Surface charge determines the lung inflammogenicity: A study with polystyrene nanoparticles

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Abstract
Surface functionalization is a routine process to improve the behavior of nanoparticles (NPs), but the induced surface properties, such as surface charge, can produce differential toxicity profiles. Here, we synthesized a library of covalently functionalized fluorescent polymeric NPs (F-PLNPs) to evaluate the role of surface charge on the acute inflammation and the localization in the lung. Guanidinium-, acetylated-, zwitterionic-, hydroxylated-, PEGylated-, carboxylated- and sulfated-F-PLNPs were synthesized from aminated-F-PLNP. The primary particle sizes were identical, but the hydrodynamic sizes ranged from 210 to 345 nm. Following surface functionalization, the F-PLNPs showed diverse zeta potentials from −41.2 to 31.0 mV, and each F-PLNP showed a single, narrow peak. Pharyngeal aspiration with these eight types of F-PLNPs into rats produced diverse acute lung inflammation, with zeta potentials of the F-PLNPs showing excellent correlation with acute pulmonary inflammation parameters including the percentage of polymorphonuclear leukocytes ($R^2 = 0.90$, $p < 0.0001$) and the levels of interleukin-1β ($R^2 = 0.83$, $p < 0.0001$) and of cytokine-induced neutrophil chemo-attractant-3 ($R^2 = 0.86$, $p < 0.0001$). These results imply that surface charge is a key factor influencing lung inflammation by functionalized polymeric NPs, which further confirms and extends the surface charge paradigm that we reported for pristine metal oxide NPs. This demonstrates that the surface charge paradigm is a valuable tool to predict the toxicity of NPs.

Introduction
Tailoring the surface of nanoparticles (NPs) by functionalization is a routine process to improve the behavior of NPs for nanomedicine application (Mout et al., 2012). Functionalization can change the surface of NPs, which is the main responder to biological systems such as molecules, cells and tissues (Nel et al., 2009). The representative physicochemical properties tunable by simple surface modification are surface charge, spacer length, hydrophobicity, aggregation propensity and so on (Cho et al., 2014; Li et al., 2013). These tunable parameters in turn influence the stability, in vitro and in vivo kinetics, and genotoxicity of NPs, as well as inflammation and even immunological response (Podila & Brown, 2013; Zhao & Castranova, 2011; Zhu et al., 2013). Therefore, surface modification is a good strategy to tune the profiles of NPs especially for biomedical applications.

One aspect of nanotoxicology, the surface charge, which is modifiable by surface functionalization, is a key factor of NP toxicity. Indeed, surface charge of NP is related to destabilization of membrane potential and destruction of the cellular membrane causing an inflammation cascade. The surface charge of metal/metal oxide NPs showed excellent correlation with hemolytic potential and lung inflammogenicity, although there was a threshold zeta potential triggering harmful effects (Cho et al., 2012). Here, eight types of functionalized polystyrene NPs, all having an identical core, showed that zeta potential positively correlated with hemolytic potential (Cho et al., 2014). Positively charged NPs are also able to depolarize the membrane potential and increase Ca$^{2+}$ influx in the cytoplasm, which can ultimately cause cell death (Brown et al., 2001; Goodman et al., 2004). Surface functionalization could also modulate genotoxicity by direct (i.e. DNA adduct formation) and indirect mechanisms (i.e. reactive oxygen species production) (Chompoosor et al., 2010; Donaldson et al., 2010). However, studies that look at the impact of surface charge on toxicity are few, with most studies performed using a limited number of functionalizations (i.e. positive, neutral and negative).

To evaluate the role of surface charge on the toxicity and inflammogenicity of NPs in the lung environment, well-engineered model NPs that are non-cytotoxic, chemically stable...
and easy to modify are preferred. Ideally, these model NPs have different surface charges due to different functionalization with an identical particle core. To minimize the influence on physicochemical properties, the particles should be prepared in a big batch. In addition, inherent fluorescence provides important information about the cellular and tissue distribution patterns. Additionally, the selection of the dose for comparison has to be considered because toxic substances are harmless in low doses and harmless substances are toxic at high doses (Donaldson et al., 2013; Duffin et al., 2007). The selection of the critical point for discriminating inflammogenic potential is essential to evaluate the impact of surface charge. In this study, we synthesized one batch of fluorescein-conjugated aminomethyl-polystyrene NPs (NH2F-PLNP), followed by conjugation with 7 kinds of functional groups: guanidinium (GDPLNP), acetyl (ACF-PLNP), zwitterionic (ZF-PLNP), hydroxyl (OHF-PLNP), PEG (PEGF-PLNP), carboxyl (COOHF-PLNP) and sulfonic acid (SO3HF-PLNP). These model NPs were aspirated into rats to evaluate the role of surface charge or of surface functionalization of NPs on pulmonary inflammogenicity and on cellular- or tissue-localization.

