Involvement of activated transcriptional process in efficient gene transfection using unmodified and mannose-modified bubble lipoplexes with ultrasound exposure

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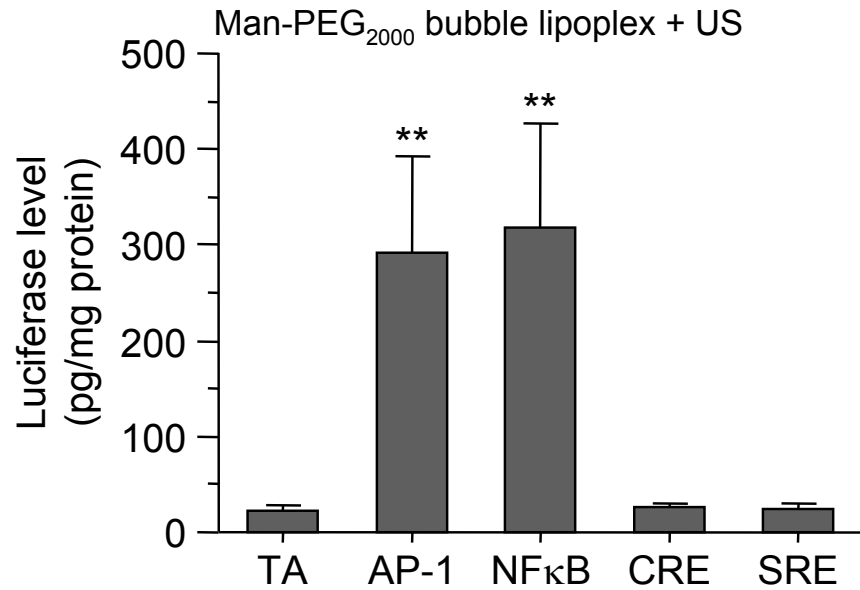E-mail: hashidam@pharm.kyoto-u.ac.jp (M. Hashida)
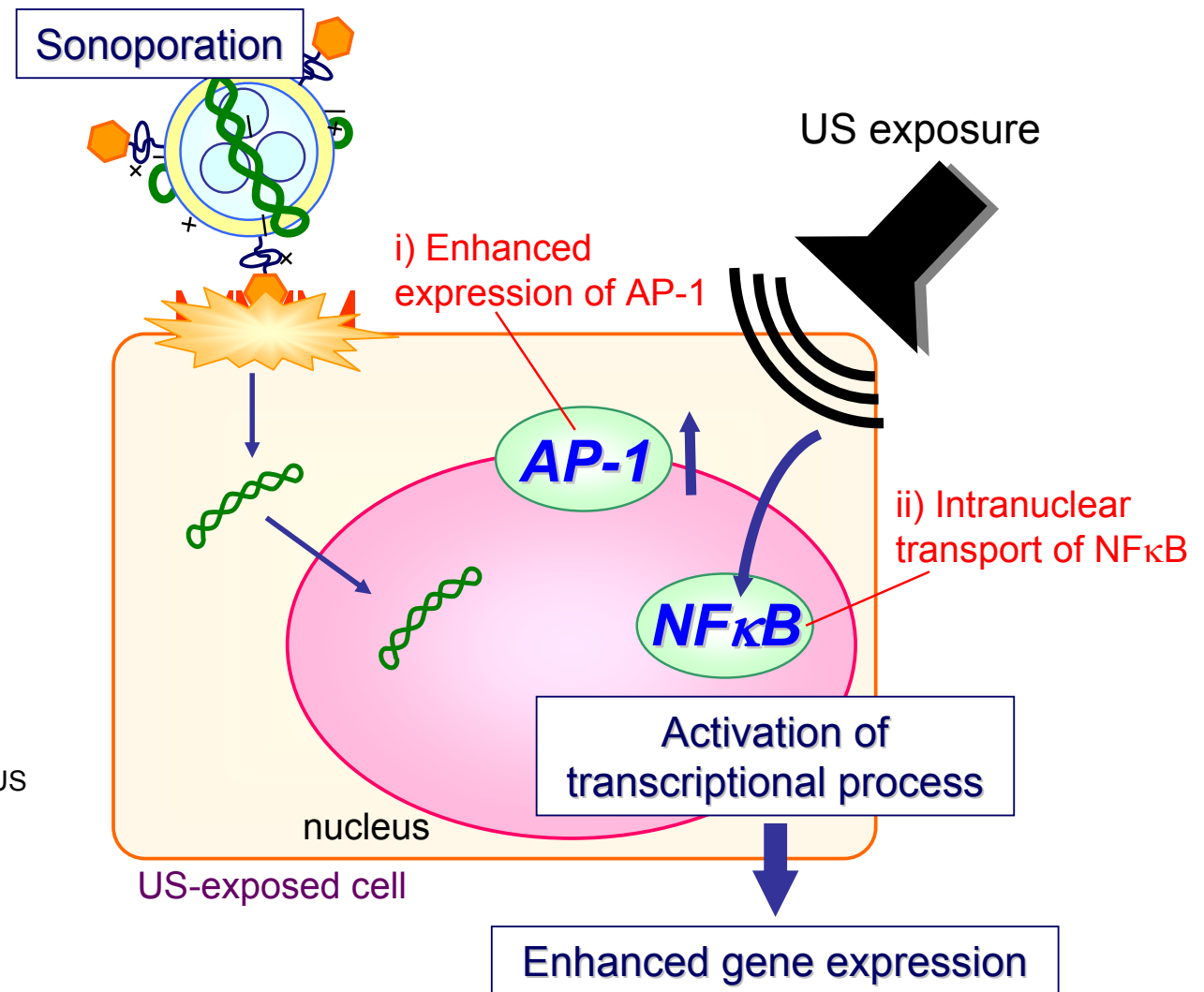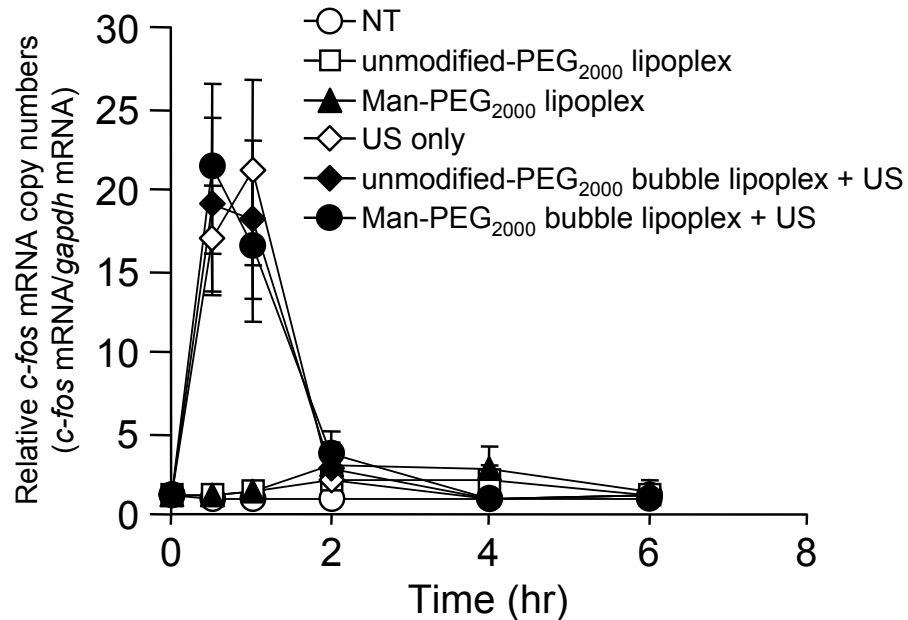E-mail: kawakami@pharm.kyoto-u.ac.jp (S. Kawakami)

