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Phospholipid polymer can reduce cytotoxicity of poly (lactic acid) nanoparticles in a high-content screening assay

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Abstract. The objective of this study was to evaluate the cytotoxicity of poly (lactic acid) (PLA) nanoparticles. We used a water-soluble, amphiphilic phospholipid polymer, poly (2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate) (PMB30W), as a stabilizer for the PLA nanoparticles. The PLA nanoparticles and PMB30W-modified PLA (PLA/PMB30W) nanoparticles were prepared by evaporating tetrahydrofuran (THF) from its aqueous solution. Precipitation of the polymers from the aqueous solution produced PLA and PLA/PMB30W nanoparticles with a size distribution of 0.4-0.5 μm. The partial coverage of PMB30W on the surface of the PLA/PMB30W nanoparticles was confirmed by X-ray photoelectron spectroscopy (XPS) and dynamic light-scattering (DLS). A high-content automated screening assay (240 random fields per group) revealed that the PLA nanoparticles induced apoptosis in a mouse macrophage-like cell line (apoptotic population: 73.9% in 0.8 mg PLA/mL), while the PLA/PMB30W nanoparticles remained relatively non-hazardous in vitro (apoptotic population: 13.8% in 0.8 mg PLA/mL). The reduction of the apoptotic population was attributed to the phosphorylcholine groups in the PMB30W bound to the surface of the nanoparticle. In conclusion, precipitation of PLA in THF aqueous solution enabled the preparation of PLA nanoparticles with similar shapes and size distribution but different surface characteristics. PMB30W was an effective stabilizer and surface modifier, which reduced the cytotoxicity of PLA nanoparticles by enabling their avoidance of the mononuclear phagocyte system.

Keywords: nanoparticles; phospholipid polymer; cytotoxicity; high-content screening assay; PLA

1. Introduction

Recent advances in nanotechnology have produced numerous nanoparticles for use in drug delivery, vaccination, and medical diagnostics. From a health and safety perspective, it is critical to be able to predict their potential toxic effects. Since the physicochemical properties of nanoparticles are diverse, a standardized design for assessing their biological and cytotoxic effects is necessary for the development of a universal database and the establishment of scientific guidelines. High-throughput systems for rapid and cost-effective screening of cytotoxic nanoparticles can meet future demands for assessment of nanotoxicity under different conditions (Geys *et al*. 2010, Jan *et al*. 2008, Lankveld *et al*. 2010).

Poly(lactic acid) (PLA) and poly(lactic acid-co-glycolic acid) have been used widely for preparing nanoparticles because they are biodegradable in nature (Soppimath and Aminabhavi,

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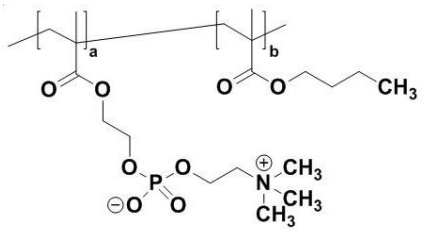
2001, Kumari *et al.* 2010). However, there is a lack of data regarding the biological effects arising from techniques used in the preparation of PLA nanoparticles (Lassale and Ferreira 2007, Wischke and Schwendeman 2008). The physicochemical properties of PLA nanoparticles strongly depend on the properties of stabilizing agents present in the medium during their preparation. These stabilizers remain attached to the PLA nanoparticles, regardless of washing and purification processes (Chen *et al.* 2001, Jeffery *et al.* 1991, Lassale and Ferreira 2007, Wischke and Schwendeman 2008). Therefore, it is important to consider the fact that the stabilizers used during preparation of PLA nanoparticles could play a critical role in the cytotoxic effects of PLA nanoparticles.

