

# Effects of alkylated-chitosan–DNA nanoparticles on the function of macrophages

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**Abstract** Chitosan could form nanoparticles with DNA through electrostatic interaction, and hence protect the DNA from enzymatic degradation. Numerous studies have been working on modifying chitosan aiming at improving its transgenic efficacy. While the modification of chitosan with alkyl group has been shown to significantly improve the cell transfection efficiency, little is known about its impact on its biocompatibility. The current study was performed to investigate the impact of alkylated-chitosan/DNA nanoparticles on the function of the murine macrophage through observing its phagocytic activity and production of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-10, IL-12 and TNF- $\alpha$ ). Our results demonstrated that the alkylated-chitosan/DNA nanoparticles at the concentration of 20  $\mu$ g/ml DNA content had no significant impact on the production of cytokines and phagocytic activity of the macrophages as compared with the unmodified chitosan/DNA nanoparticles and negative control even after 24 h co-incubation. It suggested that the modification of chitosan with alkyl group should not have negative impact on the function of the macrophages.

## 1 Background

Lack of appropriate gene carriers has been one of the major drawbacks to medical application of gene therapy. Viruses

are among the most stable and effective carriers for transferring genes into eukaryotic cells. However, endogenous virus recombination, oncogenic effects, and immunological reaction remain to be the top issues of concern when viruses are used as gene carriers.

Chitosan (CS) is one of the potential candidates as gene carriers because of its excellent biocompatibility and biodegradability. It is able to form nanoparticles with DNA through electrostatic interaction, and hence protect the DNA from enzymatic degradation [1–4]. However, the transgenic efficacy of CS was cell-dependent and still considerably lower as needed for a successful gene delivery [4–6]. Numerous investigations have been conducted to chemically modify the CS for the purpose of improving its transgenic efficacy [7–9]. Alkylated-CS has been proved to be a more efficient gene carrier [10, 11], but little is known about the negative impact of the alkyl group modification on the biocompatibility of the CS.

Macrophages are among the major cells mediating the inflammatory responses to foreign substances, especially the particulate substances such as nanoparticles, through phagocytosis and secretion of proinflammatory cytokines, and thus could impact on the biocompatibility of the nanoparticles. The size, chemical property, and the surface charge of the particles are the major factors determining their impact on the function of macrophages. Kanchan et al showed that immunization of the macrophages with PLA nanoparticles (200–600 nm) induced higher levels of IFN- $\gamma$  production, whereas immunization with microparticles (2–8  $\mu$ m) promoted IL-4 secretion [12]. Rothen-Rutishauser et al found that there was a 2–3 fold increase of tumour necrosis factor- $\alpha$  in the supernatants after incubating macrophages with 1  $\mu$ m polystyrene particles and gold nanoparticles, but not with polystyrene and titanium dioxide nanoparticles [13]. In the in vitro studies between

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suspension macrophages and core-shell nanoparticles performed to determine how the hydrophilicity and the charge on the nanoshell can promote or reduce the cellular uptake, AS Zahr et al showed that after 24 h the cellular uptake was decreased three-fold when PEGs of 2,000 and 20,000 Da were chemisorbed to the nanoshell, as opposed to the nanoshell with either a positive or highly negative charge, and in the protein adhesion studies with bovine serum albumin to determine the relationship between surface charge and opsonization of core-shell nanoparticles, they found that a hydrophilic surface with a low negative charge reduced protein adsorption and cellular uptake [14]. To date, only one study has been reported on the impact of CS–DNA nanoparticles on the biological behavior of macrophages and it showed that CS–DNA nanoparticles had no significant effect on the production of proinflammatory cytokines by murine macrophage cell line THP-1 [15]. It is of great interest to learn about the impact of alkylated-CS on the function of macrophages. The present study was designed to investigate the effect of the alkylated-CS/DNA nanoparticles on the function of the macrophage by looking at its phagocytic activity and production of pro-inflammatory cytokines.