Abstract

Recently, our group developed ultrasound (US)-responsive and mannose-modified gene carriers (Man-PEG$_{2000}$ bubble lipoplexes), and successfully obtained a high level of gene expression in mannose receptor-expressing cells following gene transfection using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. We also reported that large amounts of plasmid DNA (pDNA) were transferred into the cytoplasm of the targeted cells in the gene transfection using this method. In the present study, we investigated the involvement of transcriptional processes on enhanced gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. The transcriptional process related to activator protein-1 (AP-1) and nuclear factor-κB (NFκB) was activated by US exposure, and was founded to be involved in enhanced gene expression obtained by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. On the other hand, activation of AP-1 and NFκB pathways followed by US exposure was hardly involved in the inflammatory responses in the gene transfection using this method. These findings suggest that activation of AP-1 and NFκB followed by US exposure is involved in the enhanced gene expression using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure, and the selection of pDNAs activated by US exposure is important in this gene transfection method.

Keywords: gene transfection; bubble lipoplex; ultrasound; transcription factor; inflammatory response

Effects of enhancer-binding site on enhanced gene expression

Man-PEG$_{2000}$ bubble lipoplex + US

Luciferase level (pg/mg protein)

TA AP-1 NFκB CRE SRE

Effects of US exposure on transcriptional process

Relative c-fos mRNA copy numbers (c-fos mRNA/$gapdh$ mRNA)

- ○ NT
- □ unmodified-PEG$_{2000}$ lipoplex
- ▲ Man-PEG$_{2000}$ lipoplex
- ◇ US only
- ◆ unmodified-PEG$_{2000}$ bubble lipoplex + US
- ● Man-PEG$_{2000}$ bubble lipoplex + US

Time (hr)

Sonoporation

US exposure

i) Enhanced expression of AP-1

AP-1

ii) Intranuclear transport of NFκB

NFκB

Activation of transcriptional process

nucleus

US-exposed cell

Enhanced gene expression

1. Introduction

Various obstacles are associated with *in vivo* gene transfection, including the control of *in vivo* distribution of nucleic acids, the improvement of intracellular/intranuclear transport of nucleic acids, and the activation of transcriptional/translational processes directly involved in the gene expression [1,2]. Viral and non-viral carriers have been studied as valuable gene carriers for *in vivo* gene transfection [3-6], with both possesses advantages and disadvantages relating to safety, productivity and gene expressing efficiency. Hama and Harashima *et. al.* have reported that the high gene expression efficiency in gene transfection using viral carrier is influenced by the high transcriptional and translational efficiency following intranuclear transport of pDNA [7,8]. Therefore, the transcriptional/translational processes associated with gene transfection of non-viral carriers are potentially controlled by improved gene expression efficiency.

Gene transfection methods using physical stimulation, such as electroporation method [9], hydrodynamic injection [10,11], tissue pressure-mediated method [12] and sonoporation method [13], enable to obtain high-level gene expression. Gene expression has also been reported to be enhanced by intracytoplasmic transfer of pDNA as a result of using these methods [14-16]. Recently, our group developed US-responsive and/or mannose-modified gene carriers (unmodified and Man-PEG$_{2000}$ bubble lipoplexes), and reported that high level gene expression can be selectively obtained in mannose receptor-expressing cells following intravenous administration of Man-PEG$_{2000}$ bubble lipoplexes and US exposure, both *in vitro* and *in vivo* [17,18]. Furthermore, we have reported that large amounts of pDNA are transferred into the cytoplasm of targeted cells in the gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with optimized US exposure under both *in vitro* and *in vivo* conditions [19].

Various types of physical stimulations, such as electric pulse, physical pressure, radiation and US exposure, can activate the transcriptional process involved in the AP-1-mediated and NFκB-mediated pathways [20-26]. It has been reported that this activation of transcription followed by physical stimulation partly contributes to the high gene expression observed when using the hydrodynamics and physical pressure-mediated methods [21,22,27]. However, there are few reports that the transcriptional process is activated by US exposure *in vivo*. Moreover, there is little information that the transcriptional activation followed by US exposure involves in the enhanced gene expression by *in vitro* and *in vivo* gene transfection using sonoporation method.