Water-soluble polymers such as poly(ethylene glycol) (PEG) are applied to stabilize the molecular assembly of polymeric nanoparticles. Numerous reports regarding the surface modification of PLA nanoparticles have been published (Tobio *et al.* 1998, *Choi et al.* 2003, Cheng *et al.* 2007). The role of PEG is to provide good dispersion characteristics and stealth property to the coated PLA nanoparticles. When the molecular weight of the PEG is lower than 2×10^4, it can escape from blood through the kidneys as urine. There are some reports regarding immunological response against PEG and PEG coated nanoparticles (Garay and Labaune 2011, Semeta *et al.* 2010, Ishida *et al.* 2007, Armstrong *et al.* 2007). The ether bonds of the PEG chain undergo auto-oxidation by active oxygen species even under physiological conditions and degrade quickly (McGay Jr 1960, Neto *et al.* 2002, Duval and Gross 2013). These two properties of PEG should be taken into consideration when PLA nanoparticles modified with PEG are introduced into the living system.

Phosphatidylcholines are major components of the eukaryotic outer cell membrane. Some drug formulations are realized using phosphatidylcholines as liposomal microspheres (Mizutani 1996). They can escape the mononuclear phagocyte system and passively target tumors (Alexis *et al.* 2010, Sengupta *et al.* 2005). However, the molecular assembly of phosphatidylcholines is unstable under biological circumstances and circulation lifetime in blood is not sufficient for effective treatment (Ishihara *et al.* 2011, Matsuno *et al.* 2011). It is well known that polymers composed of 2-methacryloyloxyethyl phosphorylcholine (MPC) units have the phosphorylcholine group in the side chain, which has the same polar group as phosphatidylcholine (Ishihara *et al.* 1990, Ueda *et al.* 1992). The MPC units in the polymer are extremely hydrophilic and poly(MPC) has a stable conformation in solutions with high salt content and over a wide pH range. The solubility of the MPC copolymers, which are synthesized by radical copolymerization of MPC and other vinyl compounds, depends on the chemical structure and composition of comonomers and molecular weight of the polymers. Tuning these parameters allowed the water-soluble MPC polymers with hydrophobic monomer units to possess an amphiphilic nature and form polymer aggregates when dissolved in aqueous medium (Ishihara *et al.* 1999). They function well as a solubilization and emulsification agent of hydrophobic compounds in aqueous medium. For instance, poorly water-soluble anticancer drug, paclitaxel, can be dissolved in the MPC polymer–aqueous solution and injected directly in the blood stream (Choi *et al.* 2014, Wada *et al.* 2007). The water-soluble and amphiphilic MPC polymers have been used as a stabilizer in the preparation and surface modification of PLA nanoparticles (Goto *et al.* 2008, Konno *et al.* 2001). The MPC polymer modified PLA nanoparticles disperse well in biological medium. Here, we report *in vitro* techniques for investigating the influence of an MPC polymer as a stabilizing agent in PLA nanoparticles on their cytotoxicity by using a high-content screening assay.



(a:b = 30:70, Molecular weight = 5 x 10^4)

Fig. 1 Chemical structure of PMB30W

2. Experimental methods

2.1 Materials

MPC was purchased from NOF Co. Ltd., which was synthesized as previously reported method (Ishihara *et al.* 1990). PLA (weight-averaged molecular weight=2×10^4) was purchased from Wako Chemicals (Osaka, Japan). The water-soluble poly(MPC-*co*-*n*-butyl methacrylate (BMA)) (PMB30W, molecular weight=3×10^4) was synthesized by conventional radical polymerization of MPC and BMA as reported (Ishihara *et al.* 1999). The chemical structure of PMB30W is shown in Fig. 1.

2.2 Preparation of PLA nanoparticles with/without PMB30W

The PLA (100 mg) was dissolved in 50 mL of tetrahydrofuran (THF). Subsequently, 100 mL of distilled water, with or without 100 mg of PMB30W, was slowly added to the PLA polymer solution with stirring. After three minutes of stirring, the solution was maintained at room temperature for two days to allow the THF to evaporate. The residual THF was completely removed under reduced pressure. To remove the residual PMB30W in the polymer particle suspension, the PLA/PMB30W solution was centrifuged at 10,000×*g* for 30 min. The supernatant was removed, and the precipitated polymer particles were resuspended in 100 mL of distilled water.