## 2 Materials and methods

### 2.1 Materials

Chitosan with molecular weight of 130,000–180,000 and degree of deacetylation of 85% was purchased from Sigma-Aldrich Company (St.Louis, MO, USA). Murine macrophage cell line RAW264.7 was obtained from ATCC (Manassas, VA, USA). FBS was obtained from Hyclone (Logan, UT). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Company (St.Louis, MO, USA). Endofree Plasmid Mega Kits was purchased from Qiagen GmbH (Hilden, Germany). Immunoassay Kits for murine cytokines (IL-1 $\beta$ , IL-6, IL-10, IL-12, and TNF- $\alpha$ ) were purchased from BioSource International, Inc (California, USA). Dialysis membrane (MWCO 12 000) was obtained from Beijing Dingguo Biotechnology Development Co., Ltd. (Beijing, China). pEGFP-C1 plasmid DNA was a product of BD Biosciences Clontech (San Jose, CA, USA).

### 2.2 Purification of plasmid DNA

The pEGFP-C1 plasmid DNA was purified using the QIAGEN Endofree Plasmid Mega Kits according to the manufacturer's instruction. The amount of LPS in the purified plasmid DNA was quantified by gel clot assay as described previously [16].

### 2.3 Synthesis of *N*-alkylated-CS

The *N*-alkylated-CS was synthesized according to the method described by Liu et al. [10]. Briefly, 5 g of CS was added into 100 ml of 2-propanol/0.4 N sodium hydroxide solution and stirred at 40°C for 30 min. The hexadecyl bromide was added drop-wise to the mixture and allowed to react for 4 h. The reaction mixture was centrifuged and the obtained precipitate was washed with ethanol and then dried at vacuum to obtain the alkylated-CS derivatives. The resultant alkylated-CS derivatives were dialyzed for 3 days using dialysis membrane (MWCO 12 000) against water.

### 2.4 Preparation of DNA nanoparticles

The DNA nanoparticles were prepared according to the method described by Mao et al [2] either using CS or alkylated-CS as condensing material. Briefly, plasmid DNA solution (200  $\mu$ g/ml) was prepared with 25 mM of sterilized sodium sulfate solution. CS stock solution (400  $\mu$ g/ml) was prepared by dissolving the polymer in 0.2 M sterilized sodium acetate buffer, (pH 5.5). Equal volume of DNA solution and CS (or alkylated-CS) solutions were mixed together after preheating at 55°C, and subsequently vortexed for 60 s. The resulting DNA nanoparticles were measured using a Brookhaven BI-9000AT digital autocorrelator at fixed scattering angle ( $\theta$ ) of 90° at room temperature. To avoid the influence of dusts on the reliability of results, the complexes were filtered through 0.45  $\mu$ m filters (millipore) and directly dropped into a clean and specially treated bottle. Each sample was carried out in triplicate.

### 2.5 ELISA for cytokines

RAW264.7 cells were grown in DMEM supplemented with 10% heat inactivated FBS. The cells were seeded in 24 well-culture-plate at the density of  $5 \times 10^5$  cells/ml, 2 ml each well, and co-incubated with different amount of CS/DNA nanoparticles (CGN) or alkylated-CS/DNA nanoparticles (ACGN) with the DNA contents being 0.1, 1, 10 and 20  $\mu$ g/well, respectively. LPS at the concentration of 1  $\mu$ g/ml was used as positive control, whereas the culture medium alone was used as negative control. At 1, 6, and 24 h post co-incubation, aliquots of the cell supernatants were harvested by centrifugation and stored at –80°C for cytokine determination. The concentration of cytokines (IL-1 $\beta$ , IL-6, IL-10, IL-12, and TNF- $\alpha$ ) in the cell supernatant was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction. Briefly, 50  $\mu$ l each of the cell supernatant and standard diluent buffer were added into each well of the micro-titer plate provided in the kit, whereas 100  $\mu$ l of standard

diluent buffer was added into the zero well and the well reserved for chromogen blank was left empty. To establish the standard curve, 100  $\mu\text{l}$  of standard diluent buffer containing different amount of cytokines was added into each well. Subsequently, 50  $\mu\text{l}$  of biotinylated anti-cytokine antibody solution was added to each well except for the chromogen blanks. After 2 h incubation, the solution was aspirated thoroughly and the wells were washed four times with washing buffer. About 100  $\mu\text{l}$  of streptavidin-HRP working solution was then added and incubated for 30 min. The wells were washed again as described above. About 100  $\mu\text{l}$  of stabilized chromogen was added into each well and incubated for 30 min before addition of 100  $\mu\text{l}$  of stop solution into each well. The OD values at 450 nm were determined by a spectrophotometer (SPECTRAMax plus 384). The amount of the cytokines in the cell supernatants was calculated by reference to the standard curve constructed with fixed concentrations of the human recombinant cytokines provided in the kits. The experiments were repeated three times with different batches of nanoparticles and each ELISA test was performed in duplicates.

