pEGFP-C1 was ordered from Clontech (BD Biosciencences) and was propagated and isolated from E.Coli DH5α. Ethidium bromide, agarose analytical grade and LB-medium were obtained from Invitrogen, Germany. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were obtained from Sigma-Aldrich, RNase-Fee DNase I from bovine pancreas (Sigma Aldrich, St. Louis, USA) and agarose were ordered from Invitrogen. All other salts and chemicals were of analytical grade.

Preparation of pEGFP plasmid

Enhanced Green Fluorescence Protein (EGFP) plasmid was used to monitor protection of chitosan complexes against endonuclease. The plasmid was propagated in E. coli DH5α and isolated and purified using an alkaline lysis method (Sambrook, *et al*., 1989). Purified pDNA was resuspended in distilled water. The pDNA concentration was determined by measuring absorbance at 260 nm utilizing an UV–vis spectrophotometer (UV-1202, Shimadzu Co., Kyoto, Japan). Purity was confirmed by gel electrophoresis with 0.8% agarose. Plasmid DNA solution was stored at -20^{0}C until further use.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were generated by complex coacervation of chitosan and chitosan-TPP with pDNA. The pDNA (25 μg) in 250 μL of 50

mM sodium sulfate and 400 µL of chitosan and chitosan/TPP solution in a 5 mM acetate buffer (pH 4.0) was heated to 50^0C for 10 min. Two hundred and fifty microliters of pDNA–sodium sulfate solution and 400 µL of chitosan solution were mixed by vortexing for 20 s. Particle suspension was examined after 30 min (Martien and Loretz, 2006 and 2007; Martien, *et al.,* 2008).

Physicochemical characterization

Particle size measurement

The resulting noncross-linked and cross-linked chitosan nanoparticles were analyzed for mean particles size by *Delsa™ Nano Submicron Particle size*. The hydrodynamic diameter of the nano-particles was determined by light scattering. *Scattering intensity* was 5694 cps. The refractive index (1.3328) and the viscosity (0.8878 cp) of distilled water were used at 25°C for measurements. The size was calculated assuming solid particles, and the number of weighting distribution was fit to a curve.

Zeta potential measurement

Zeta potential measurement of the chitosan and crosslinked chitosan nanoparticles dispersion were performed using a *Delsa™ Nano Submicron Particle size* at 25°C. The zeta potential value was measured at the default parameters of dielectric constant, refractive index, and viscosity of water. The sampling time was set to automatic.

Electron microscopical characterization of nanoparticles

Drops of 10 mL freshly prepared nano-particle solution were placed on pioloform coated grids and air dried for 15 min. The redundant fluid was removed while the dried nanoparticles remained on the grids. These grids were dried and examined by transmission electron microscope (JOEL-JEM 1400, Japan) with an in-column energy filter (EFTEM). Thus, nanoparticles were analyzed 120kV energy loss, depending on their density. Digital micrographs were obtained from STEM digital imaging/scanning circuitry which displays STEM images (BF/DF) on the standard GUI.

Nanoparticles stability assay against DNase I degradation

Two hundred microlitres unmodified chitosan and crosslinked chitosan nanoparticles dispersion contain 7.69 µg pDNA were separately incubated with 1 µL of DNase I (1 U) and 20 µL of DNase reaction buffer (400 mM Tris-HCl (pH 8.0), 100 mM MgSO4 and 10 mM CaCl2) at 37°C for 1 h. Ten microlitres of 20 mM EDTA pH 8.0 were added to stop the reaction. Free plasmid DNA (pEGFP) served as positive control. Degradation of particles was investigated by 0.8% agarose gel electrophoresis. Gel was visualized under UV light and documented (Martien and Loretz, *et al.,* 2006).