2.3 Physicochemical properties

The morphology of the polymer particles was determined using a scanning electron microscope (SM-200, Topcon, Tokyo, Japan). To determine the zeta potential, 1 mg/mL polymer particle suspension was dialyzed for 1 day against 25 mM HEPES buffer using a dialysis membrane (molecular weight cut-off=10 kDa, Thermo Fisher Scientific, Rockford, IL). The size distribution and zeta potential were measured by electrophoretic dynamic light-scattering (DLS, Zetasizer Nano-ZS, Green Badge, ZEN3500, Malvern, UK). The presence of PMB30W and its density in the PLA/PMB30W particles were characterized by X-ray photoelectron spectroscopy (XPS, AXIS-HSi, Kratos/Shimadzu, Kyoto, Japan) with a magnesium Kα (energy=1253.6 eV) radiation source. The photoelectron take-off angle was 90°. In order to evaluate the surface coverage of the MPC polymer on the PLA nanoparticle, the phosphorus/carbon (P/C) atomic percentage ratios were

calculated. The integrated peak area was calculated by applying the sensitivity factors supplied by the manufacturer.

2.4 High-content screening assay

A mouse macrophage-like cell line (J774A.1) was incubated in a 96-well plate with RPMI-1640 containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin before activation with 10 ng/mL tumor necrosis factor-α and 1 ng/mL interferon-γ for 1 day. The polymer nanoparticles were sterilized using overnight ultraviolet irradiation adjusted according to the concentration of PLA (mg/mL) suspended in Hank's balanced salt solution (HBSS) containing 5% FBS. After removal of the culture media, the attached J774A.1 cells were incubated in the polymer nanoparticle suspension. The negative control group was HBSS containing 5% FBS without the polymer nanoparticles. After 24 h, the cells were washed, stained with fluorescein isothiocyanate (FITC)-labeled annexin-V in HEPES buffer solution for 10 min, washed again, and then stained with Hoechst 33342 (H33342, 2 μg/mL) in HBSS containing 5% FBS for 20 min in the incubator. The stained cells were washed, fixed with 4% paraformaldehyde/PBS for 15 min, washed again, and then scanned with an automated cell high-content screening system (HCS, IN Cell Analyzer 1000, GE Healthcare, Buckinghamshire, UK). The HCS scanned through the bottom of the plate and focused on random fields on the plate. The H33342 and FITC-labeled annexin-V stains were visualized using a dichroic mirror. The experiment was conducted with three independent runs. In each run, 10 randomized fields from each well were imaged using a 20× objective. Cells were classified as either viable or apoptotic by using the classification algorithm of the image analysis software (IN Cell Investigator, GE Healthcare). The nucleus intensity represents the average pixel intensity in the nuclear region, and the cell intensity refers to the average pixel intensity in the cytoplasmic region.

3. Results and discussion

3.1 Physicochemical properties

The PLA nanoparticles with or without PMB30W were of similar shape and size (Fig. 2). Their surfaces were characterized using XPS and DLS. The PLA nanoparticles modified with PMB30W (PLA/PMB30W nanoparticles) had specific peaks that were attributed to the component atoms, such as a strong carbon peak at 285 eV, a nitrogen peak at 402 eV, an oxygen peak at 530 eV, and a phosphorus peak at 133 eV (Fig. 3). The detected nitrogen and phosphorus atomic components were attributed to the phosphorylcholine groups of PMB30W. The zeta potential of the PLA nanoparticles and the PLA/PMB30W nanoparticles were −62.9±6.4 and −53.5±6.2, respectively. The zeta potential of the MPC polymer surface was almost zero (Ueda *et al.* 1995) because of the presence of zwitterionic phosphorylcholine groups containing a cationic trimethylammonium group and an anionic phosphate group in the side chain. In the case of PLA/PMB30W, the values were closer to zero, but still negative. Thus, it is possible that the PMB30W partially covered the surface of the PLA/PMB30W nanoparticles. The XPS results further support this idea. The P/C ratio on the PMB30W surface calculated from the molecular structure was 3.3%. On the surface of the PLA/PMB30W nanoparticles, however, the P/C ratios were 0.90 ± 0.09%. The physicochemical properties of PLA and PLA/PMB30W nanoparticles are summarized in Table 1.