## 2.6 Detection of phagocytic activity of the macrophages

Nanoparticles were suspended at the concentration of 50  $\mu\text{g}/\text{ml}$  in 0.1 M HAc/NaAc buffer containing 12.5 mM  $\text{Na}_2\text{SO}_4$ , pH 5.5. Thirty mice (25 g, male) were randomly divided into five groups (six/group). Group 1, 2, 3 and 4 were peritoneally injected with 1 ml each CGN suspension, ACGN, HAc/NaAc/  $\text{Na}_2\text{SO}_4$  buffer, and LPS (1  $\mu\text{g}/\text{ml}$ ) solution, respectively. Group 5 received no rejection. About 2 ml of 0.9% NaCl solution was injected into the murine peritoneal cavity 24 h after the nanoparticle injection and the solution was collected. The cells were harvested by centrifugation and suspended by 0.9% NaCl solution. About 50  $\mu\text{l}$  of the cell suspension containing  $2 \times 10^6$  cells/ml was incubated with 50  $\mu\text{l}$  of freshly prepared 5% chicken red blood cells (CRBC) suspension for 30 min at 37°C. The phagocytosis of CRBC by macrophages was observed under microscope with the magnification of 1,000 $\times$  after Wright–Giemsa staining. The phagocytic rate (PR) and phagocytic index (PI) were calculated with the following formulas: PR = (the number of the macrophages containing CRBC/the total number of the macrophages)  $\times$  100%; PI = the total number of the phagocytosed CRBC/the total number of macrophages.

## 2.7 Statistical analysis

Data were presented as the mean of six individual observations with standard deviation. The statistical analysis was

performed using the ANOVA (a one-way analysis of variance), followed by Bonferroni *t*-test for comparison with the control group. Statistical significance was determined at  $P < 0.05$ .

## 3 Results

### 3.1 Content of bacterial endotoxin in the plasmid DNA

The amount of LPS in the purified plasmid DNA was 0.0094 EU/ml which is usually regarded as endotoxin-free.

### 3.2 Characterization of CGN and ACGN by DSL

As shown in Table 1, the average diameter of CGN was 115.6 nm, ranged from 74.14 to 136.28 nm. The average diameter of ACGN was 174.8 nm, ranged from 92.83 to 214.73 nm.

### 3.3 ELISA for cytokines

As shown in Fig. 1, no statistical significance was observed between the negative control (no injection group) and the exposure groups (CGN or ACGN), whereas the positive control (LPS group) induced significantly higher production of IL-1 $\beta$ , IL-6, IL-10, IL-12, and TNF- $\alpha$ . It might suggest that the exposure of RAW264.7 cells to either CS-DNA nanoparticles or Arg-CS-DNA nanoparticles do not induce the production of IL-1 $\beta$ , IL-6, IL-10, IL-12, and TNF- $\alpha$  in the cell culture medium even at the concentration of nanoparticles containing 20  $\mu\text{g}$  DNA/well and up to 24 h co-incubation.