Our present study investigated the involvement of transcriptional processes in enhanced gene expression obtained by transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. We examined the gene transfection efficiency obtained by US-mediated gene transfection using pDNAs controlled by various transcription factors including AP-1, NFκB, cyclic adenosine 3',5'-monophosphate response element (CRE) and serum response element (SRE), in RAW264.7 cell lines, primary mouse cultured macrophages, and mice. Then, we evaluated the gene expression and intranuclear transport of transcription factors, such as AP-1 [28] and NFκB [29,30], followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure, both *in vitro* and *in vivo*. Finally, the involvement of activated transcription on inflammatory cytokine production was also examined, since activation of specific transcriptional factors might contribute to the inflammatory responses [31,32],

2. Materials and Methods

2.1. Materials

1,2-Distearoyl-*sn*-glycero-3-trimethylammoniumpropane (DSTAP), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (NH_2-PEG_{2000}-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), Sigma-Aldrich (St. Louis, MO, USA) and NOF (Tokyo, Japan), respectively. RPMI-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan) and fetal bovine serum (FBS) was purchased from Japan Bioserum (Hiroshima, Japan). All other chemicals were of the highest purity available.

2.2. pDNA, cell lines and mice

pCMV-Luc was constructed as described previously [33]. Briefly, the HindIII/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) was sub-cloned into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). Pathway profiling luciferase systems (pTA/Luc, pAP-1/Luc, pNFκB/Luc, pCRE/Luc and pSRE/Luc) were purchased from Clontech Laboratories (Mountain View, CA, USA). pDNA were amplified in the *Escherichia coli* strain DH5α, isolated and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN, Hilden, Germany). RAW264.7 cells, from a murine macrophage-like cell line, were cultured in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were plated onto 24-well culture plates at a density of 5×10^4 cells/1.88 cm^2 at 37°C in 5% CO_2, and incubated for 48 hr prior to experiments. Female ICR mice (4-week-old) and female C57BL/6 mice (6-week-old) were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care, as adopted and propagated by the U.S. National Institutes of Health and the Kyoto University Guidelines for Animal Experiments.

2.3. Construction of Man-PEG_{2000} bubble lipoplexes

Man-PEG_{2000} bubble lipoplexes were constructed according to our previous report [17]. Briefly, DSTAP, DSPC, and NH_2-PEG_{2000}-DSPE or mannose-modified PEG_{2000}-DSPE were mixed in chloroform at a molar ratio of 7:2:1 to produce the liposomes for bubble lipoplexes. The liposome construction mixture was dried by evaporation and vacuum desiccated before the resultant lipid film was resuspended in sterile 5% dextrose. After hydration for 30 min at 65°C, the dispersion was sonicated for 10 min in a bath sonicator and 3 min in a tip sonicator for liposome production. Liposomes were then sterilized by passage through a 0.45 μm filter (PALL, East Hills, NY, USA). Lipoplexes were prepared by gently mixing with equal volumes of pDNA and liposome solution at a charge ratio of 1.0:2.3 (-:+). Prepared lipoplexes were pressurized with perfluoropropane gas (Takachiho Chemical Industries, Tokyo, Japan) and sonicated using a bath-type sonicator (AS ONE, Osaka, Japan) for 5 min to enclose US imaging gas. Particle sizes and ζ-potentials of the liposomes/lipoplexes were determined using a Zetasizer Nano ZS instrument (Malvern Instrument, Worcestershire, UK).

2.4. Harvesting of mouse peritoneal macrophages

Mouse peritoneal macrophages were harvested and cultured as previously described [34]. Briefly, the macrophages were harvested from the peritoneal cavity of female ICR mice, before being washed and suspended in RPMI-1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, and plated onto 24-well culture plates at a density of 2×10^5 cells/1.88 cm^2. After incubation for 2 hr at 37°C

in 5% CO_2, non-adherent cells were washed off with culture medium, and the macrophages were incubated for another 72 hr.

2.5. In vitro gene transfection

After RAW264.7 cells and macrophages were plated and incubated for 48 and 72 hr, respectively, the culture medium was replaced with Opti-MEM I containing bubble lipoplexes (5 μg pDNA).   Cells were exposed to US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2) for 20 sec using a Sonopore-4000 sonicator (NEPA GENE, Chiba, Japan) with a 6 mm diameter probe placed in each well at predetermined times after the addition of bubble lipoplexes.   At 1 hr after addition of bubble lipoplexes, the incubation medium was replaced with RPMI-1640 and incubated for an additional time.   Subsequently, the cells were scraped from the plates and suspended in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8).   The cell suspension was shaken, and centrifuged at 10,000×g, 4°C for 10 min.   Luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan) was mixed with the supernatant and the luciferase activity was measured in a luminometer (Lumat LB 9507; EG&G Berthold, Bad Wildbad, Germany).   Luciferase activity was normalized against the cellular protein content.   Protein concentration was determined with a Protein Quantification Kit (Dojindo Molecular Technologies, Tokyo, Japan).

2.6. In vivo gene transfection

Mice were intravenously injected with 400 μL of bubble lipoplexes *via* the tail vein using a 26-gauge syringe needle at a dose of 50 μg pDNA.   At predetermined times after the injection, US (frequency, 1.045 MHz; duty, 50%; burst rate, 10 Hz; intensity 1.0 W/cm^2; time, 2 min) was exposed transdermally to the abdominal area using a Sonopore-4000 sonicator (NEPA GENE) with a 20 mm diameter probe.   At predetermined times after injection, mice were sacrificed and organs were collected for each experiment.   Organs were washed twice with cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8).   Lysis buffer was added at a weight ratio of 5 mL/g for the liver or 4 mL/g for other organs. After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000×g at 4°C for 10 min.   Luciferase activity of the resulting supernatant was determined by above-mentioned luciferase assay.