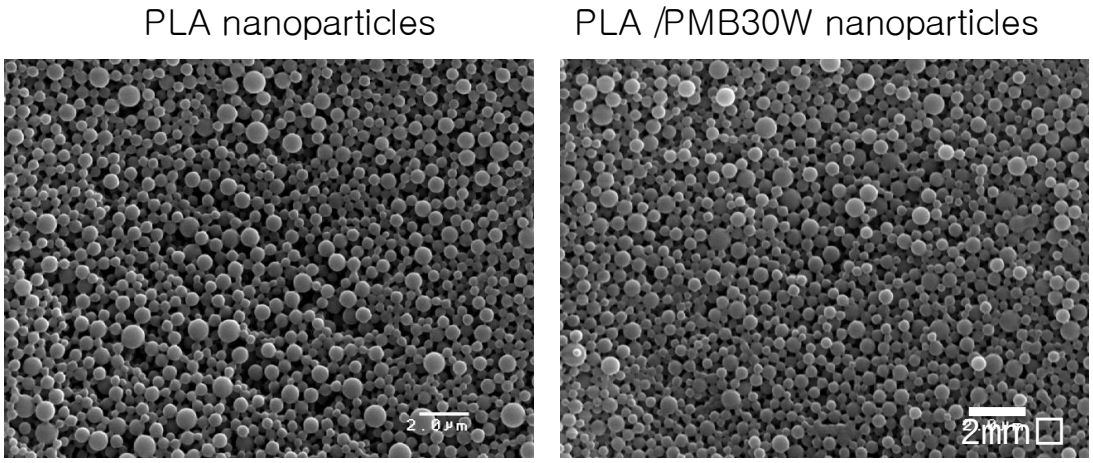


Fig. 2 Scanning electron microscope images of PLA and PLA/PMB30W nanoparticles

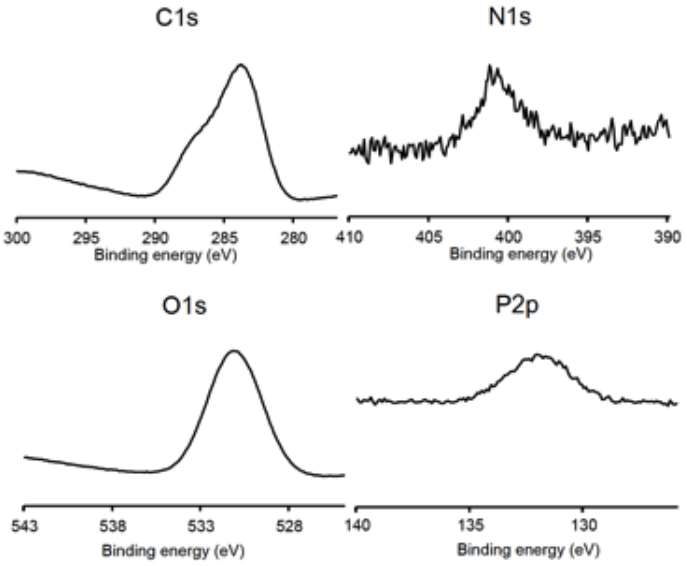


Fig. 3 XPS images of PLA/PMB30W nanoparticles

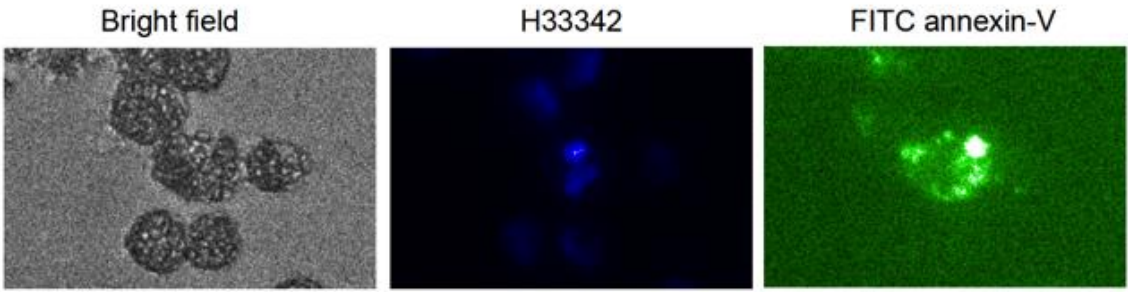


Fig. 4 Bright field and fluorescence field imaging of J774A.1 cells stained with H33342 and FITC-labeled annexin-V after 24 h induction with polymer nanoparticles