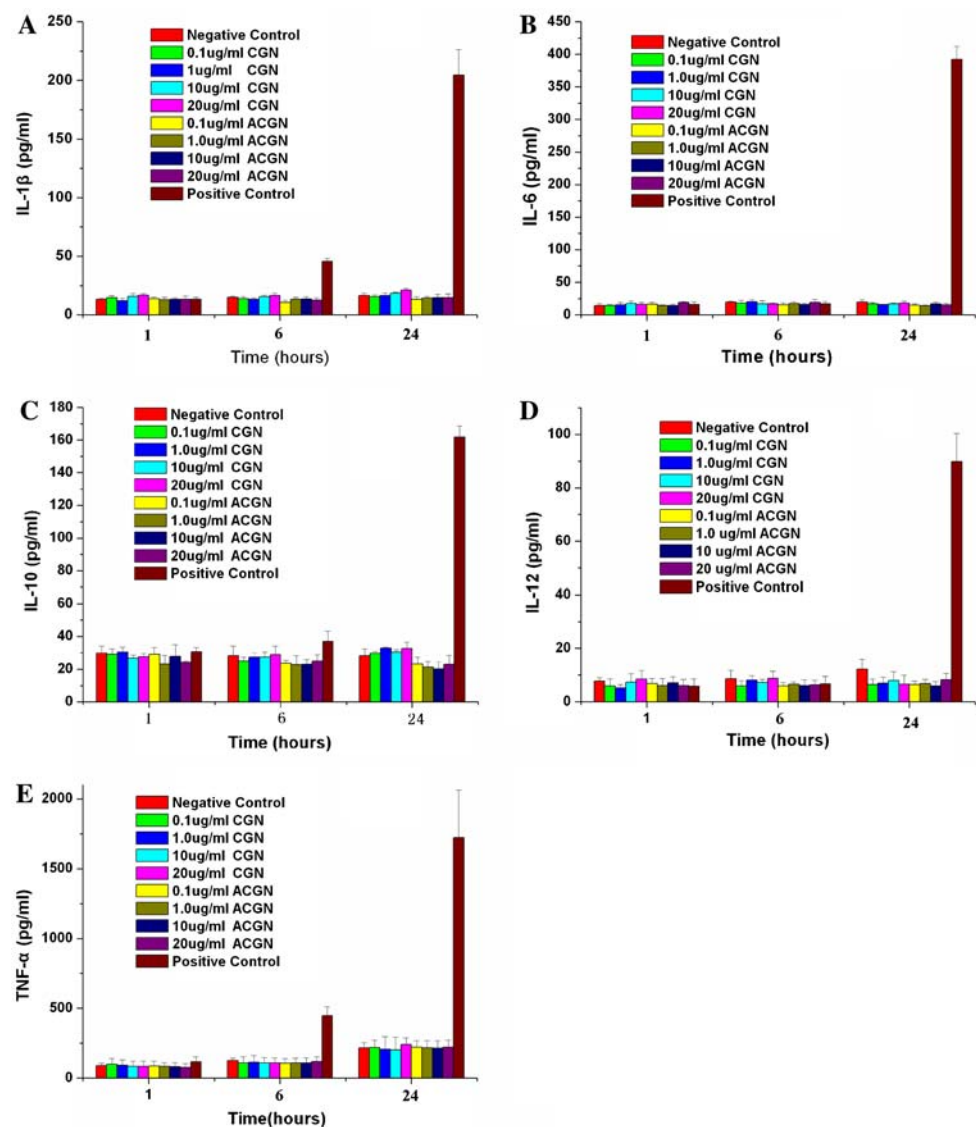
### 3.4 Phagocytic activity

The PR was  $53.17 \pm 5.53\%$ ,  $24.17 \pm 4.26\%$ ,  $26.50 \pm 3.21\%$ , and  $25.83 \pm 3.43\%$  for the LPS stimulation group, negative control group, GCN group, and ACGN group, respectively. The PI was  $2.877 \pm 0.213$ ,  $0.765 \pm 0.064$ ,  $0.767 \pm 0.097$ , and  $0.812 \pm 0.099$  for the LPS stimulation group, negative control group, GCN group, and ACGN group, respectively, as shown in Table 2. There was no statistic significance among the negative control, CGN exposure group, and ACGN exposure group, in terms of both PR and PI, whereas the LPS exposure group showed a significant elevated PR and PI.

