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Activated human neutrophil response to perfluorocarbon nanobubbles: Oxygen-dependent and -independent cytotoxic responses

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ABSTRACT

Nanobubbles, a type of nanoparticles with acoustically active properties, are being utilized as diagnostic and therapeutic nanoparticles to better understand, detect, and treat human diseases. The objective of this work was to prepare different nanobubble formulations and investigate their physicochemical characteristics and toxic responses to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-activated human neutrophils. The nanobubbles were prepared using perfluoropentane and coconut oil as the respective core and shell, with soybean phosphatidylcholine (SPC) and/or cationic surfactants as the interfacial layers. The cytotoxic effect of the nanobubbles on neutrophils was determined by extracellular $O_2^{\bullet-}$ release, intracellular reactive oxygen species (ROS), lactate dehydrogenase (LDH), and elastase release. Particle sizes of the nanobubbles with different percentages of perfluorocarbon, oil, and surfactants in ranged 186–432 nm. The nanobubbles were demonstrated to inhibit the generation of superoxide and intracellular ROS. The cytotoxicity of nanobubbles may be mainly associated with membrane damage, as indicated by the high LDH leakage. Systems with Forestall (FE), a cationic surfactant, or higher SPC contents exhibited the greatest LDH release by 3-fold compared to the control. The further addition of an oil component reduced the cytotoxicity induced by the nanobubbles. Exposure to most of the nanobubble formulations upregulated elastase release by activated neutrophils. Contrary to this result, stearylamine (SA)-containing systems slightly but significantly suppressed elastase release. FE and SA in a free form caused stronger responses by neutrophils than when they were incorporated into nanobubbles. In summary, exposure to nanobubbles resulted in a formulation-dependent toxicity toward human neutrophils that was associated with both oxygen-dependent and -independent pathways. Clinicians should therefore exercise caution when using nanobubbles in patients for diagnostic and drug targeting aims.

1. Introduction

Nanotechnology is at the leading edge of rapidly developing new therapeutic and diagnostic concepts in all areas of medicine (Shubayev et al., 2009). Nanoparticles are increasingly used in different applications, including bioimaging, diagnostic technology, and drug/gene delivery (Kim et al., 2006). A prerequisite for human use of nanoparticles is an acceptably low toxicity. Hence a significant concern exists regarding the potential toxicity of nanoparticles. Possible undesirable side effects of nanoparticles are harmful interactions with biological systems and the environment, with the potential to generate toxicity (Nel et al., 2006). Regulatory agencies, researchers, and health and environmental watchdogs are investigating how nanoscale materials affect human health (Service, 2004). However, there is a lack of information regarding the health implications of manufactured nanomaterials toward humans.

Among the medical applications of nanotechnology, microbubbles are one of the several examples of nanoparticles which are already being used in clinical settings. It would therefore be very useful to establish minimal safety standards for the delivery of microbubbles. Microbubbles are comprised of spherical voids or cavities filled by perfluorocarbon gas. Microbubbles can be stabilized by a shell of proteins, polymers, or surfactants (Fang et al., 2007). When these microbubbles are subjected to acoustic pulses produced by an ultrasound transducer, the pressure waves cause

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the bubbles to undergo volumetric oscillations that subsequently produce a secondary acoustic pulse detectable by the imaging system. This acoustic property makes microbubbles highly detectable with clinical ultrasound systems (Dayton et al., 2001). Currently, microbubbles are marked as contrast agents for ultrasound diagnostics and imaging. Microbubbles also represent a new class of parenteral formulations with drug therapeutic applications such as anticancer drugs and genes (Zhao et al., 2005; Lentacker et al., 2010). An increasing number of recent studies concentrated on the development of microbubble structures with nano-sized particles (Fang et al., 2009; Tinkov et al., 2009), which are called nanobubbles. They provide better drug-loading potential and have the ability to extravasate through leaky tumor microvasculature and accumulate in tumor interstitium.

An important aspect of toxicology is the burden of multiple dosing of nanoparticles. In the case of bioimaging, exposure to microbubbles repeatedly occurs with each scanning session (Lewinski et al., 2008). Most microbubbles/nanobubbles are applied via a parenteral route into the bloodstream. Once nanoparticles are translocated into the bloodstream, they instantly encounter a complex environment of proteins and phagocytic cells such as neutrophils (Gonçalves et al., 2010a). Polymorphonuclear neutrophils are primordial players in innate immunity and provide a pivotal role in the defense of the human body against infections. Microbubbles were previously shown to be phagocytosed intact by activated neutrophils (Lindner et al., 2000; Dayton et al., 2001). Most research on microbubbles/nanobubbles has focused on the beneficial effects for experimental and clinical uses. A systematic approach to assess their toxicity has not been established.

The aim of this work was to examine the responses of human neutrophils treated with nanobubbles. In the present study, we designed nanobubbles with various additives to explore the effects of the formulation variables on physicochemical characteristics and neutrophil behaviors. Ultrasound contrast agents are often administered to critically ill patients. In the predisposing inflammatory disorders, the number of neutrophils markedly increases (Gonçalves et al., 2010b). We utilized a bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) to activate neutrophils in order to simulate an ex vivo inflammatory condition. In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as reactive oxygen species (ROS), granule proteases, and lipids (Hwang et al., 2006b). We examined superoxide anions ($O_2^{\bullet -}$), lactate dehydrogenase (LDH), and elastase released by neutrophils after nanobubble incubation. It is our intention to suggest the feasible and less-toxic materials for nanobubble production based on the data shown in this investigation.

2. Materials and methods

2.1. Materials

Coconut oil, cholesterol, stearylamine (SA), and dihydrorhodamine 123 (DHR123) were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). Perfluoropentane (96%) was obtained from Strem Chemicals (Newburyport, MA, USA). Hydrogenated soybean phosphatidylcholine (SPC, Phospholipon® 80H) was supplied by American Lecithin (Oxford, CT, USA). Soyaethyl morpholinium ethosulfate (Forestall®) was from Croda (East Yorkshire, UK). 2-(4-Indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.2. Preparation of nanobubbles

SPC, cholesterol, and/or cationic surfactants were dissolved in 5 ml of a chloroform: ethanol (2:1) solution. The organic solvent was evaporated in a rotary evaporator at 50 °C, and solvent traces were removed by maintaining the lipid film under a vacuum overnight. The film was hydrated with double-distilled water using a probe-type sonicator (VCX600, Sonics and Materials, Newtown, CT, USA) at 35 W for 10 min at 60 °C. Then coconut oil was added to the system, followed by high-shear homogenization (Pro250, Pro Scientific, Monroe, CT, USA) for 4 min. The resulting dispersion was cooled to 20 °C, after which perfluoropentane was incorporated into the system and sonicated at 35 W for 10 min. The nanobubbles developed in this work are listed in Table 1.