Incubation with artificial intestinal fluids

Incubation with Artificial Intestinal Fluid. To evaluate the stability of unmodified chitosan/pDNA and chitosan–TPP/pDNA nanoparticles against the salts solution, they were incubated with artificial intestinal fluid. Artificial intestinal fluid (AIF) is a physiological salt solution containing 20 mM bicarbonate, 139 mM chloride, 5 mM potassium, 140 mM sodium, 4 mM calcium, and 3 mM magnesium adjusted to pH 7.0 with acetic acid. The test was performed with a mixture of 300 µL of particles suspension (11.5 µg/mL) and 150 µL of AIF, incubated at 37°C while shaking at 400 rpm (Thermomixer comfort 2 ml, Eppendorf, Germany). After 4 h incubation, each particle suspension was investigated via agarose gel electrophoresis. Agarose gel electrophoresis was performed in a 0.8% (w/v) gel for 30 min at 100 V using ethidium bromide for visualization. Gel was visualized under UV light and documented (Martien, *et al.,* 2006).

Cytotoxicity assay

Viable cells in the samples were measured using the MTT staining method (Mosmann, 1983). MTT was dissolved in phosphate buffered saline (PBS, pH 7.4) at 5 mg/mL as stock solution and filtered to be sterilized. The T47D cells were grown in 96 well plates at an initial seeding density of $5x10^3$ cells per well in 200 µL of supplemented MEM medium for 24 h. Then cells were incubated with pellet of 650 µL chitosan nanoparticles (both noncross-link and cross-link system) in 200 µL MEM medium for 24 h. Untreated cells were used as negative control. After incubation, medium was replaced by 100 µL of MTT stock solution per well. The cells were incubated for 3 h along with the reagent at 37°C in 5% CO_2 incubator. One hundred microliter stopper reagent (SDS 10%) added to every well to dissolve the blue-violet crystals. Absorbance of the dye was measured at the wavelength of 550-600 nm with ELISA Reader (Bio-Rad Model 680XR, Japan). The survival rate could be calculated according to [a]/[b] multiplied by 100, where [a] and [b] are absorbance of the dye in treated cells and untreated cells, respectively.

***In vitro* Transfection assay**

T47D cells were seeded on a 6 well plate at an initial concentration of $1x10^5$ cells per well and incubated for 24 h before addition of the nanoparticle solution. The condition of incubation set up was the same as described above. Chitosan/pDNA and chitosan/TPP nanoparticles were tested for gene delivery using plasmid DNA

expressing a green fluorescent protein (GFP). The culture medium was removed and substituted with fresh medium containing a 200 µL of chitosan and chitosan/TPP nanoparticles which was prepared as described above. pDNA were used as a control. After 24 h incubation, culture medium was changed with fresh medium. Culture cells were harvested for GFP assay (Supriatno, *et al.,* 2005; Martien, *et al.,* 2006; Indrawati, 2010, Mutmainah, 2010). GFP expression was evaluated under fluorescent microscope (Carl Zeiss Axiolab HB50, Germany).

Results and Discussion

Plasmid preparation

The EGFP-C1 plasmid was maintained and propagated in the *E. coli* strain DH5α. It was isolated by alkaline lysis method. Result of plasmid electrophoresis using 0.8% w/v agarose gel shown by the figure 1. Purity of plasmid DNA was certified by $OD_{260/280}$ ratio (higher than 1.8). The concentration of plasmid DNA was determined using 1 (OD_{260}) = 50 µg of DNA (Sambrook, *et al*., 1989). The results of plasmid isolation with alkaline lysis method get the purity as indicated by the ratio of 1.75 and plasmid concentration is 4380.1µg/mL. The plasmid DNA was stored at – 20 °C before use.

Agarose gel visualization showed three bands of the plasmid from the fast to the slow supercoiled, linear and nicked open circular.

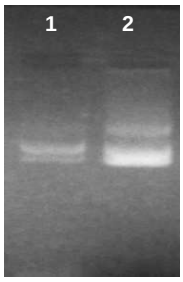

Figure 1. Agarose gel electrophoresis for the result of plasmid isolation. *Lane 1*. pEGFP-C1 1,05 ug, *lane 2*. pEGFP-C1 19,65 ug

Formulation of chitosan/pDNA nanoparticles

The complex formation between plasmid DNA and chitosan is electrostatic by the attraction between the anionic DNA and the cationic carrier material. Complex formation can be evaluated with electrophoresis gel agarose 0.8% w/v (Figure 2).