2.7. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total ribonucleic acid (RNA) was isolated from the cells and organs using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich).   Reverse transcription of messenger RNA (mRNA) was carried out using PrimeScript® RT reagent Kit (Takara Bio, Shiga, Japan). The detection of complementary deoxyribonucleic acid (cDNA) (*c-fos*, *c-jun*, *p105*, *p65* and *gapdh*) was conducted using real-time PCR using SYBR® Premix Ex Taq (Takara Bio) and a Lightcycler Quick System 350S (Roche Diagnostics, Indianapolis, IN, USA).   Primers for *c-fos*, *c-jun*, *p105*, *p65* and *gapdh* cDNA were synthesized by Sigma-Aldrich as follows: *c-fos*, 5'-CCA GTC AAG AGC ATC AGC AA-3' (forward) and 5'-AAG TAG TGC AGC CCG GAG TA-3' (reverse); *c-jun*, 5'-TCC CCT ATC GAC ATG GAG TC-3' (forward) and 5'-TGA GTT GGC ACC CAC TGT TA-3' (reverse); *p105*, 5'-CCT GGA TGA CTC TTG GGA AA-3' (forward) and 5'-TCA GCC AGC TGT TTC ATG TC-3' (reverse); *p65*, 5'-TAG CAC CTG ATG GCT GAC TG-3' (forward) and 5'-CGT TCC ACC ACA TCT GTG TC-3' (reverse); *gapdh*, 5'-TCT CCT GCG ACT TCA ACA-3' (forward) and 5'-GCT GTA GCC GTA TTC ATT GT-3' (reverse).   mRNA copy number was calculated for each sample from the standard curve using the thermal-cycler software ('Arithmetic Fit Point analysis' for the

Lightcycler).   Results were expressed as relative copy number calculated relative to *gapdh* mRNA (*c-fos, c-jun, p105, p65* mRNA copy number/*gapdh* mRNA copy number).

2.8. Measurement of the level of intranuclear protein

Cells and tissues were collected at predetermined times after gene transfection, and the nuclear extract from cells and tissues was prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA).   Nuclear protein was divided into aliquots and stored at -80 °C for later use.   The protein concentration was measured with a Protein Quantification Kit. The amounts of p50 and p65, which are the components of NFκB in the cellular nuclear extract was measured using a NFκB (p50) Transcription Factor Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a NFκB (p65) transcription Factor Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), respectively, according to the manufacturer's protocols.

2.9. Measurement of inflammatory cytokines

At predetermined times after the *in vitro* and *in vivo* gene transfection, the supernatants and serum were collected and the cytokine levels (TNF-α, IFN-γ, and IL-6) were determined with a commercial enzyme-linked immunosorbent assay (ELISA) Kit (Bay Bioscience, Hyogo, Japan) according to the recommended procedures.

2.10. Statistical analysis

Results were presented as the mean±S.D. of more than three experiments.   Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Two-group comparisons were performed by Student's *t*-test.   Multiple comparisons between control and test groups were performed by Dunnett's test and multiple comparisons between all groups were performed uding the Tukey-Kramer test.

3. Results

3.1. Physicochemical properties of lipoplexes and bubble lipoplexes used in this study

The physicochemical properties of lipoplexes and bubble lipoplexes constructed with various pDNAs used in all experiments were evaluated by measuring the particle sizes and ζ-potentials. Mean particle sizes and ζ-potentials of unmodified and Man-PEG$_{2000}$ lipoplexes were approximately 137 nm and +48 mV, respectively (Supplementary Table 1). Moreover, mean particle sizes and ζ-potentials of unmodified and Man-PEG$_{2000}$ bubble lipoplexes were approximately 550 nm and +48 mV, respectively (Supplementary Table 1). These results correspond to previous reports [17-19]; suggesting that these pDNA had no effect on the physicochemical properties of lipoplexes and bubble lipoplexes.

3.2. Involvement of transcriptional process on enhanced gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure in vitro

The involvement of transcription on enhanced gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure was investigated in mouse primary cultured macrophages. First, we examined gene expression levels using unmodified/Man-PEG$_{2000}$ lipoplexes or bubble lipoplexes constructed with luciferase expressing-pDNA controlled by various transcription factors, including AP-1, NFκB, CRE and SRE. Gene expression levels obtained by Man-PEG$_{2000}$ lipoplexes only or Man-PEG$_{2000}$ bubble lipoplexes with US exposure were higher than those by unmodified-PEG$_{2000}$ formulations (Fig. 1), since mouse cultured macrophages express the mannose receptors abundantly. Moreover, although the level of gene expression obtained by both lipoplexes was similar in all pDNAs, the level of gene expression obtained by both bubble lipoplexes and US exposure was enhanced approximately 10-fold by gene transfection using pAP-1/Luc and pNFκB/Luc, compared with that using pTA/Luc, which is a pDNA without transcription factor-binding site within the enhancer region (Fig. 1). Similar results were observed in the murine macrophage-like RAW264.7 cells (Supplementary Fig. 1).
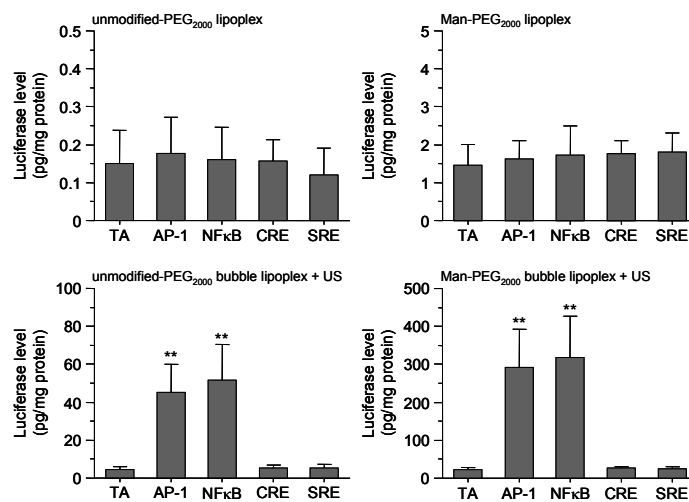

Figure 1. The effect of transcriptional factors on gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure in mouse cultured macrophages. Luciferase expression levels obtained by unmodified-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, unmodified-PEG$_{2000}$ bubble lipoplexes with US exposure, and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (5 μg of pDNA) at 24 hr after transfection in mouse primary cultured macrophages. Lipoplexes were constructed with pDNAs controlled by various transcription factors. Each value represents the mean+S.D. (n=4). Key: TA; pTA/Luc, AP-1; pAP-1/Luc, NFκB; pNFκB/Luc, CRE; pCRE/Luc, SRE; pSRE/Luc. [**]p<0.01, compared with the corresponding TA group.