2.3. Determination of the particle size and zeta potential

The mean particle size (z-average) and zeta potential of nanobubbles were measured by a laser-scattering method (Nano ZS® 90, Malvern, Worcestershire, UK). The nanobubbles were diluted 100-fold with double-distilled water in order to measure the size and surface charge. The determination was repeated three times per sample for three independent batches.

2.4. Preparation of human neutrophils

Blood was taken from healthy human donors (20–30 years old) by venipuncture, using a protocol approved by the Institutional Review Board at Chang Gung Memorial Hospital. Healthy adult volunteers provided written informed consent in order to participate. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes (Boyum et al., 1991). Purified neutrophils that were comprised of >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in Ca^{2+}-free Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY, USA) buffer at pH 7.4 and maintained at 4 °C before use. The morphological assessment of neutrophils using Wright-Giemsa stain was observed by optical microscope. Our result showed that 99% of purified cells are polymorphonuclear leukocytes, which often refer specifically to neutrophil granulocytes.

2.5. Measurement of $O_2^{\bullet -}$ generation by WST-1

The assay of the generation of $O_2^{\bullet -}$ was based on the superoxide dismutase (SOD)-inhibitable reduction of WST-1, as reported previously (Tan and Berridge, 2000). Major advantages of the WST-1 assay include its high specificity and sensitivity. The reaction is inhibited by SOD, thus indicating that the WST-1 assay is specific for $O_2^{\bullet -}$ generation. The cell proliferation reagent WST-1 is a ready-to-use solution, containing WST-1 and an electron coupling reagent. On the contrary, the electron coupling reagent is not included in the assay of the generation of $O_2^{\bullet -}$. After supplementation with 0.3 mM WST-1 and 1 mM Ca^{2+}, freshly isolated neutrophils (6×10^5 cells/ml) were equilibrated at 37 °C for 2 min and incubated with nanobubbles (1:50 dilution in suspensions) for 5 min. Cells were activated with fMLP (100 nM). Cytochalasin B at 1-μg/ml was incubated for 3 min before cell activation. Changes in absorbance with the reduction of WST-1 at 450 nm were continually monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (U-3010, Hitachi, Tokyo, Japan). The materials of nanobubbles were also tested alone at equivalent concentrations to those used in the nanosystems.

2.6. Determination of intracellular ROS by a flow cytometric assay

The intracellular production of ROS in stimulated neutrophils was quantified in individual cells by flow cytometry. DHR123 (2 μM) in a 1 mM $CaCl_2$ HBSS solution was loaded into the neutrophils and incubated for 10 min at 37 °C. The nanobubbles were then added to the suspensions for 5 min. After that, 0.3 μM fMLP was added as a neutrophil-stimulating agent and incubated for 15 min. Ice-cold HBSS was added to the suspensions to stop the reaction. DHR123 was converted to rhodamine 123 after oxidation. Intracellular rhodamine 123 fluorescence of neutrophils was determined by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA).

2.7. Measurement of lactate dehydrogenase (LDH) release

Release of LDH to the cell medium indicates cell membrane damage. LDH release was determined by a commercially available method (Promega, Madison, WI, USA). Cytotoxicity was represented by LDH release to cell-free medium as a percentage of the total LDH released. The total LDH released was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37 °C.

2.8. Measurement of elastase release

Elastase release measurement was used to detect degranulation of azurophilic granules as described previously with some modifications (Hwang et al., 2006a). Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), neutrophils (6×10^5 cells/ml) were equilibrated at 37 °C for 2 min and incubated with nanobubbles for 5 min. Cells were activated by 100 nM fMLP and 0.5 μg/ml cytochalasin B, and changes in absorbance at 405 nm were continually monitored to assay elastase release. Results are expressed as a percent of the initial rate of elastase release in the fMLP/cytochalasin B-activated nanobubble-free control group.

2.9. Statistical analysis

The statistical analysis of differences among various treatments was performed using an analysis of variance (ANOVA) test. A 0.05 level of probability was taken as

Table 1
The compositions and their percentages (%, w/w) of perfluorocarbon nanobubbles used in this study.

Code	Characterization	Perfluoropentane	Oil	SPC[a]	Cholesterol	FE[b]	SA[c]
P1	Typical	12.0	4.0	3.5	1.0	–	–
P2	More pentane	15.0	4.0	3.5	1.0	–	–
P3	More oil	12.0	10.0	3.5	1.0	–	–
P4	More SPC	12.0	4.0	4.5	1.0	–	–
P5	Less SPC	12.0	4.0	2.5	1.0	–	–
P6	With FE	12.0	4.0	3.5	1.0	0.6	–
P7	With SA	12.0	4.0	3.5	1.0	–	0.6
P8	More SA	12.0	4.0	3.5	1.0	–	1.0

[a] SPC, soybean phosphatidylcholine.
[b] FE, Forestall.
[c] SA, stearylamine.

the level of significance. The unpaired Student's *t*-test was also used if necessary. Statistically different samples compared to the control are indicated by asterisks in the figures: *data which were lower ($p < 0.05$) compared to the control; **data which were higher ($p < 0.05$) compared to the control.

3. Results

3.1. Determination of the particle size and zeta potential

We designed a sophisticated nanobubble system of perfluoropentane stabilized with SPC and/or cationic surfactants. Coconut oil was used as the oil phase in the present work. Based on the additives and preparation procedures, the interior of the nanobubbles was loaded with perfluorocarbon, which was then surrounded by coconut oil. SPC and cationic surfactants as emulsifiers were located in the oil/water interface. A similar construction was reported by Liu et al. (2006). On visual inspection, the nanobubbles were white and homogeneous. Table 2 summarizes the physicochemical properties of the nanobubbles developed in this study (P1∼P8). There were significant differences in particle sizes among these formulations. The standard nanobubbles (P1) had a particle size of 246 nm. An increment of incorporated perfluoropentane percentage from 12% (P1) to 15% (P2) in the nanobubbles resulted in a size increase to 286 nm ($p < 0.05$). Also, an increment in the oil phase from 4% to 10% (P3) led to an enlargement of size (433 nm, $p < 0.05$). Both the addition (3.5–4.5%) (P4) and reduction (3.5–2.5%) of SPC (P5) increased the particle sizes to 298 and 345 nm, respectively ($p < 0.05$). The results clearly showed that the cationic surfactants in the interface modified the properties of the nanobubbles. The incorporation of FE (P6) and SA (P7) significantly reduced ($p < 0.05$) the particle size compared to the typical formulation (P1). However, further addition of SA from 0.6% to 1% (P8) greatly increased the size from 209 to 387 nm ($p < 0.05$). The polydispersity index (PDI) could be well controlled to a narrow range of <0.3, except for systems with a higher oil percentage (P3), for which the PDI was 0.73. The PDI results indicated a quite-homogeneous population of particles for most of the formulations.