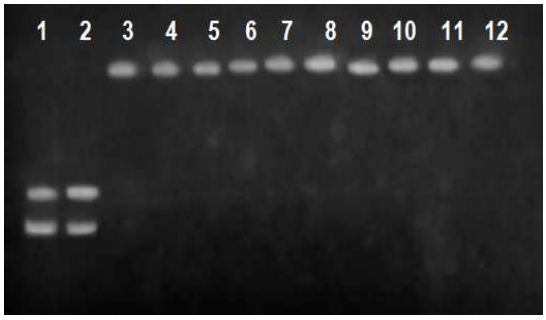

Figure 2. Agarose gel electrophoresis for complex formation between pDNA and chitosan.

Lane 1: pEGFP in H_2O; 2: pEGFP in acetate buffer pH 4.0; 3: chitosan nanoparticle 0.02%; 3: chitosan nanoparticle 0.04%; 4: chitosan nanoparticle 0.06%; 5: chitosan nanoparticle 0.06%; 6: chitosan nanoparticle 0.08%; 7: chitosan nanoparticle 0.1%; chitosan nanoparticle 0.02%; 8: chitosan nanoparticle 0.04% +TPP; 9: chitosan nanoparticle 0.06%+TPP; 10: chitosan nanoparticle 0.06%+TPP; 11: chitosan nanoparticle 0.08%+TPP; 12: chitosan nanoparticle 0.1%+TPP

After storage at room temperature for 7 days, only chitosan nanoparticle with concentration of chitosan 0.02% to 0.04% which stable. The next evaluation used this concentration for the formation of nanoparticle.

Nanoparticle characterization

Dynamic light scattering measure the zeta potential and particle size distribution.

Table I. Dynamic Light Scattering and Zeta Potential Analysis of Chitosan Nanoparticle

Nanoparticle chitosan-pEGFP-C1	Size (nm) X ± SD	Zeta Potential (mV)
0.02%	229.0±52.9	14.57 ± 1.56
0.04%	282.8±66.6	16.60 ± 0.33
0.02%+TPP	59.05±16.0	12.83 ± 0.01
0.04%+TPP	86.20±24.4	14.03 ± 0.09

The mean particle size for nanoparticle chitosan-TPP/pDNA are on the range of 50-100nm, whereas for nanoparticle chitosan/ pDNA are on the range of 200-300nm. The net charge on chitosan-TPP/pDNA and chitosan/ pDNA nanoparticle was found to be positive.

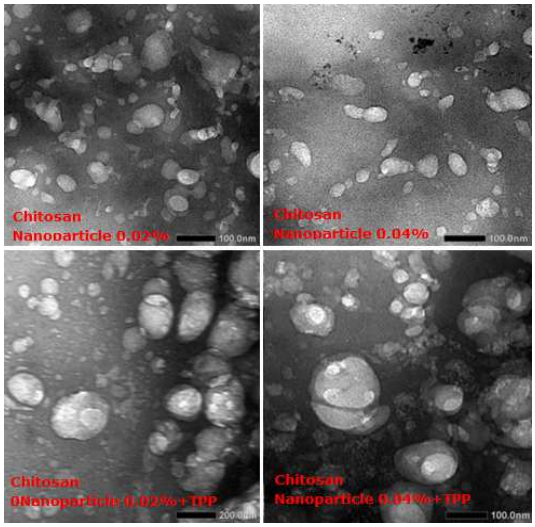

Figure 3. Electron micrograph of noncross-linked chitosan and chitosan nanoparticle cross=linked by TPP, 120kV, x40.000 magnification.

The charge value of chitosan-TPP/pDNA nanoparticle was less than that of chitosan/pDNA nanoparticle because the addition of TPP can neutralize positive charge of chitosan. The negative charge of chitosan nanoparticle is primarily based on pDNA but to some extent is also based on TPP being ionically attached to chitosan. To determine the morphology of nanoparticles, TEM was performed. Result showed that the shape of the nanoparticles is fairly spherical (Figure 3).

Particle size determination was confirmed by TEM image via light scattering analysis.