3.3. Involvement of transcriptional process on enhanced gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure in mice

Next, we investigated the level of gene expression by *in vivo* gene transfection using unmodified/Man-PEG$_{2000}$ lipoplexes and bubble lipoplexes constructed with luciferase expressing-pDNA controlled by various transcription factors. Gene expression levels in the
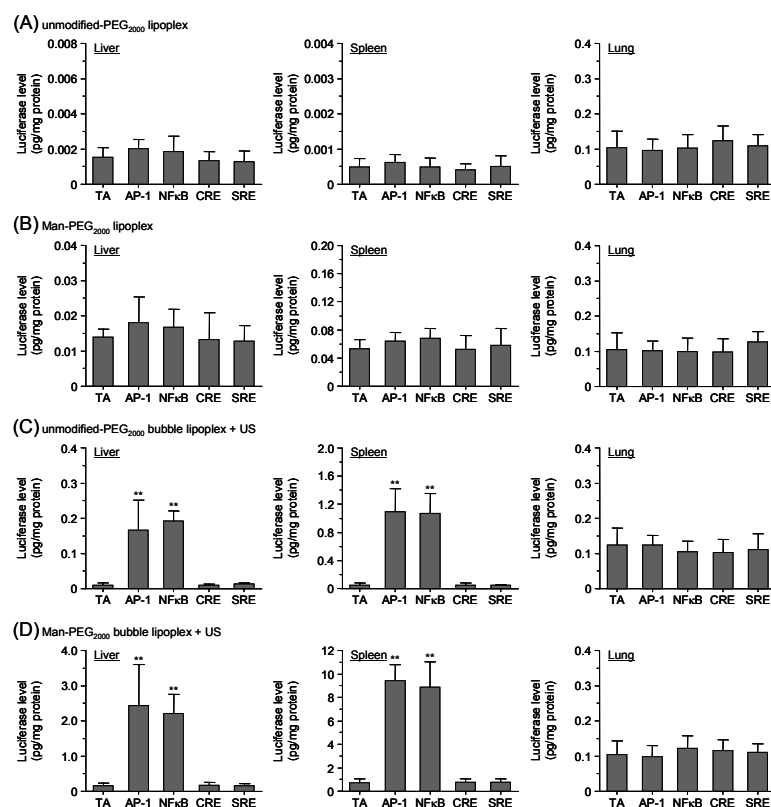
Figure 2. The effect of transcriptional factors on gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*. Luciferase expression levels obtained by unmodified-PEG$_{2000}$ lipoplexes (A), Man-PEG$_{2000}$ lipoplexes (B), unmodified-PEG$_{2000}$ bubble lipoplexes with US exposure (C), and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (D) (50 μg of pDNA) in the liver, spleen and lung at 6 hr after transfection. Lipoplexes were constructed with pDNAs controlled by various types of transcriptional factors. Each value represents the mean+S.D. (*n*=4). Key: TA; pTA/Luc, AP-1; pAP-1/Luc, NFκB; pNFκB/Luc, CRE; pCRE/Luc, SRE; pSRE/Luc. $^{**}p<0.01$, compared with the corresponding TA group.

liver and spleen obtained by Man-PEG$_{2000}$ lipoplexes only or Man-PEG$_{2000}$ bubble lipoplexes with US exposure were higher than those by unmodified-PEG$_{2000}$ formulations (Fig. 2), since liver and spleen are the major target organ of mannose-modified carriers. Although the level of gene expression obtained by both lipoplexes was similar in all pDNAs (Fig. 2A and B), gene expression levels in the liver and spleen obtained by both bubble lipoplexes and US exposure were enhanced approximately 10-fold by gene transfection using pAP-1/Luc and pNFκB/Luc, compared with that using pTA/Luc (Fig. 2C and D). On the other hand, enhanced gene expression followed by gene transfection using bubble lipoplexes constructed with pAP-1/Luc or pNFκB/Luc was not observed in the lung.

3.4. The effect of in vitro gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure on AP-1 and NFκB

Following examination of the expression properties for c-fos and c-jun, which are the components of AP-1, *c-fos* and *c-jun* mRNA expression was enhanced transiently in mouse primary cultured macrophages by not only the gene transfection using bubble lipoplexes and US exposure, but also US exposure alone (Fig. 3A). Moreover, enhanced expression of *c-fos* and *c-jun* mRNA was not observed in the gene transfection using lipoplexes only (Fig. 3A). Evaluation of the expressing properties and intranuclear transporting properties of NFκB followed by gene transfection revealed that *p105* (precursor of p50) and *p65* mRNA expression in mouse primary cultured macrophages was not enhanced in all of groups, which differed from the results obtained for *c-fos* and *c-jun* mRNA (Supplementary Fig. 2). In