Table 2
The characterization of the perfluorocarbon nanobubbles by particle size, polydispersity index (PDI), and zeta potential.

Code	Size (nm)	PDI	Zeta potential (mV)
P1	246.0 ± 12.3	0.26 ± 0.02	−60.8 ± 3.2
P2	286.2 ± 14.2	0.30 ± 0.02	−62.5 ± 5.4
P3	432.8 ± 35.0	0.73 ± 0.25	−79.1 ± 1.3
P4	297.9 ± 10.2	0.24 ± 0.01	−60.3 ± 4.6
P5	344.5 ± 13.9	0.18 ± 0.05	−57.0 ± 9.5
P6	185.5 ± 0.5	0.18 ± 0.02	51.6 ± 3.8
P7	209.3 ± 1.2	0.17 ± 0.01	61.6 ± 1.7
P8	387.1 ± 5.6	0.29 ± 0.09	65.4 ± 0.7

Each value represents the mean ± SD ($n = 3$).

The absolute zeta potential of the standard formulation (P1) was −61 mV. The change in the zeta potential was not significant ($p > 0.05$) after incorporation of perfluoropentane (P2). The negative zeta potential increased as the volume of the oil phase increased (P3, $p < 0.05$). Changing the SPC contents did not modify the surface charge of the interface (P1 vs. P4 vs. P5, $p > 0.05$). FE and SA provided a positive charge to the nanobubbles, with the systems with a greater SA ratio (P8) showing the highest positive zeta potential.

3.2. Measurement of $O_2^{\bullet -}$ generation by WST-1

To investigate whether nanobubbles influenced oxygen-dependent pathways of neutrophils in response to fMLP, the extracellular amount of $O_2^{\bullet -}$ was determined as shown in Fig. 1A. Compared to the $O_2^{\bullet -}$ generated from activated neutrophils of the control group, lower amounts of the $O_2^{\bullet -}$ were detected in cells treated with nanobubbles ($p < 0.05$) except for P3. Treatment with nanobubbles containing more oil (P3) had no effect ($p > 0.05$) on $O_2^{\bullet -}$ production by neutrophils. Further addition of perfluoropentane (P2) did not change $O_2^{\bullet -}$ production ($p > 0.05$) compared to the typical systems (P1). It was found that $O_2^{\bullet -}$ downregulation was strengthened following an increase in the amount of SPC, although the difference was not significant ($p > 0.05$) among the $O_2^{\bullet -}$ levels of these nanobubbles (P1 vs. P3 vs. P4). The incorporation of FE (P6), a cationic surfactant, inhibited $O_2^{\bullet -}$ generation to a greater degree as compared to the standard P1 formulation ($p < 0.05$). A greater amount of SA did not further reduce ($p > 0.05$) superoxide production (P7 vs. P8). The $O_2^{\bullet -}$ levels of fMLP-stimulated neutrophils were also tested with pure compounds of the interfacial components at an equivalent dose with those used in the nanobubbles. As shown in Fig. 1B, SPC did not alter the amount of superoxide anions ($p > 0.05$) compared to the control, while FE and SA completely inhibited $O_2^{\bullet -}$ production ($p < 0.05$).

3.3. Determination of intracellular ROS by a flow cytometric assay

In order to examine the production of total intracellular ROS by neutrophils, we stained cells with DHR123, a marker for ROS. Within cells, DHR123 is oxidized to rhodamine 123, a highly fluorescent compound. Fig. 2 displays the representative flow cytometric diagrams of stimulated neutrophils. Fig. 2A and B are the diagrams of neutrophil fluorescence at the basal level (with no treatment) and fMLP treatment, respectively. It can be seen that the signal had shifted to the right after fMLP treatment, indicating an increase in the ROS amount within cells. Four nanobubble formulations (P1, P4, P6, and P7) were incubated with neutrophils with fMLP treatment (Fig. 2C–F). The fluorescence signal of neutrophils with nanobubbles was left-shifted compared to that of fMLP-activated neutrophils with no incubation. It was noted that the total numbers of living cells (total counts within the circle in the left figure) were fewer for cationic surfactant-containing systems

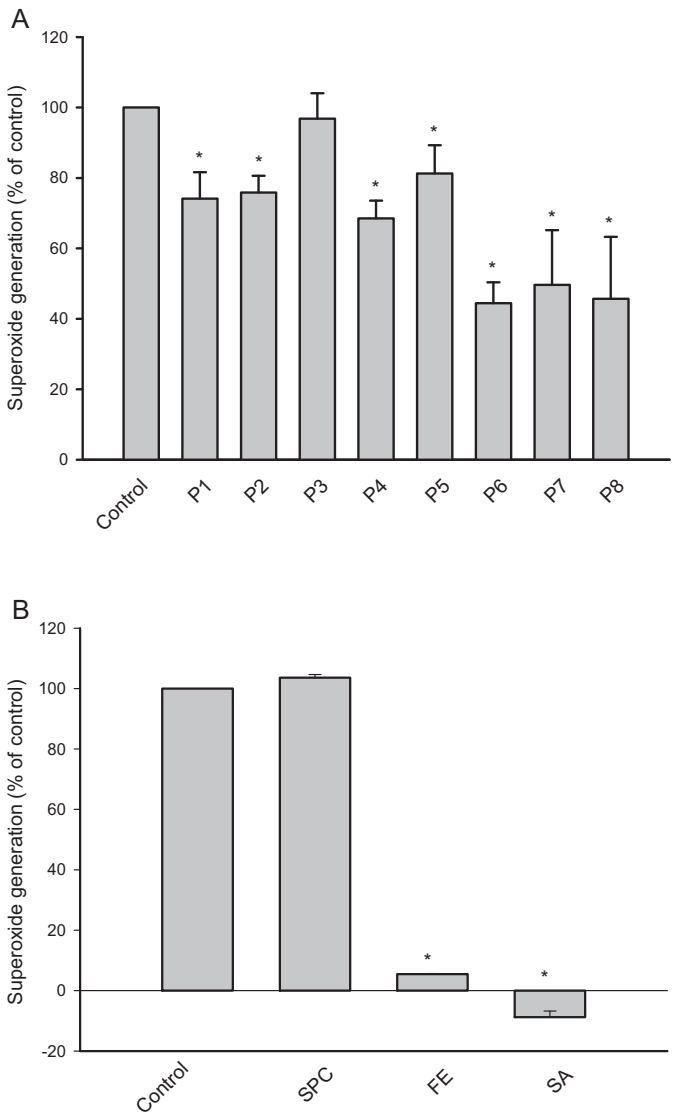

Fig. 1. Effects of various nanobubble formulations (A) and emulsifiers (B) on $O_2^{\bullet-}$ generation in fMLP-activated neutrophils. All data are expressed as the mean ± S.E.M. (n = 4–8). *The value was lower ($p < 0.05$) compared to the control. SPC, soybean phosphatidylcholine; FE, Forestall®; SA, stearylamine.