Nanoparticle stability assay against DNase I

An advantage of the encapsulated DNA approach for gene delivery is the protection against nucleases. Plasmid DNA encapsulated in chitosan and chitosan-TPP remained intact in the presence of DNase I for up to 1 h of incubation. This result demonstrated that chitosan and chitosan-TPP could protect encapsulated plasmid DNA from nuclease digestion (figure 4). Chitosan have an activity as chelatting agent and DNase I is Ca^{2+}/Mg^{2+} dependent nuclease (Counis and Torriglia, 2000). Inhibition mechanism of chitosan polymer is based on chelation of the divalent cation from the enzyme structure. Crosslinked chitosan nanoparticles with TPP displayed a protective effect in the same range as that of non-crosslinked particles.

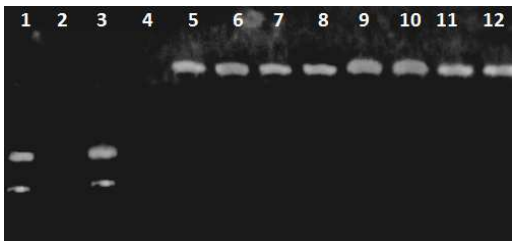

Figure 4. Agarose gel electrophoresis for DNase I protection assay. DNA and nanoparticles were incubated with 1 U/mL of DN ase I for 1 h at 37 °C.

Lane 1: untreated control DNA in H_2O; 2: untreated control DNA in H_2O+DNase I; 3: untreated control DNA in acetate buffer pH 4.0; 4: untreated control DNA in acetate buffer pH 4.0+DNase I; 5: chitosan nanoparticle 0.02%; 6: chitosan nanoparticle 0.02%+DNase I; 7: chitosan nanoparticle 0.04%; 8: chitosan nanoparticle 0.04% + DNase I; 9: chitosan-TPP nanoparticle 0.02%; 10: chitosan-TPP nanoparticle 0.02%+DNase I; 11: chitosan-TPP nanoparticle 0.04%; 12: chitosan-TPP nanoparticle 0.04%+DNase I.

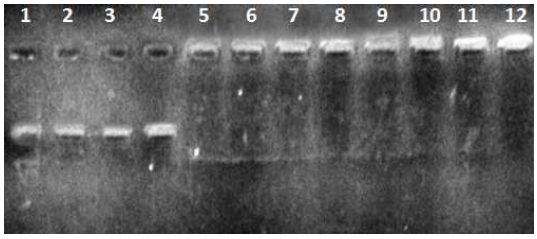

Figure 5. Agarose gel electrophoresis for stability on artificial intestinal fluid pH 7.0. DNA and nanoparticles were incubated with 150 µL of artificial intestinal fluid for 4h.

Lane 1: untreated control DNA in H_2O; 2: untreated control DNA in H_2O+AIF; 3: untreated control DNA in acetate buffer pH 4.0; 4: untreated control DNA in acetate buffer pH 4.0+ AIF; 5: chitosan nanoparticle 0.02%; 6: chitosan nanoparticle 0.02%+ AIF; 7: chitosan nanoparticle 0.04%; 8: chitosan nanoparticle 0.04%+ AIF; 9: chitosan-TPP nanoparticle 0.02%; 10: chitosan-TPP nanoparticle 0.02%+ AIF; 11: chitosan-TPP nano-particle 0.04%; 12: chitosan-TPP nanoparticle 0.04% + AIF.

Nanoparticle stability against AIF

Electrostatic interaction stability between chitosan charge and pDNA charge appears to be strong enough so that chitosan/DNA complex does not dissociate upon the incubation with AIF. This properties can give benefit for the protection of pDNA so that the complex does not dissociate until it has entered the cell (Roy, *et al.,* 1999). Chitosan nanoparticle and chitosan-TPP nanoparticle made at pH 4.0 were resistant toward dissociation during incubation with artificial intestinal fluid for 4 h (Figure 5).

Cytotoxicity assay

MTT cytotoxicity test were utilized to evaluate the effect of nanoparticles on viability of cell (Vihola, *et al.,* 2005). MTT test for chitosan nanoparticle at pH 4.0 and crosslinked chitosan-TPP showed that in a concentration of pDNA of 25µg/mL, nanoparticles are not harmful to T47D cells (Fig. 6). The viabilities of the cells were around 100%. The viability of the cells was > 90% as compared to the control. Cross-linked by TPP did not have an effect on the cell culture. Overall, neither of the methods gave any alarming evidence for cytotoxicity, hence nanoparticles can be regarded relatively nontoxic for T47D cells during the exposure time.