Methods

Synthesis of polystyrene NPs

Fluorescein-conjugated aminomethyl-polystyrene NPs (NH2F-PLNPs) were synthesized as core NPs, and seven different functional groups (guanidinium, acetyl, zwitterionic, hydroxyl, PEG, carboxyl and sulfonic acid) were conjugated to provide F-PLNPs having diverse surface charges. Because the methods for synthesis of F-PLNP overlapped with our previous literature (Thielbeer et al., 2011a,b), the detailed methods are presented in the Supplementary Material.

Physicochemical characterization of NPs

The qualitative and quantitative content of free amino groups was determined with the Kaiser test as reported previously (Gude et al., 2002; Kaiser et al., 1970). The sample was prepared by washing an emulsion of NPs (3 mg) with MeOH (2 × 500 µl) and then removing the supernatant. Formation of a deep blue color indicated the presence of free primary amines. The solid content of the NPs was determined by drying the NP suspension (100 µl; n = 3) under vacuum (<20 bar) at 40°C overnight and weighing. To measure the primary diameter and morphology of NPs, a solution of a 10-µl particle suspension in water (200 µg, solid content 20 mg/ml) was dried onto carbon-coated stubs under vacuum (<20 bar) at 40°C overnight and was gold coated by sputtering (~20 nm layer) before analysis on a Phillips XL30CP scanning electron microscope (Eindhoven, The Netherlands). The hydrodynamic diameter and zeta potential of the NPs were determined with a Malvern Zetasizer Nano-ZS (Malvern, Malvern Hills, UK) according to the manufacturer’s recommendations. A suspension of NPs (20 µg, 1 µl, solid content 20 mg/ml) was diluted in 10% PBS (pH = 7.4, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na2HPO4 and 0.2 g/l KH2PO4) in distilled water (DW, 1 ml), vortexed, briefly sonicated with a bath sonicator (Saehan-Sonic, Seoul, Korea) and transferred into either a 4-ml polystyrene cuvette (FB55143, Fisher Scientific, Loughborough, UK) or a 1-ml clear zeta potential cuvette (DTS1060, Malvern). The electrophoretic mobility of the sample was measured and converted into the zeta potential by applying the Henry equation. The data were collected and analyzed with the Zetasizer software (v7.03 Malvern), producing histograms for the NP size as a number distribution or diagrams for the zeta potential as a distribution versus total counts. The level of endotoxin was measured in NPs suspensions in 0.9% sterile saline at 2 mg/ml using an endpoint chromogenic limulus amebocyte lysate assay (Cambrex, Walkersville, MD). The detection limit was 0.1–1.0 EU/ml.

Animal handing and protocols

Specific pathogen-free female Wistar rats aged at 6 weeks were purchased from Samtako Ltd. (Osan, Korea) and were acclimated for 7 days before experiment. Rats were maintained in an animal room at a controlled temperature (21–24°C), humidity (45–60%) and light cycle (12 h light/dark). Water and diet (LabDiet 5002, PMI nutrition, Richmond, IN) were provided ad libitum. Animal experiment protocols were reviewed and were approved by the Animal Care and Use Committee of Dong-A University, Korea.

Preparation of NP for pharyngeal aspiration

To discriminate the role of surface functionalization, dispersion medium, such as serum and surfactant, was excluded because corona formation on the surface of the NP can change surface properties (Cho et al., 2014). NP stock solutions were concentrated at a range of 18–24 mg/ml in DW. To destruct agglomeration, stock solutions were sonicated for 10 min with a bath sonicator (Saehan-Sonic). Then working concentrations were prepared with sterile 0.9% saline and were sonicated again for 5–10 min using a bath sonicator (Saehan-Sonic) just before pharyngeal aspiration.