Table 1 Characterization of nanoparticles

Nanoparticles	P/C ratio (%)	Zeta potential (mV)	Mean diameter (nm)	Polydispersity index
PLA	0	-62.9±6.4	414	0.063
PLA/PMB30W	0.90±0.09	-53.4±6.2	478	0.132

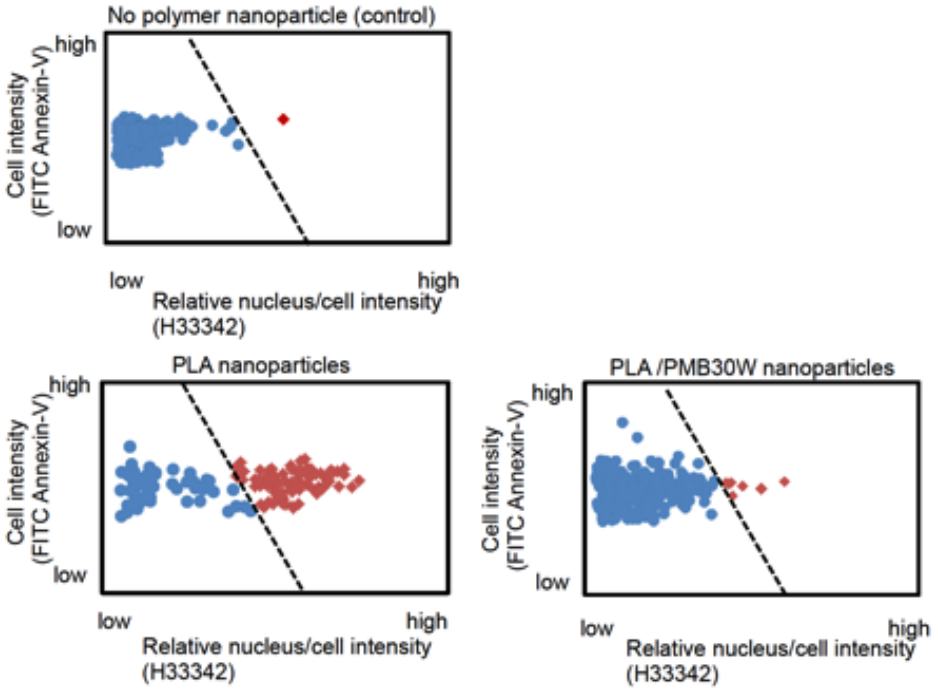


Fig. 5 Classification of J774A.1 cells after a 24 h induction with polymer nanoparticles (0.8 mg/mL), using a high-content screening assay. Data were acquired from 10 random fields per well of a 96-well plate (8 wells per group). Blue circles indicate viable cells and red squares indicate apoptotic cells. The linear threshold (dashed line) on the 2D plot was manually determined after the microscopic examination on each plot in the border zone. The threshold remained consistent during the experiments

3.2 High-content screening assay

After the introduction of PLA or PLA/PMB30W nanoparticles into the mouse macrophage-like cell line (J774A.1) in the presence of FBS for 24 h, the apoptotic population of J774A.1 cells was analyzed using HCS. HCS is a recent advancement towards the integration and automation of quantitative fluorescence microscopy and image analysis. HCS has been approved as a tool for the evaluation of particle immunotoxicity (Jan *et al.* 2008, Lankveld *et al.* 2010). The FITC-labeled annexin-V conjugate binds to phosphatidylserine, which shifts to the outer cell membranes during apoptosis. H33342 stains the nuclei in viable and apoptotic cells (Fig. 4). The cells in each image (n = 80 per group, per run) were divided into viable and apoptotic cell populations by the relative nucleus/cell intensity with the nucleus intensity (H33342) on the X-axis and the cell intensity (FITC Annexin-V) on the Y-axis (Fig. 5). Apoptotic cells demonstrate nuclear condensation, which