(P6 and P7) compared to the other formulations. Fig. 3 shows the DHR123 oxidation level of neutrophils treated with nanobubbles. fMLP served as a positive control. The level of DHR123 oxidation can be an indicator of ROS in the interior of cells. ROS amounts of neutrophils treated with nanobubbles were relatively lower ($p < 0.05$) than those without treatment, with FE-containing systems (P6) showing the lowest value. A further increase in the SPC percentage (P4) did not alter the intracellular ROS amounts ($p > 0.05$). Free radicals both outside and inside of cells were reduced after nanobubble treatments based on $O_2^{\bullet-}$ detection and flow cytometric profiles, respectively.

3.4. Measurement of LDH release

The cell membrane damage induced by nanobubbles was monitored by an LDH leakage assay, since LDH, a stable cytosolic enzyme in normal cells, can leak into the extracellular fluid only after membrane damage. As depicted in Fig. 4A, significantly greater LDH levels were observed in nanobubble-exposed groups than in the control group ($p < 0.05$) except for P3. Treatment with the formulations with abundant oil (P3) did not affect ($p > 0.05$) cell viability, as assayed by LDH release. The standard nanobubbles (P1) caused a 60% increase in the LDH level. More perfluoropentane (P2) did not further promote LDH release ($p > 0.05$). More SPC challenge (P4) significantly elevated ($p < 0.05$) LDH compared to challenge with less SPC (P1 and P5). The LDH level was greater in FE-containing nanobubbles (P6) than in the standard group (P1, $p < 0.05$). A 160% increment in LDH release was detected for this formulation. On the other hand, SA addition (P7 and P8) increased LDH to a level comparable ($p > 0.05$) to that of the standard formulation without SA (P1). When tested alone at equivalent concentrations to those used in the nanomaterials, the surfactants, used as components of the nanobubbles, resulted in enhanced LDH. This upregulation was especially significant for cationic surfactants. A 6-fold increase in LDH release was observed for both cationic surfactants.

3.5. Measurement of elastase release

The elastase derived from activated neutrophils treated with nanobubbles was determined as shown in Fig. 5A. Elastase release from fMLP-stimulated neutrophils did not change after treatment with standard nanobubbles (P1, $p > 0.05$). Exposure to nanobubbles with extra perfluoropentane and oil (P2 and P3) resulted in a greater elastase release ($p < 0.05$) than did exposure to the standard systems. The extra SPC (P4) also produced a higher level of elastase ($p < 0.05$). On the other hand, the formulation with less SPC (P5) did not alter the basal level of elastase release ($p > 0.05$) by human neutrophils. Some minor effects in reducing elastase release ($p < 0.05$) were seen after treatment with nanobubbles incorporating SA (P7 and P8). The influence of neat materials in nanobubbles on elastase was also tested as shown in Fig. 5B. From the experimental results, SPC was considered to be fairly non-cytotoxic ($p > 0.05$) according to elastase release. FE showed a slight and non-significant increase ($p > 0.05$) in elastase compared to the control group. The same as when it was incorporated in nanobubbles, SA in the free form significantly decreased ($p < 0.05$) elastase release.

4. Discussion

As with any new technology or science, developing a framework for selecting appropriate nanomaterials and making decisions with uncertainty and incomplete information are current challenges in the field of nanotechnology. Understanding nanomaterial toxicity requires multiple sets of information because of the complexity of nanoparticles and the limited database of relevant experimental studies (Linkov et al., 2008). Lipid microbubbles/nanobubbles can be retained within the circulation of inflamed tissue because of their attachment to neutrophils adherent to venular walls (Lindner et al., 2000). Different cellular responses and functions were investigated in this work. Moreover, the physicochemical properties of nanobubbles were characterized to correlate them with the toxicity to neutrophils.

We developed eight nanobubble formulations with different additives and/or percentages (Table 1). A higher perfluoropentane ratio (P2) and oil ratio (P3) led to increases in the size of the particles. The monolayer shell composed of SPC and cholesterol imparts stability to the nanobubbles. Since the contents of emulsifiers in these systems were the same, the increase in diameter may have resulted from impoverishment of the emulsifiers at the interface with an increasing volume of the dispersed oil and perfluorocarbon phases (Hwang et al., 2009). Another reason for the greater size of the systems with a greater oil percentage is that the viscosity of the coconut oil should be higher than those of the other ingredients used in these nanobubbles. It was established that increasing the viscosity of oil-in-water or perfluorocarbon emulsions leads to an