contrast, the amount of intranuclear p50 and p65 increased transiently by not only the gene transfection using bubble lipoplexes and US exposure, but also US exposure alone (Fig. 3B). On the other hand, enhanced intranuclear transport of p50 and p65 was not observed in the gene transfection using lipoplexes only (Fig. 3B). Moreover, these transient AP-1 expression and intranuclear transport of NFκB followed by US exposure were also observed in RAW264.7 cells in this gene transfection method (Supplementary Fig. 3). These results suggest that transcription activation, such as increased AP-1 expression and enhanced intranuclear transport of NFκB, is partly involved in enhanced gene expression produced by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure.
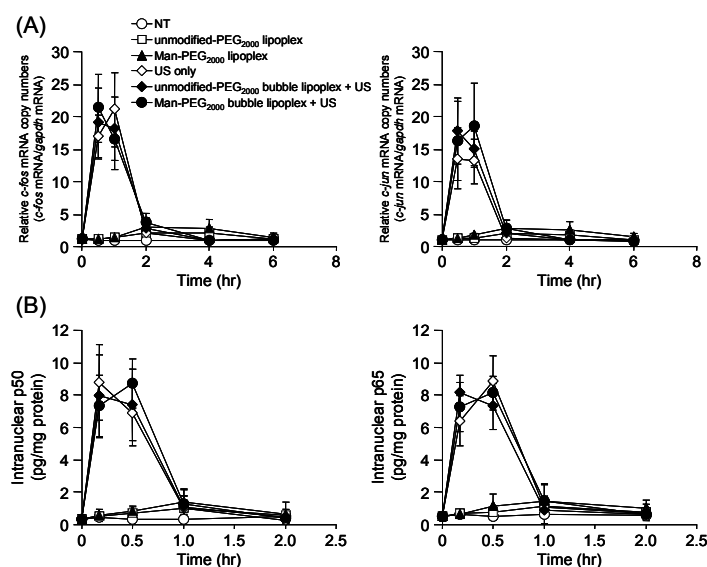

Figure 3. Enhanced *c-fos/c-jun* mRNA expression and intranuclear transport of p105/p65 followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure in mouse primary cultured macrophages. Time-course of *c-fos/c-jun* mRNA expression levels (A) and intranuclear p105/p65 levels (B) followed by various transfection methods (5 μg of pCMV-Luc) in mouse primary cultured macrophages. Each value represents the mean±S.D. (*n*=4). $^{**}p<0.01$, compared with the corresponding non-treatment (NT) group.
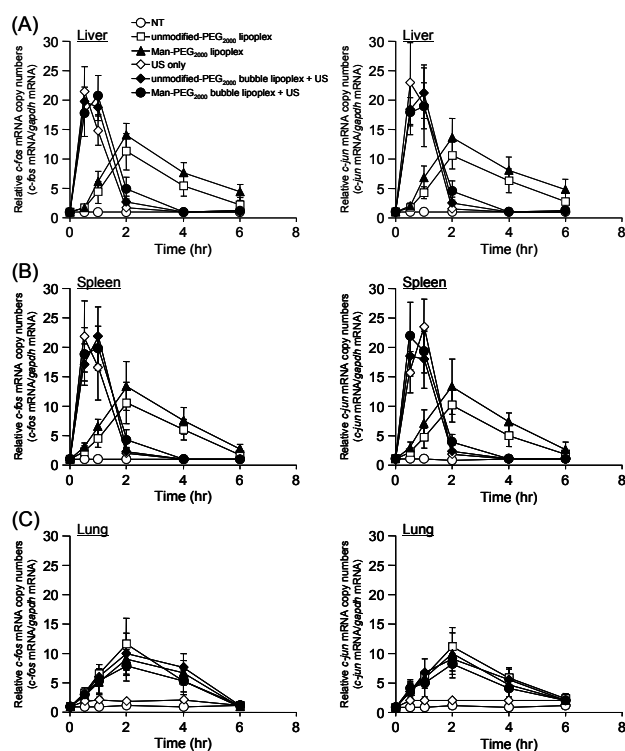

Figure 4. Enhanced *c-fos/c-jun* mRNA expression followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*. Time-course of *c-fos* and *c-jun* mRNA expression levels in the liver (A), spleen (B), and lung (C) followed by various transfection methods (50 μg of pCMV-Luc) in mice. Each value represents the mean±S.D. (*n*=4). NT; non-treatment.

3.5. The effect of in vivo gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure on AP-1 and NFκB

c-fos/c-jun mRNA expression and the intranuclear amount of p50/p65 were enhanced transiently by not only the gene transfection using bubble lipoplexes and US exposure, but also US exposure alone in both the liver and spleen (Figs. 4 and 5). On the other hand, these phenomena were not observed in the lung (Figs. 4C and 5C). In addition, *c-fos* and *c-jun* mRNA expression levels in the liver and spleen followed by US exposure were dependedent on the US intensity (Supplementary Fig. 4).
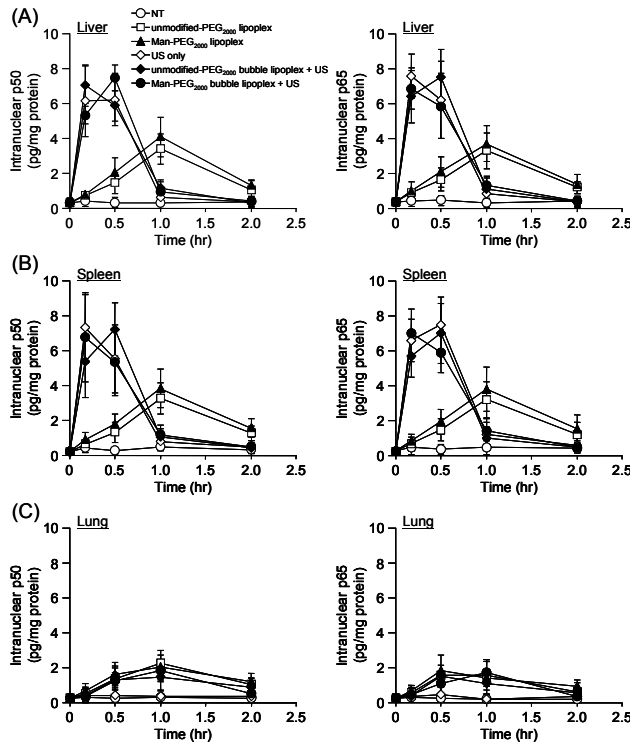

Figure 5. Enhanced intranuclear transport of p50 and p65 followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*. Time-course of intranuclear p50 and p65 levels in the liver (A), spleen (B), and lung (C) followed by various transfection methods (50 μg of pCMV-Luc) in mice. Each value represents the mean±S.D. (*n*=3). NT; non-treatment.