Pharyngeal aspiration and bronchoalveolar lavage fluid analysis

The pharyngeal aspiration was performed according to the previously described method with slight modification (Sarlo et al., 2009). Briefly, rats were fasted overnight to exclude possible aspiration pneumonia and were anesthetized by intraperitoneal injection of Tiletamine-Zolazepam (20 mg/kg body weight) plus Xylazine (2 mg/kg body weight). Then, the tongue was gently held in full extension while 250 µl NP suspension was loaded onto the base of the tongue. The tongue was held extended, and the nose was covered until the NP suspension was fully aspirated to stimulate gas reflex. Rats were recovered fully, and diet was supplied ad libitum. After 24-h post-aspiration, rats were sacrificed by intraperitoneal injection of Tiletamine-Zolazepam (40 mg/kg) plus Xylazine (5 mg/kg), followed by exsanguination via the abdominal vein. Then, the bronchoalveolar lavage (BAL) fluid was collected and prepared according to the previously described method (Cho et al., 2010). Briefly, the lungs were lavaged 4 times with 8 ml of ice-cold sterile saline. The first lavage was kept separately for lactate dehydrogenase (LDH), total protein and cytokine measurements. Then, the subsequent lavages were pooled for cell counts, and 4 × 105 cells were centrifuged onto the glass slides. The cytospin slides were fixed with methanol and stained with Diff-Quik (Thermo Scientific, Waltham, MA). More than 300 cells were counted for differential cell count using a light microscope (Nikon Instruments Inc., Melville, NY).

Dose-range finding study of F-PLNPs

To discriminate the role of surface functionalization on the toxicity of NPs, the selection of the critical point of dose through a dose-range finding study is essential. Because all the functionalized F-PLNPs had the same primary size and surface area, NPs were treated at a mass metric. To evaluate the dose–response, each NP suspension was aspirated at 100 µg (29.6 cm2) per rat, and acute lung inflammogenicity was evaluated by counting cells in the BAL fluid. Then, the significantly inflammogenic NPs (NH2F-PLNP, GD-F-PLNP and ZF-F-PLNP)
were aspirated at 20 μg (5.9 cm²) per rat. Likewise, the non-inflammogenic NPs (PEG-F-PLNP, OH-F-PLNP, AC-F-PLNP, COOH-F-PLNP and SO3H-F-PLNP) were aspirated at 500 μg (148.0 cm²) per rat. As a result, inflammogenic NPs at 100 μg/rat showed no significant inflammation (<5% polymorphonuclear leukocytes (PMN)) at 20 μg/rat, and non-inflammogenic NPs at 100 μg/rat showed significant inflammation (43–52% PMN) at 500 μg/rat. Therefore, the critical point of dose for comparison was selected as 100 μg (29.6 cm²)/rat.

**Detection of fluorescent polystyrene NPs in BAL cells and tissues**

The NPs are expected to have different behaviors such as phagocytic efficacy, cellular uptake and tissue localization owing to their diverse surface functionalities. Therefore, we evaluated the cellular localization of fluorescent polystyrene NPs in the cytosin slide and lung tissues. BAL cells were attached onto glass slides by cytosin and fixed with methanol. Before observation using a fluorescence microscope (Leica DM 5500 B; Leica Microsystems, Wetzlar, Germany), cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and images were acquired using Leica Application Suite, Advanced Fluorescence 1.8 software (Leica Microsystems). Lung tissues were fixed with 10% neutral-buffered formalin, and routine histological processes, including trimming, processing and embedding, were performed. Tissue blocks then were sectioned by 3 μm, de-waxed with xylene (Sigma–Aldrich, St. Louis, MO) and hydrated with series of ethanol. Slides were stained with DAPI and were observed under a fluorescence microscope (Leica Microsystems).

**Measurement of LDH and total proteins in the BAL fluid**

The levels of LDH in the BAL fluid were measured using the Cytoxicity Detection Kit (Roche Diagnostics, Seoul, Korea). The levels of total protein in the BAL fluid were measured using the bicinchoninic acid assay kit (Thermo Fisher Scientific, Rockford, IL). Undiluted BAL fluid samples were used for LDH and total protein assays according to the instruction manuals.

**Measurement of pro-inflammatory cytokines by enzyme-linked immunosorbent assay**

To evaluate the pro-inflammatory cytokine expression in the BAL fluid, Duoset enzyme-linked immunosorbent assay kits for rat interleukin-1β (IL-1β) and cytokine-induced neutrophil chemotactant-3 (CINC-3) were purchased from the R&D systems (Minneapolis, MN). All the assays were performed according to the instruction manuals using non-diluted BAL fluids.