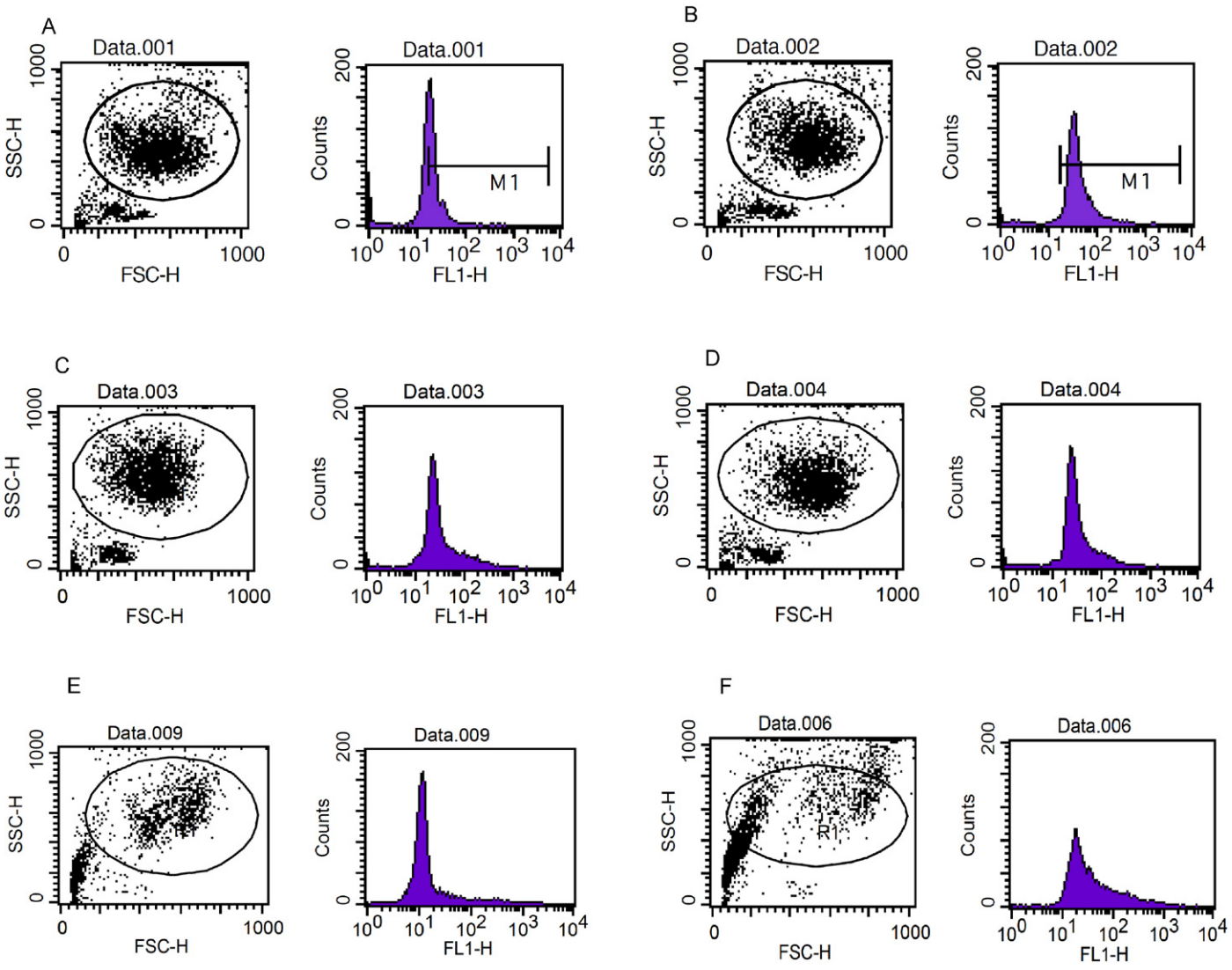

Fig. 2. Flow cytometric diagrams of dihydrorhodamine 123 (DHR123) oxidation in fMLP-activated neutrophils after treatment with various nanobubble formulations. (A) Basal level without fMLP activation; (B) control group with fMLP activation but no nanobubble treatment; (C) P1 treatment; (D) P4 treatment; (E) P6 treatment; and (F) P7 treatment.

increase in particle diameter (Fang et al., 2009). The insufficiency of the emulsifiers and the viscosity of the oil of the systems with a greater oil percentage may have contributed to the instability of this formulation as shown by the PDI profiles.

Phosphatidylcholine is used as a part of the shell of nanobubbles for commercial products such as Imagent®, Definity, and SonoLysis (Narayan et al., 2001; Zhao et al., 2005; Pancholi et al., 2008). A decrease in the SPC percentage in nanobubbles increased their size, which was due to an insufficiency of SPC for stabilizing the system. An increase in the SPC percentage also increased the particle size, although the increment was lower than that of the formulation with less SPC. This may have been due to the spacious lipophilic chains of SPC, which has two fatty acid chains. The additional SPC located in the oil/water interface increased the surface area of each particle (Wang et al., 2006), thus increasing its size. Abundant surface electrostatic charges can also disturb the formulations and stability of the monolayer shell by causing lateral electrostatic repulsion within the monolayer (Borden et al., 2007). A similar result was observed for nanobubbles with greater SA (P8). However, incorporation of the cationic surfactant at a feasible content (0.6%) reduced the particle size (P6 and P7). This was due to the emulsifier activities of FE and SA in the systems.

The zeta potential is an important characteristic of nanobubbles and provides information on the colloidal dispersion stability, biological characteristics, and cell interactions (Tinkov et al., 2009). The negative charge of nanobubbles was predominantly provided by SPC. The anionic fractions, such as phosphatidylserine and phosphatidylglycerol, in SPC with 80% phosphatidylcholine (Phospholipon® 80H) are responsible for the negative charge. Phosphatidylcholine itself exhibits no net charge (Fang et al., 2007). A higher negative charge was observed for the formulation with a greater oil content (P3). The larger surface area of the larger particles may explain this phenomenon. A further increase in SPC concentrations did not change the zeta potential, which may have been due to saturation of SPC molecules in the interface. The addition of cationic surfactants reversed the negative charge of the nanobubble surface to a positive charge. The cationic nanocarriers were found to efficiently target the brain because of binding to the paracellular area of the blood–brain barrier (BBB), which is beneficial for brain imaging with ultrasound (Unger et al., 2004). Regarding the long-term stability, the zeta potential of a stable colloidal system should not fall below ±30 mV (Buszello et al., 2000). In that case, the nanobubbles developed here would likely show acceptable stability in normal use.

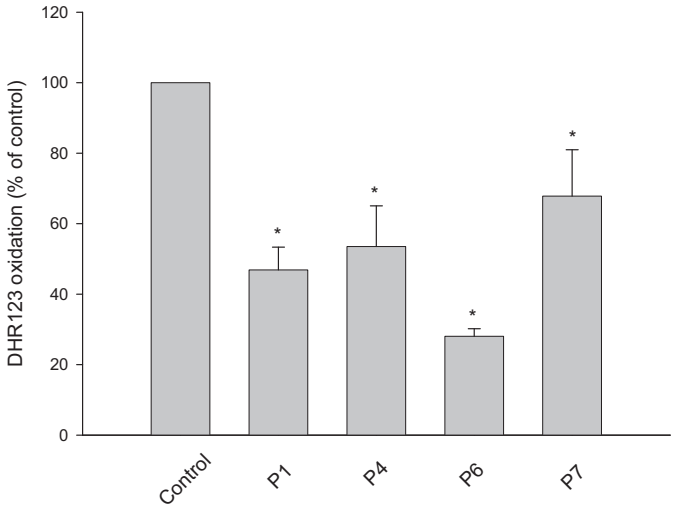

Fig. 3. Effects of various nanobubble formulations on DHR123 oxidation in fMLP-activated neutrophils determined by flow cytometry. All data are expressed as the mean ± S.E.M. (n = 4–8). *The value was lower (p < 0.05) compared to the control. DHR123, dihydrorhodamine 123.