3.6. The effect of in vitro and in vivo gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes and US exposure on inflammatory cytokine production

Increased AP-1 expression and intranuclear transport of NFκB followed by US exposure were demonstrated to be involved in the enhanced gene expression by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. On the other hand, since these phenomena are potentially involved in the production of inflammatory cytokines [31,32], the
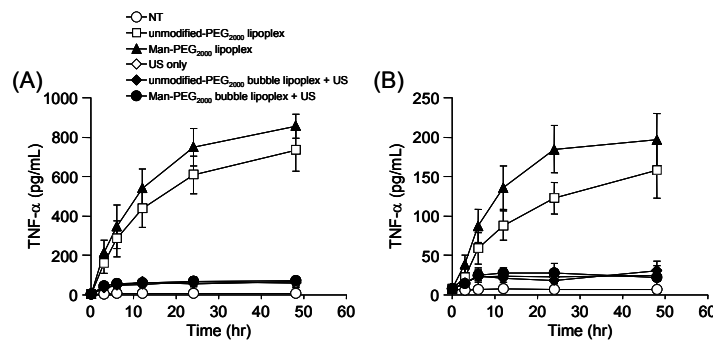

Figure 6. Evaluation of TNF-α secretion followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vitro*. TNF-α concentration in the supernatant was measured following various transfection methods (5 μg of pDNA) at predetermined times in RAW264.7 cells (A) and mouse primary cultured macrophages (B). Each value represents the mean±S.D. (*n*=4).

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production properties of inflammatory cytokines followed by gene transfection were investigated *in vitro* and *in vivo*. Although TNF-αproduction followed by gene transfection using only lipoplexes was significantly increased time-dependently in RAW264.7 cells and mouse primary cultured macrophages, only a slight increase in TNF-α production was observed followed by gene transfection using bubble lipoplexes and US exposure (Fig. 6).

While the inflammatory cytokines (TNF-α, IFN-γ, and IL-6) in the serum followed by *in vivo* gene transfection exhibited transient and significant increases in all of gene transfection methods (Fig. 7), the maximum amount of secreted inflammatory cytokines followed by gene transfection using bubble lipoplexes and US exposure was 3-fold lower than that using lipoplexes only. Moreover, the time-to-maximum concentration of secreted inflammatory cytokines followed by gene transfection using bubble lipoplexes and US exposure was earlier than that using only lipoplexes (Fig. 7). These results suggest that the production properties of inflammatory cytokine are different between conventional lipofection methods and US-mediated gene transfection methods, and that inflammatory cytokines have a minor effect on enhanced AP-1 expression/NFκB intranuclear transport followed by US exposure.
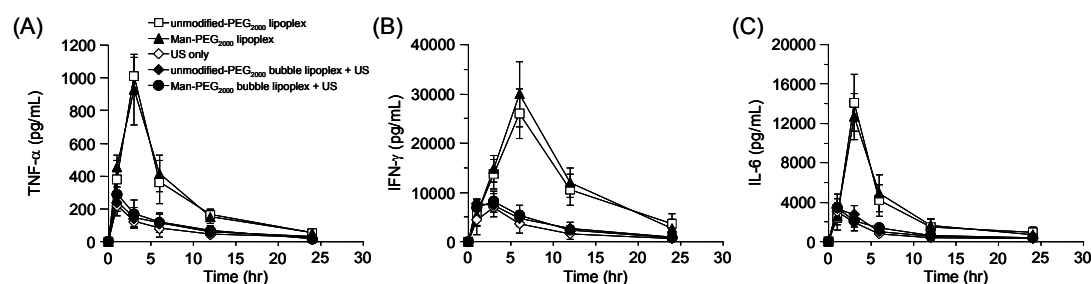

Figure 7. Evaluation of pro-inflammatory cytokine secretion in serum followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*. TNF-α (A), IFN-γ (B), and IL-6 (C) concentrations in the serum were measured following various transfection methods (50 μg of pDNA) at predetermined times in mice. Each value represents the mean±S.D. (*n*=4).

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4. Discussion

We recently reported that large amounts of pDNA are directly transferred into the cytoplasm in the gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure [17,19]. However, this enhanced gene expression followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure may not correspond to the increase of intracellular pDNA by targeted delivery of pDNA and intracytoplasmic transfer of pDNA; suggesting the involvement of the other factors on the enhanced gene expression in the gene transfection using both bubble lipoplexes and US exposure. It has been reported that the transcriptional process following intranuclear transport of pDNA is important factor in gene transfection efficiency [7,8]; therefore, we investigated the involvement of transcriptional processes in gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure.

Following examination of gene expression levels using luciferase-expressing pDNAs controlled by various transcription factors, including AP-1, NFκB, CRE and SRE, we found that the level of gene expression obtained by both lipoplexes *in vitro* (Fig. 1 and Supplementary Fig. 1), and in mouse liver and spleen (Fig. 2A and B), was similar in all pDNAs studied. On the other hand, gene expression levels using pAP-1/Luc and pNFκB/Luc were approximately 10-fold higher than those using other pDNAs in the gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure *in vitro* (Fig. 1 and Supplementary Fig. 1), and in mouse liver and spleen (Fig. 2C and D). These results strongly suggest that AP-1 and NFκB were involved in the enhanced gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. Therefore, we further investigated the AP-1/NFκB gene expression and intranuclear transport followed by this gene transfection method.

c-fos/*c-jun* mRNA expression (Figs. 3A and 4) and intranuclear p50/p65 levels (Figs. 3B and 5) were enhanced transiently by not only the gene transfection using both bubble lipoplexes and US exposure, but also US exposure alone *in vitro*, and in mouse liver and spleen. It has been reported that US exposure induced the enhanced expression of c-fos and c-jun *via* phosphorylation of ERK, p38 and JNK [25,26], and our results partially correspond to these reports. These observations led us to believe that the activation of AP-1 and NFκB-mediated transcriptional processes followed by US exposure is involved in the enhanced gene expression using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure.