**Statistical analysis**

Data were analyzed with GraphPad Prism Software (Version 5; GraphPad Software Inc., La Jolla, CA). One-way analysis of variance with post hoc Tukey’s pairwise comparisons was applied to compare each treatment group. The correlation of zeta potentials of F-PLNPs and parameters for pulmonary inflammation was evaluated by Pearson correlation test, and significance was evaluated by linear fit model. We considered p < 0.05 to be statistically significant.

**Results**

**Surface modification of F-PLNPs**

The NPs in this study were synthesized with high efficiency and were characterized during each step of the synthetic pathway by Kaiser test and zeta potential, which demonstrated surface modifications (Thielbeer et al., 2011b). The functional groups were derived from glutaric anhydride, 4-sulfobenzoic acid and suitably protected amino acids that represented a wide range of zeta potentials (–41.2 to 31.0 mV) with acidic, neutral and basic charges on the NP surface (Figure 1). The zeta potential of the starting aminomethyl NPs (NH2-F-PLNP) was +19.8 mV and changed to +31.0 mV during conversion to the more basic guanidinium group. A strongly negative zeta potential was found after conversion to carboxylic acid (–28.0 mV), and, as expected, even stronger negative values were obtained after attachment of the sulfonic acid (–41.2 mV).

**Physicochemical properties of F-PLNPs**

The morphology and primary particle size of NH2-F-PLNP analyzed by scanning electron microscopy showed a uniform spherical shape with a diameter of 193.8 ± 18.5 nm (Supplementary Figure 1). The hydrodynamic size of NH2-F-PLNP was 215.5 nm, which is the smallest among NPs, while the size of PEG-F-PLNP was 344.5 nm (Table 1). The polydispersity index (PDI) was <0.2 for all the F-PLNPs, which means that all the F-PLNPs had narrow size distribution. The endotoxin levels of all the F-PLNPs were below the detection limit (0.1 EU/ml).

**Acute lung inflammogenicity of F-PLNPs**

Based on the dose-range finding study, we selected the critical point of dose as 100 μg/rat, which can discriminate the role of functionalization. When F-PLNPs were treated at 100 μg/rat, the percent of PMNs, a marker for acute lung inflammation, was significantly increased by GD-F-PLNP and NH2-F-PLNP, while those of PEG-F-PLNP, ZI-F-PLNP, OH-F-PLNP, AC-F-PLNP, COOH-F-PLNP and SO3H-F-PLNP showed no statistical significance compared to vehicle control (Figure 2). Although treatment of PEG-F-PLNP, ZI-F-PLNP, OH-F-PLNP and AC-F-PLNP did not reach statistical significance, the percent PMN in those groups showed >10%.

**Levels of LDH and total protein in the BAL fluid**

Levels of LDH, a marker of cell death and total protein, a marker of vascular permeability, showed no significant differences between each treatment (Figure 3).

**Pro-inflammatory cytokine expression**

Levels of IL-1β were increased significantly by treatment with GD-F-PLNP, NH2-F-PLNP, ZI-F-PLNP and OH-F-PLNP compared...
with vehicle control (Figure 4A). Levels of CINC-3 showed significant increases by treatment with GD-F-PLNP, NH2-F-PLNP, PEG-F-PLNP and ZI-F-PLNP, while others showed no significant differences compared with vehicle control (Figure 4B).

Table 1. Physicochemical characterization of the F-PLNPs.

| NPs Structure | SEM (nm) | DLS (nm) | PDI    | Zeta potential (mV) |
|---------------|----------|----------|--------|---------------------|
| GD-F-PLNP     | 218 ± 27 | 242.9 ± 17 | 0.144 | 31.0 ± 1.0          |
| NH2-F-PLNP    | 194 ± 18 | 215.5 ± 24 | 0.093 | 19.8 ± 1.6          |
| PEG-F-PLNP    | 320 ± 46 | 344.5 ± 33 | 0.197 | −10.3 ± 0.8         |
| ZI-F-PLNP     | 216 ± 25 | 250.7 ± 27 | 0.129 | −15.0 ± 1.2         |
| OH-F-PLNP     | 224 ± 20 | 260.7 ± 24 | 0.097 | −17.9 ± 1.4         |
| AC-F-PLNP     | 209 ± 9  | 227.3 ± 15 | 0.037 | −20.3 ± 1.6         |
| COOH-F-PLNP   | 214 ± 15 | 228.0 ± 7  | 0.064 | −28.0 ± 2.2         |
| SO3H-F-PLNP   | 220 ± 11 | 230.4 ± 13 | 0.053 | −41.2 ± 2.4         |

DLS, dynamic light scattering; PDI, polydispersity; SEM, scanning electron microscopy. Data represent the mean ± SD of n = 3 for DLS and n = 10 for SEM.