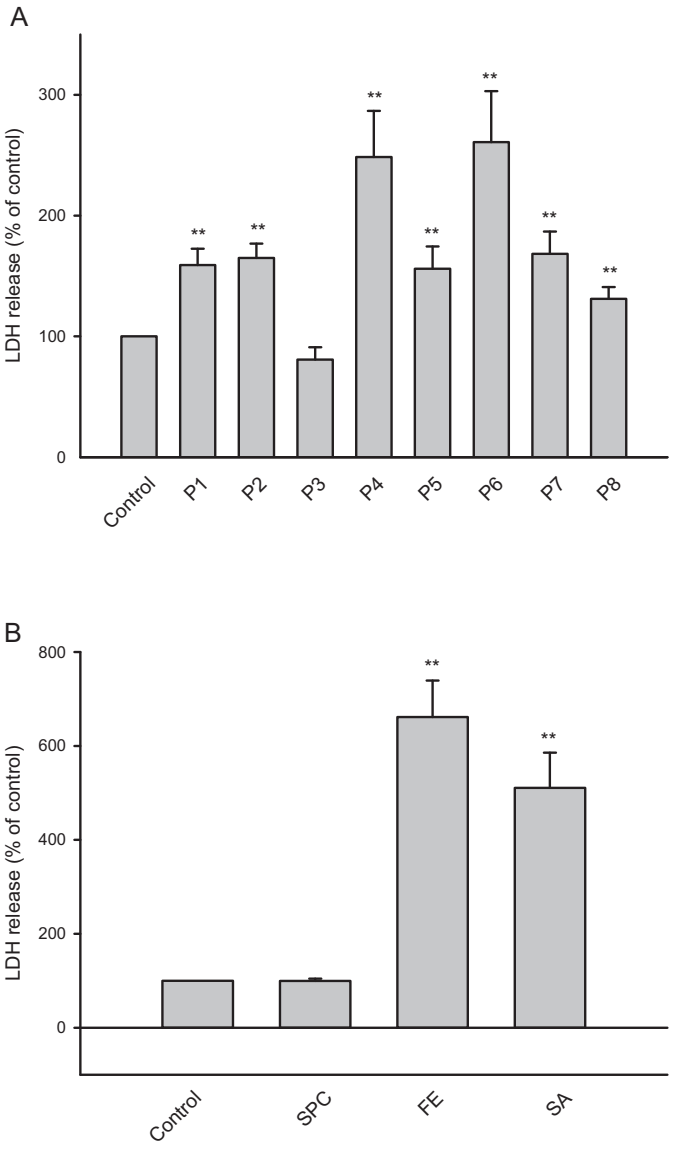

Fig. 4. Effects of various nanobubble formulations (A) and emulsifiers (B) on LDH release in fMLP-activated neutrophils. All data are expressed as the mean ± S.E.M. (n = 4–8). **The value was higher (p < 0.05) compared to the control. SPC, soybean phosphatidylcholine; FE, Forestall®; SA, stearylamine; LDH, lactate dehydrogenase.

Neutrophils are partitioned in the blood between a circulating pool, present in large blood vessels and in the axial stream of small vessels, and a marginating pool. In the absence of inflammation, the marginating pool is comprised of granulocytes transiently arrested in narrow, mainly pulmonary, capillaries. Conversely, in inflamed organs, neutrophil traffic involves selectin- and integrin-dependent sequestration in capillaries and post-capillary venules (Witko-Sarsat et al., 2000). Soluble activating agents, including the chemoattractant peptide, fMLP, induce tyrosine phosphorylation in neutrophils that parallels activation of host defense responses in these cells (Fialkow et al., 2007). Incubation of cells with fMLP causes a significant increase in ROS and proteases secretion compared to non-activated cells. The prompt and vigorous formation of $O_2^{\bullet -}$ by NADPH oxidase in activated human neutrophils, so-called respiratory burst, is an important inflammatory response (Cowburn et al., 2008). In regions of inflammation, contrast agents such as microbubbles are captured and phagocytosed by stimulated neutrophils adherent to venular walls (Dayton et al., 2001; Jing et al., 2008). Cytotoxic effects can be caused by particles adhering to cell membranes and being internalized by cells (Müller et al., 1997). As cells are seen to readily internalize nanoparticles, the number of internalized nanoparticles is correlated to the cytotoxicity (Lewinski et al., 2008).

Respiratory bursts are a characteristic property of phagocytes. Oxidative stress is the result of an imbalance in homeostasis. $O_2^{\bullet -}$ formation is directly or indirectly linked to damage to or destruction of surrounding tissues (Hwang et al., 2010). Hence, $O_2^{\bullet -}$ production may play a role in nanobubble toxicity. The oxidant production in response to nanobubbles was characterized by the production of superoxide radicals. ROS, such as superoxides, hydrogen peroxide, hydroxyl, and other oxygen radicals, are capable of directly oxidizing DNA, proteins, and lipids. There is much evidence showing that nanoparticles increase ROS production in different types of cells (Huang et al., 2009; Shubayev et al., 2009; Wang et al., 2009). A previous study (Mainardes et al., 2009) also showed that nanoparticles activated neutrophils by producing more ROS, as detected by the oxidation of luminol. However, that was not the case in the present study. Superoxide anions were generally reduced to a certain level by incubating nanobubbles with human neutrophils. A possible reason is the reduction of cell viability, thus decreasing the total production of $O_2^{\bullet -}$.Several studies showed that some nanoparticles can induce oxidative stress, ultimately leading to cell death (Huang et al., 2009; Khatchadourian and Maysinger, 2009). This oxidative stress-induced death might not have occurred in this study since the intracellular ROS determined by flow cytometry also decreased by treatment with nanobubbles.