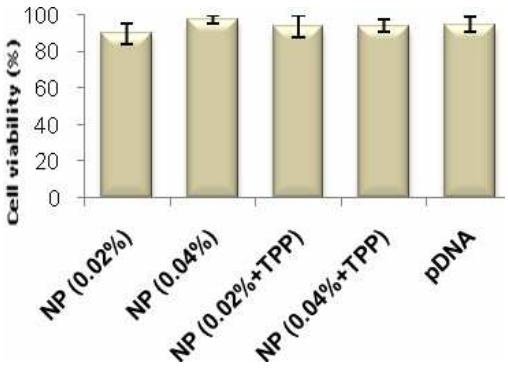

Figure 6. Viability of T47D cells in vitro cell culture with non cross-linked chitosan nanoparticles and cross-linked chitosan nanoparticles and incubation time OVN. Viability of untreated cells was considered 100%. Each point represents the mean±SD of three determinations.

Cell transfection study

Transfection was carried out using chitosan nanoparticles with non cross-linked and cross-linked chitosan nanoparticle. The human breast cancer cells (T47D) were transfected using 7.7 µg/mL of nanoparticles solution at 24 h of transfection. Qualitative analysis was used in this study. The qualitative analysis of EGFP-C1 expression upon transfection in T47D cells was evaluated by fluorescence microscopy (Fig. 7). It was shown by fluorescence imaging that within 24 h of transfection with chitosan/pDNA nanoparticles, cells were able to express EGFP-C1. These nanoparticles protect plasmid DNA during its transport which are later taken up by the cells through the endosomal pathway and released into the cytosol (Kommaredy and Amiji, 2005).

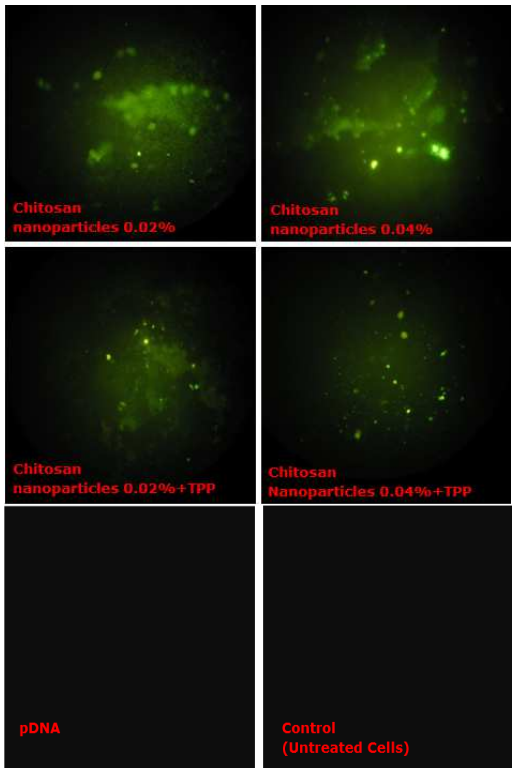

Figure 7. Expression of green fluorescence protein in T47D cells after 24 h transfection with nanoparticles. Pictures were taken after 48 h post transfection under fluorescence microscope.

Among the cross-linked and non cross-linked chitosan nanoparticles, non crosslinked chitosan nanoparticles showed higher transfection than cross-linked chitosan nanoparticles. The result can be explain from the zeta potential value from non cross-linked chitosan nanoparticle which higher than cross-linked chitosan nanoparticles. Positive charge will promote the internalization of nanoparticles by endocitocys pathway. Beside that's cross-linked chitosan nanoparticles forming an aggregates that lead to less internalization.

Conclusion

Chitosan nanoparticle and chitosan–TPP conjugates have the capability to protect pDNA nanoparticles from enzymatic degradation of DNase I and stable in artificial intestinal fluid. Transfection studies with T47D cell line showed that chitosan and chitosan–TPP nanoparticles can transfect the cells and give an expression of EGFP. According to these results it seems that encapsulation of pDNA in chitosan and chitosan-TPP is a promising strategy for the development of efficient gene delivery systems.

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