Cellular localization of F-PLNPs

Cellular localization of F-PLNPs in the BAL cells was evaluated by fluorescence microscopy. Although some F-PLNPs were scattered outside of cells, most of the F-PLNPs generally were engulfed by alveolar macrophages and some neutrophils (Figure 5). However, there were no differences in the pattern of localization between each treatment group (Figure 5).

Tissue localization of fluorescent F-PLNPs

In the lung tissue, most of the NPs were localized into the alveoli, while airways, including bronchioles, had few F-PLNPs (Figure 6). Consistent with the BAL slides, F-PLNPs were phagocytosed by alveolar macrophage-like cells, but some F-PLNPs were still lined on the surface of alveolar epithelium. There were no observable differences in distribution patterns of F-PLNPs between functional groups (Supplementary Figure 2).

Correlation between surface charge and acute lung inflammogenicity

To evaluate the role of surface charge or of surface functionalization on the acute lung inflammogenicity of F-PLNPs, the Pearson correlation test and the linear fit model were applied between physicochemical properties by F-PLNPs and parameters for acute lung inflammation. Because all the F-PLNPs had the same composition, primary size and treatment dose, zeta potentials of F-PLNPs can be plotted solely against the acute lung inflammation parameters such as %PMNs, LDH, total proteins and pro-inflammatory cytokines. The zeta potential of F-PLNPs showed a positive correlation against the %PMN in the BAL (Figure 7). Zeta potentials of F-PLNPs showed good correlation with the
%PMN in the BAL ($R^2 = 0.90, p < 0.0001$), IL-1β ($R^2 = 0.83, p < 0.0001$) and CINC-3 ($R^2 = 0.86, p < 0.0001$).

**Discussion**

To investigate the role of surface charge or of surface functionalization on acute lung inflammogenicity and on localization in cells or tissues, aminomethyl-functionalized fluorescent polystyrene NPs (NH$_2$F-PLNPs) were prepared and subsequently modified with various functional groups. The F-PLNPs were synthesized by an emulsifier-free emulsion polymerization of the monomers styrene, aminomethyl-functionalized styrene, a polymerizable fluorescein derivative and the cross-linker divinylbenzene to give uniform fluorescent NPs, avoiding the need for additional steps of fluorophore labeling (Thielbeer et al., 2011a).

Fluorescent NPs synthesized by this approach generate a truly homogeneous distribution of fluorescence, while giving NPs with a high number of available surface functionalities and long-term fluorescence stability, which are highly desirable for studying NPs in a biological environment. Furthermore, the NPs are functionalized with aminomethyl groups for subsequent transformations into various functionalities in two reaction steps. Hydrodynamic sizes of the F-PLNPs indicated that they are slightly agglomerated. PEG-F-PLNP showed a slightly larger size than the others because of the hydrophilic PEG layer can be measurable by dynamic light scattering.

To evaluate the correlation between surface charge and acute lung inflammogenicity, selection of dose at the steepest section, reflecting the maximum responses per unit dose, is critical (Rushton et al., 2010). The highest dose of some F-PLNPs at...
500 μg/rat caused inflammation near the saturation phase, but the dose at 20 μg/rat did not show inflammation for any F-PLNPs. Therefore, 100 μg/rat was selected as the critical dose for comparison between F-PLNPs lung inflammogenicity properties.