Cell viability can be evaluated by several cytotoxic assays including LDH (Dias et al., 2009). The amount of LDH released is proportional to the number of cells damaged or lysed (Haslam et al., 2000). We found that there was an inverse correlation between $O_2^{\bullet -}$ amounts and LDH release. It is well documented that LDH levels, as a marker of necrosis, in the cell medium are upregulated after cells are exposed to nanoparticles (Lin et al., 2006). The membrane damage can lead to cell death, which results in blockage of signal transduction and a subsequent reduction in ROS generation. It was our inference to explain the inverse relationship between ROS and LDH release. A previous study (Cury-Boaventura et al., 2006) proved that treatment with soybean oil emulsions reduced the number of neutrophils. The emulsions cause mitochondrial depolarization in circulating neutrophils. Fatty acids contained in the emulsions can

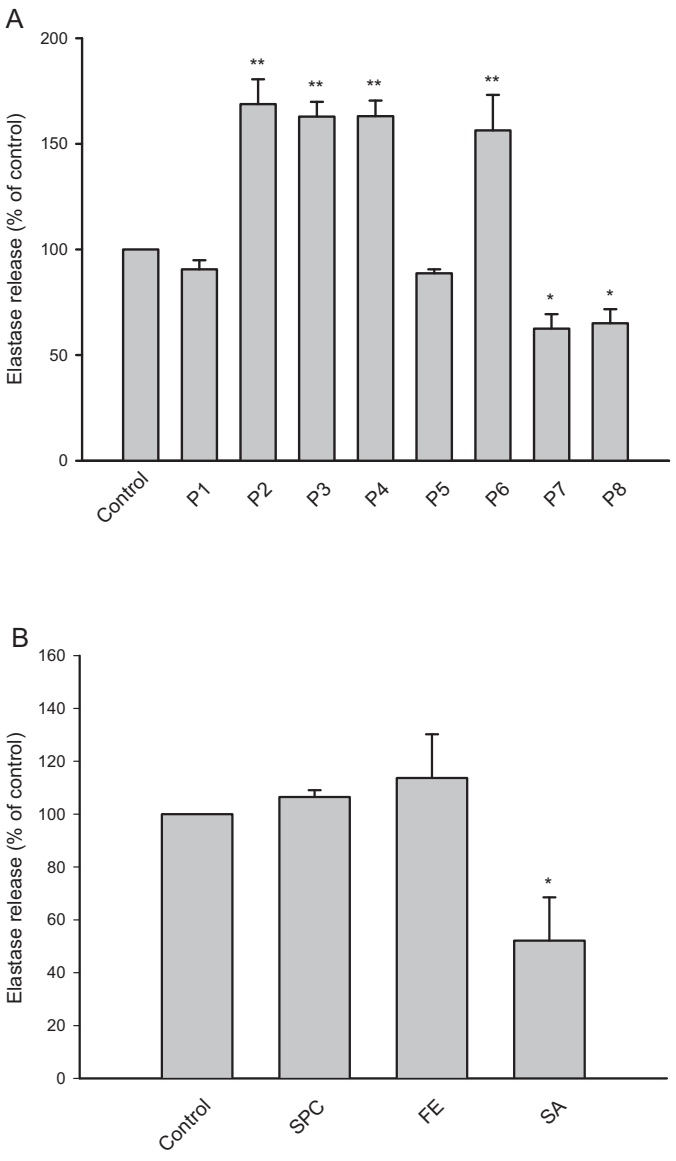

Fig. 5. Effects of various nanobubble formulations (A) and emulsifiers (B) on elastase release in fMLP-activated neutrophils. All data are expressed as the mean ± S.E.M. (n = 4–8). *The value was lower ($p < 0.05$) compared to the control; **The value was higher ($p < 0.05$) compared to the control. SPC, soybean phosphatidylcholine; FE, Forestall®; SA, stearylamine.

induce mitochondrial depolarization either directly or via changes in gene/protein expressions.

Both soybean oil and coconut oil are glycerides with fatty acids. The typical composition of soybean oil is mainly long-chain triglycerides (LCTs) including oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). Coconut oil is a composition of saturated fatty acids from medium-chain triglycerides (MCTs, C8:0∼C14:0) (Hung et al., 2006), which is used for preparing nanobubbles (Díaz-López et al., 2010). A previous study (Wanten et al., 2002) revealed that MCTs inhibited fMLP-induced neutrophil activation more potently than LCTs. Hence the oil composition in nanobubbles could be a risk factor for inducing neutrophil toxicity. However, nanobubbles with a higher oil loading (P3) were not cytotoxic to neutrophils by an oxygen-dependent pathway according to the $O_2^{\bullet-}$ and LDH assays. The large particle size may be the reason for the low toxicity. Mass is the most important parameter in toxic interactions, while area should be most important in cell surface binding (Ehrenberg et al., 2009). It was noted that the smaller the particle, the stronger the particles were and the greater specific surface area they had per unit mass; and this property makes nanoparticles very reactive in the cellular environment and promotes easier penetration into cells (Medina et al., 2007). The low cytotoxicity of the formulation with the higher oil content (P3) can possibly be attributed to a low uptake by neutrophils because it had the largest diameter among the nanobubbles developed. Another contribution to the toxicity of nanobubbles is the presence of perfluoropentane. Some commercial products such as EchoGen® have used perfluoropentane as the core material (Rapoport et al., 2007). Much of the function of nanoparticles is due to their core structure (Lewinski et al., 2008). fMLP-activated neutrophils incubated with perfluorocarbons exhibit diminished respiratory bursts and an attenuated inflammatory response (Fernandez et al., 2001). Perfluorochemicals can also induce the necrosis of neutrophils and reduce neutrophil counts in the circulation and lung tissues (Schoof et al., 2002; Qazi et al., 2009). This may explain the decreased $O_2^{\bullet-}$ and increased LDH with nanobubble treatment. Perfluorocarbons are capable of partitioning into cell membranes when the cells and liquid are brought into close proximity (Fernandez et al., 2001).

Nanoparticles can stimulate and/or suppress immune responses, and their compatibility with the immune system is largely determined by their surface chemistry (Dobrovolskaia and McNeil, 2007). The main component in the particulate surface of nanobubbles was SPC. The findings in this work indicate that the increase in SPC concentrations could trigger responses of $O_2^{\bullet-}$ and LDH to more-significant levels. Because of their shell surface properties, phospholipid-containing microbubbles such as Sonazoid® tend to be taken up by circulating and phagocytizing immune cells (Tinkov et al., 2009). Lindner et al. (2000) suggested that the presence of phospholipids in the microbubble shell resulted in greater accumulation of microbubbles within activated neutrophils. SPC can suppress $O_2^{\bullet-}$ release by activated neutrophils (Senda et al., 2008; Hartmann et al., 2009). The same phenomenon may exist for SPC that resides in nanobubbles. The choline constituent of SPC may participate in a wide range of responses, including interference with the mechanisms of neutrophil activation. SPC and choline metabolites have the ability to counteract intracellular ROS production. Our results from flow cytometry demonstrate this. ROS produced by activated neutrophil granulocytes promote the formation of oxidized phospholipids. The oxidized phospholipids specifically inhibit oxidative bursts by inhibiting the assembly of phagocyte oxidase complexes (Blüml et al., 2008). It was noted that free SPC utilized in this work did not show the ability to inhibit superoxide anions. The upregulated release of LDH by free SPC was also limited. This possibly indicates that SPC should be included in nanobubbles because of its ability to modulate cell responses.