**Table 1** Diameters of the DNA nanoparticles

	Mean size(nm)	Size range(nm)	Polydispersity
CGN	$115.6 \pm 3$	74.14–136.28	$0.160 \pm 0.01$
ACGN	$174.8 \pm 5$	92.83–214.73	$0.215 \pm 0.02$

**Fig. 1** The concentration of cytokines in RAW264.7 cell supernatants after incubation with different concentrations of CS–DNA nanoparticles and alkylated-chitosan–DNA nanoparticles and LPS. **a** IL-1 $\beta$ , **b** IL-6, **c** IL-10, **d** IL-12, **e** TNF- $\alpha$



**Table 2** The phagocytic percentage and phagocytic index of macrophage

	Phagocytic rate(%)	Phagocytic index
Negative control	24.17 $\pm$ 4.26	0.765 $\pm$ 0.064
CGN	26.50 $\pm$ 3.21	0.767 $\pm$ 0.097
ACGN	25.83 $\pm$ 3.43	0.812 $\pm$ 0.099
LPS	53.17 $\pm$ 5.53*	2.877 $\pm$ 0.213*

\*  $P < 0.05$

#### 4 Discussion

Nanoparticles have been attracting intense research effort because of their unique properties that make them suitable for many uses in biomedicine and pharmacology. However, some of the possible interactions between the nanoparticles and the immune system may result in some forms of tissue

injury. The response of macrophages to the nanoparticles is of special interest because they are the first cells which intercept the nanoparticles after the administration in vivo [17]. Activated macrophages respond to the particulate substances via phagocytosis and secretion of pro-inflammatory cytokines, which may lead to a cascade of adverse reactions which cause severe damage to the host.

Five different cytokines were selected in this study for assessing the impact of CGN or ACGN on the inflammatory activity of the macrophages. Four of them, i.e., IL-1 $\beta$ , IL-6, IL-12, and TNF- $\alpha$ , are pro-inflammatory cytokines which up-regulate the inflammatory response. These four cytokines are responsible for both localized and systemic inflammatory responses [18]. IL-10, an anti-inflammatory cytokine, could down-regulate the cytokines secretion [19, 20]. In the current study, the release of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-12 was not



induced following the exposure of the macrophages to the nanoparticles up to 24 h even at the concentration of nanoparticles containing 20 µg DNA/well, which is much higher than the amount sufficient for the effective cell transfection. The release of the anti-inflammatory cytokine IL-10 was not affected by the exposure to the nanoparticles either. Since LPS is a strong inflammation stimulating agent which is able to induce the release of the inflammatory cytokines by macrophages, it was utilized to confirm the reactivity of the macrophages to the inflammatory stimuli. The result in this study showed that LPS induced significantly higher release of all the five cytokines fore-mentioned and confirmed the reactivity of the RAW264.7 cells.

It is also vital to learn if the nanoparticles would affect the phagocytic activity of the macrophages in vivo. The result in the present study showed that peritoneal injection of the nanoparticles did not activate the murine peritoneal macrophages, whereas LPS dramatically elevated the phagocytic activity of the peritoneal macrophages.

The size and the chemical properties of the nanoparticles are among the key factors which determine the nanoparticles' impact on the immune system [21, 22]. It has been reported that PTFE particles of diameter below 20 nm could dramatically activate the macrophages in mice, whereas PTFE particles of diameter above 100 nm showed no obvious effect on the macrophages [23]. Since the average diameters of the CS–DNA nanoparticles are usually above 100 nm, as shown in the current study, it is widely accepted that CS–DNA nanoparticles would not have obvious negative impact on the function of macrophages. This view is supported by the investigation conducted by Chellat et al, which demonstrated that CS–DNA nanoparticles did not induce the production of the inflammatory cytokines by THP-1 cells [15]. While there have been evidences showing that introducing alkyl group to the molecule of CS could dramatically improve its transgenic efficacy, it is of particular importance for us to learn whether it might bring any negative impact on the biocompatibility of the CS. Results of the present investigation revealed that the modification of the CS with alkyl group did not appear to bring negative impact on the inflammatory activity of the macrophages.

## 5 Conclusions

The interaction between macrophages and CS–DNA nanoparticles is one of the major factors which determine the biocompatibility of the nanoparticles. While the modification of CSs with alkyl group was shown to be able to elevate the transgenic efficacy, its impact on the macrophages should be carefully evaluated before being applied

for medical uses. The present study demonstrated that the modification of CS with alkyl group neither induced the release of cytokines IL-1β, IL-6, IL-10, IL-12, and TNF-α by the macrophages, nor elevated the phagocytic activity of the murine peritoneal macrophages. This preliminary data suggested that the alkylated-CS may be considered a safe material for gene delivery. However, further investigation should be conducted to look into its interaction with other immune cells before a full conclusion could be made.

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