Aspirated F-PLNPs were well-distributed into the lung, which was confirmed by fluorescence imaging in the BAL cells and lung tissues. Interestingly, there were no differences in the pattern of cellular localization between each functional group. This might be because of the protein corona by the lung surfactant that masks the surface properties (Kim et al., 2014; Wang et al., 2013b). However, when NPs are localized inside of phagolysosomes, the enzymatic degradation and acidic pH in the phagolysosomes causes unmasking of the surface properties and re-establishes the zeta potential of NP (Wallace et al., 1992; Wang et al., 2013b). Unmasking of the physicochemical properties in the phagolysosomes can damage the lysosomal membrane resulting in an inflammation cascade (Wang et al., 2013b). In our previous study, incubation of phospholipase A₂ and proteinase K, a set of

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Figure 5. Observation of F-PLNPs in the BAL cells at 24 h after pharyngeal aspiration of F-PLNPs into rats. Nuclei stained by DAPI staining show blue fluorescence, and F-PLNPs show green fluorescence. Note that most of F-PLNPs are engulfed by alveolar macrophages and there are no differences in the pattern of localization between each treatment group.

Figure 6. Representative fluorescent images of lung tissues at 24 h after pharyngeal aspiration of F-PLNPs. (B) Representative inflammatory lung tissues by cOO-F-PLNP and (C) representative non-inflammatory lung tissues by cSO3H-F-PLNP. Nuclei stained by DAPI staining show blue fluorescence, and F-PLNPs show green fluorescence. Fluorescent images for all the F-PLNPs are presented in Supplementary Figure 2.
lyosomal enzymes, with functionalized-polystyrene NPs demonstrated the inability to restore the surface charge (Cho et al., 2014). Although this limited set of enzymes and environment for this reaction failed to achieve restoration of surface charge, the active environment in the lung tissues can produce restoration of surface charge and destabilization of the lysosomal membrane. Similarly, several studies showed that cationic NPs are more cytotoxic in vitro by lysosomal damage than neutral or anionic NPs (Asati et al., 2010; Nagy et al., 2012; Nel et al., 2009; Wang et al., 2013a). Intratracheal instillation of 50 or 200 nm sized plain or aminated PLNPs at 30 \( \mu \)g/rat showed no differences on neutrophils influx within each group, but this might be because of very low-dose administration (Brown et al., 2014).

Aspiration of F-PLNPs showed differential inflammogenicity by their functional groups. Among inflammation markers, the percentage of PMN and pro-inflammatory cytokines showed significant changes, while other types of cells, LDH and total proteins did not show any significant changes. This might be due to the inflammation produced by F-PLNPs is not as cytotoxic as NPs, such as ZnO and CuO (Cho et al., 2010), and because we selected the optimal dose at the steepest section in the dose–response curve. Zeta potential of F-PLNPs showed excellent correlation with several acute inflammation markers, such as percentage of PMN and levels of IL-1\( \beta \) and CINC-3, in the BAL fluid. There are several physicochemical factors that can influence the acute inflammation in vivo (i.e. shape, size, surface area, charge, composition and dissolution rate). When high aspect ratio nanomaterials were injected into the pleural space, the length of nanomaterials showed clear response with the acute pleural inflammation, and there was a threshold length triggering pleural inflammation (Poland et al., 2012; Schinwald et al., 2012). Intratracheal instillation of low-toxicity, low-solubility NPs at the same mass dose showed that smaller sizes of NPs produce higher inflammatory responses, but this size effect disappeared when inflammatory responses were correlated with the surface area dose (Duffin et al., 2007; Ober dorster et al., 2000). Surface charge measured by zeta potential showed significant correlation with hemolytic potential and lung inflammation when low-solubility pristine metal oxide NPs were instilled into the lungs of rats (Cho et al., 2012). The pulmonary inflammogenicity of high-solubility NPs can be predicted by the toxicity of their ions (Cho et al., 2012; Zhang et al., 2012). Therefore, the excellent correlation between surface charge and lung inflammation shown with the F-PLNPs in this study confirmed that surface charge plays an important role in the lung inflammation not only for the pristine metal oxide NPs but also for functionalized polymeric NPs.

**Conclusion**

The surface properties of well-engineered F-PLNPs showed excellent correlation with acute lung inflammatory parameters, such as percentage of PMN and levels of IL-1\( \beta \) and CINC-3, in BAL fluid. This result implies that the surface charge paradigm can be applied not only to non-functionalized metal oxide NPs but also to functionalized polystyrene NPs. In addition, the confirmed particle pathogenicity paradigm can be a utility for predicting toxicity of NPs. Further studies with different sets of functionalized NPs are needed to validate the results shown in this study.

**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article. This study was supported by the National Research Foundation of Korea (NRF-2013R1A1A1011330) and the European Commission for Marie Curie International Incoming Fellowship to S.V.C. (Project No. PIIF-GA-2009-254238).

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