The experimental results demonstrated that cationic surfactants modified oxygen-dependent pathways to a more-significant level compared to other additives of the nanobubbles. Cationic lipids or surfactants are often utilized to render a positive surface for nanobubbles such as FluoroGene® (Unger et al., 2004). FE- and SA-containing systems (P6 and P7) possessed smaller sizes than the other systems. As discussed above, the enhancing effects of nanomaterials on toxicology tend to be greater with smaller nanomaterials than with larger ones. On the other hand, nanobubbles with a higher SA content (P8) were larger (387 nm), resulting in lower LDH release compared to the other formulations with cationic surfactants. In addition to the influence of size, surface charge may play an important role in the neutrophil response. Nanoparticles with cationic surfaces were more toxic than anionic ones, and neutral surfaces were the most biocompatible (Goodman et al., 2004). This may be due to electrostatic interactions of cationic particles with negatively charged cell membranes (Schöler et al., 2001). This interaction ensures a high efficiency of intracellular

particle uptake. An increased SA percentage in nanobubbles (P8) rendered a slightly but significantly higher zeta potential than those of the other cationic surfactant-containing systems. However, this increment did not further modulate neutrophil responses. This is due to the saturation of binding sites and/or uptake and collection pathways (Ehrenberg et al., 2009). FE and SA with long alkyl chains may have increased the overall lipophilicity of the nanobubbles. When present on the surface of a particle, the increased lipophilicity has the capability to promote hydrophobic interactions that occur between the particles and neutrophils (Mainardes et al., 2009). The free FE and SA exhibited a critical manner of influencing the release of $O_2^{\bullet -}$ and LDH. This suggests that the cytotoxicity of nanobubbles was due to the presence of cationic surfactants. The free FE and SA even reduced $O_2^{\bullet -}$ production to a more-significant level compared to the encapsulated form in nanobubbles at an equivalent dose. The properties of the nanomaterials substantially differed from those of the bulk materials at the same composition. It is possible that binding of a surfactant to lipid nanoparticles can shield the surfactant's cytotoxicity (Müller et al., 1997). This behavior was quite different from that of SPC.

In addition to respiratory bursts which are an oxygen-dependent response, degranulation also plays a pivotal role in most neutrophil functions. Elastase is a major secreted product of stimulated neutrophils and a major contributor to destruction of tissues in inflammatory diseases (Pham, 2006; Hwang et al., 2010). Exposure of neutrophils to fMLP resulted in the rapid mobilization of azurophil granules to the cell surfaces and the release of granule proteins. The results of elastase release indicated that some nanobubble formulations (P2, P3, P4, and P6) activated neutrophils by non-oxidized pathways. Upon ingestion of foreign particles, glycolysis is markedly accelerated, leading to the release of proteases from granules (Hirsch and Cohn, 1960). According to the elastase profiles, the further addition of perfluorocarbon, oil, and SPC to the nanobubbles may have accelerated this effect of releasing most of the elastase. Among the ingredients, the ability of SPC to enhance elastase release was proven by Tronchère et al. (1995). The fMLP can trigger phosphatidylcholine breakdown into phosphatidic acid. As a lipid, phosphatidic acid remains associated with the membrane where it participates in activation of elastase release (Tamura et al., 1998). SPC in nanobubbles should achieve a sufficient concentration of 4.5% to trigger the activation of neutrophils by non-oxygen mechanisms. Lower SPC concentrations did not induce a further release of elastase. The comparable elastase levels between the control group and free SPC treatment at a concentration of 3.5% confirmed this.

Degranulation may imply the enhancement and activation of neutrophilic inflammation, although the nanobubbles may have inhibited respiratory bursts ($O_2^{\bullet -}$) to attenuate the inflammatory response. This demonstrates that nanobubbles had different functions for the oxygen-dependent and -independent pathways. Another possibility for the enhanced elastase release is membrane disruption by the nanobubbles according to the LDH results. The granules inside the cells were released to the extracellular medium after this disruption. Cowburn et al. (2008) suggested that neutrophil necrosis contributes far more to elastase release than does degranulation. Interestingly, SA in both the free form and incorporated in the nanobubbles inhibited elastase release from fMLP-stimulated neutrophils. SA might disrupt the cell membrane in a different manner than FE and the other additives in the nanobubbles. It was inferred that SA can diminish cell viability and subsequent neutrophil degranulation. A previous study (Macdowell and Peters, 2007) demonstrated that neutrophil numbers in asthma correlate well with markers of neutrophil degranulation. Further studies are necessary to explore the actual mechanisms of SA. Elastase can be expressed at the plasma membrane of activated neutrophils, which appear to be bound via an ionic interaction (Owen et al., 1995). Different additives to the nanobubbles may affect this ionic interaction.

The activation of neutrophils has to be carefully controlled to balance the desired activity against invading pathogens, while avoiding overwhelming activation leading to host tissue damage or insufficient activation leading to severe infection. According to the experimental results in this study, the nanobubbles affected the homeostasis of the immune system. The nanobubbles generally decreased extracellular and intracellular free radicals. The increase in LDH by nanobubbles indicated disruption of cell membranes and/or cell death. Most nanobubbles produced a higher level of elastase release. Among all nanobubbles, the systems with cationic surfactants showed the most significant response to activate neutrophils, especially FE-containing nanobubbles. The flow cytometric profiles also revealed significant cell death and cell debris of neutrophils treated with cationic surfactant-incorporating systems. Further in-depth investigations are required to explore the detailed mechanisms of neutrophil toxicity by nanobubbles.

5. Conclusions

The establishment of principles and test methods to ensure the safe manufacture and use of nanomaterials in the marketplace and in clinical settings is urgently required and achievable. Development of novel nanoparticles for pharmacology, therapeutics, and diagnostics must proceed in tandem with assessments of any toxicological side effects of these particles. The toxicity and response of nanobubbles to stimulated neutrophils were examined by detecting $O_2^{\bullet -}$, LDH, and elastase. Nanobubbles have great potential to induce a variety of different cell functions. By changing the compositions of the nanobubble core and shell, the avidity of nanobubbles for activated neutrophils changed. In general, nanobubbles with a greater oil content in the shell displayed minimum toxic effects toward human neutrophils according to the $O_2^{\bullet -}$ production and LDH profiles. A high ratio of SPC in the nanobubbles caused more-severe cell membrane damage and degranulation. Nanobubbles should be used with caution with cationic surfactants, since they exhibited the greatest modulation of neutrophil responses, although cationic nanoparticles are useful for drug targeting and brain imaging. It is suggested that nanobubbles can affect the homeostasis and immunological responses of activated neutrophils.

Conflict of interest

None.

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