Since the activation of transcription factors such as AP-1 [28] and NFκB [29,30] is involved in the induction of inflammatory responses [31,32], we investigated the production properties of inflammatory cytokines followed by this gene transfection method. The production levels of TNF-α, IFN-γ or IL-6 followed by gene transfection using both bubble lipoplexes and US exposure were substantially lower than that using both lipoplexes *in vitro* (Fig. 6) and *in vivo* (Fig. 7). We previously reported that the inflammatory responses were significantly suppressed in the gene transfection method using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure, because a large amount of pDNA was transferred into the cytoplasm directly through the transient pores created by the destruction of both bubble lipoplexes followed by US exposure [19], suggesting that pDNA is hardly interacted with endosomal TLR-9. On the other hand, it was reported that the phosphorylation of AP-1 and NFκB was induced via the activation of p38, ERK and JNK-mediated pathways followed by US exposure [25,26], and we showed that these AP-1 and NFκB activation was transiently in our sonoporation method and condition in this study (Figs. 3-5 and Supplementary Fig. 3). Although these activation of AP-1 and NFκB leads to the inflammatory cytokine production

[31,32], the inflammatory responses induced by AP-1 and NFκB activation followed by US exposure were low under *in vitro* and *in vivo* condition (Figs. 6 and 7).   We previously have reported that the activating level of transcriptional factors, such as c-fos and c-jun, in tissue pressure-mediated transfection method was approximately one-fifth, compared with that in hydrodynamics method [22].   Moreover, the production of inflammatory cytokines under *in vivo* condition followed by tissue pressure-mediated transfection method was much lower than those by conventional lipofection method [35].   The activating levels of transcriptional factors followed by our sonoporation method using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure were almost the same with that by tissue pressure-mediated transfection method.   Therefore, the contribution of the inflammatory response induced by AP-1 and NFκB activation followed by our sonoporation method may be negligible.   These results suggest that the transient expression of AP-1 and the transient intranuclear transport of NFκB followed by US exposure might be minimally involved in the inflammatory responses in the gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure.

5. Conclusion

Our results suggest that the activated AP-1 and NFκB followed by US exposure is involved in the enhanced gene expression using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure.   These results suggest that enhanced gene expression in the gene transfection using our sonoporation method was obtained by applying pDNA controlled by the specific transcriptional factors.   Therefore, the selection of suitable pDNA with specific promoter regions activated by US stimulation is one of the important factors for efficient gene expression in our gene transfection method.   In addition, the transient expression of AP-1 and the transient intranuclear transport of NFκB followed by US exposure were not substantially involved in the inflammatory responses in this gene transfection method.   These findings may help in the development of an effective gene transfection method using US-exposing system.

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Figure captions

Figure 1. The effect of transcriptional factors on gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure in mouse cultured macrophages. Luciferase expression levels obtained by unmodified-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, unmodified-PEG$_{2000}$ bubble lipoplexes with US exposure, and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (5 μg of pDNA) at 24 hr after transfection in mouse primary cultured macrophages. Lipoplexes were constructed with pDNAs controlled by various transcription factors. Each value represents the mean+S.D. (n=4). Key: TA; pTA/Luc, AP-1; pAP-1/Luc, NFκB; pNFκB/Luc, CRE; pCRE/Luc, SRE; pSRE/Luc. $^{**}p$<0.01, compared with the corresponding TA group.

Figure 2. The effect of transcriptional factors on gene expression obtained by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*. Luciferase expression levels obtained by unmodified-PEG$_{2000}$ lipoplexes (A), Man-PEG$_{2000}$ lipoplexes (B), unmodified-PEG$_{2000}$ bubble lipoplexes with US exposure (C), and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (D) (50 μg of pDNA) in the liver, spleen and lung at 6 hr after transfection. Lipoplexes were constructed with pDNAs controlled by various types of transcriptional factors. Each value represents the mean+S.D. (n=4). Key: TA; pTA/Luc, AP-1; pAP-1/Luc, NFκB; pNFκB/Luc, CRE; pCRE/Luc, SRE; pSRE/Luc. $^{**}p$<0.01, compared with the corresponding TA group.

Figure 3. Enhanced *c-fos*/*c-jun* mRNA expression and intranuclear transport of p105/p65 followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure in mouse primary cultured macrophages. Time-course of *c-fos*/*c-jun* mRNA expression levels (A) and intranuclear p105/p65 levels (B) followed by various transfection methods (5 μg of pCMV-Luc) in mouse primary cultured macrophages. Each value represents the mean±S.D. (n=4). $^{**}p$<0.01, compared with the corresponding non-treatment (NT) group.

Figure 4. Enhanced *c-fos*/*c-jun* mRNA expression followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*. Time-course of *c-fos* and *c-jun* mRNA expression levels in the liver (A), spleen (B), and lung (C) followed by various transfection methods (50 μg of pCMV-Luc) in mice. Each value represents the mean±S.D. (n=4). NT; non-treatment.

Figure 5. Enhanced intranuclear transport of p50 and p65 followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*. Time-course of intranuclear p50 and p65 levels in the liver (A), spleen (B), and lung (C) followed by various transfection methods (50 μg of pCMV-Luc) in mice. Each value represents the mean±S.D. (n=3). NT; non-treatment.

Figure 6. Evaluation of TNF-α secretion followed by gene transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vitro*. TNF-α concentration in the supernatant was measured following various transfection methods (5 μg of pDNA) at predetermined times in RAW264.7 cells (A) and mouse primary cultured macrophages (B). Each value represents the mean±S.D. (n=4).

Figure 7. Evaluation of pro-inflammatory cytokine secretion in serum followed by gene

transfection using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure *in vivo*.   TNF-α (A), IFN-γ (B), and IL-6 (C) concentrations in the serum were measured following various transfection methods (50 μg of pDNA) at predetermined times in mice.   Each value represents the mean±S.D. (*n*=4).