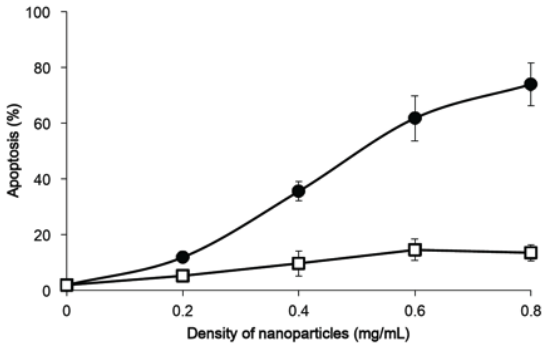


Fig. 6 Apoptosis of J774A.1 cells after a 24-h induction with polymer nanoparticles. Circles indicate PLA nanoparticles and squares indicate PLA/PMB30W nanoparticles

resulted in increased X-values.

Since increased levels of unbound FITC annexin-V cause viable cells to exhibit increased cell intensity, the X-axis provided good separation between the two subpopulations and compensated for any variation in the fluorescence intensity within a well. Thus, the apoptotic cells were identified by the increased cell intensity (FITC Annexin-V) and the viable cells were identified by the increased nucleus/cell intensity (H33342) values. PLA nanoparticles induced apoptosis in J774A.1 cells in a dose-dependent manner, while the PLA/PMB30W nanoparticles did not induce substantial apoptosis of J774A.1 cells (Fig. 6). When the density of nanoparticles was 0.8 mg/mL in the media, the apoptotic population after the introduction of PLA nanoparticles alone was 73.9%, compared to the 13.4% observed after the introduction of PLA/PMB30W nanoparticles.

4. Discussion

Oil-in-water emulsion based solvent evaporation for preparation of PLA nanoparticles requires a stabilizer. In conventional emulsion-based methods, PLA is dissolved in a water-immiscible organic solution (oil), and the stabilizer is dissolved in water. The emulsified oil drops containing the polymer form nanophases in the continuous aqueous phase, and the nanoparticles can be obtained by the elimination of the organic solvent. Higuchi *et al.* (2006) prepared polystyrene nanoparticles by evaporating an organic solvent (THF) after the addition of water into the water-miscible polymer solution. This precipitation method was introduced and modified here to prepare PLA nanoparticles with and without a stabilizer. A small amount of distilled water with or without the PMB30W was slowly dropped into THF containing PLA. Gradual evaporation of THF at room temperature caused the precipitation of finely shaped nanoparticles.

Although numerous efforts have been made to prepare PLA nanoparticles and use them in biomedicine, the data concerning their possible health hazards is limited (Bergsma *et al.* 1995, De Jong *et al.* 2005, Kim *et al.* 2009, 2011). The addition of a stabilizer during the preparation of PLA nanoparticles has further complicated the interpretation of the data concerning their toxicity. This insufficiency of data prompted us to develop a preparation method for PLA nanoparticles and to evaluate the influence of a stabilizer on their cytotoxicity. The cytocompatibility of synthetic nanoparticles is influenced both by their size and by surface properties. (Champion *et al.* 2008, Fadeel and Garcia-Bennett 2010). In order to investigate the effect of surface modification of

nanoparticles on cytotoxicity, their size should be controlled. Since the precipitation of PLA nanoparticles depends on the evaporation of THF from the aqueous solution, the size of the nanoparticles can be controlled by manipulating the temperature and PLA concentration. PLA and PLA/PMB30W nanoparticles with a similar size distribution were produced by maintaining similar temperatures and PLA concentration in all experiments.

The mechanisms underlying nanoparticle cytotoxicity are not fully understood (Hackenberg *et al.* 2011). Hydrophobic PLA particles were opsonized and easily recognized by phagocytes because of their hydrophobicity, which is a universal danger sign identified by pattern recognition receptors (Hubbell *et al.* 2009). The internalized PLA nanoparticles induce oxidative stress in phagocytes, and cause an increase in the levels of the intracellular reactive oxygen species that could induce inflammation and apoptosis (Horie *et al.* 2010, Kim *et al.* 2011). In contrast, the PLA/PMB30W nanoparticles could escape the mononuclear phagocyte system because of the high free-water fraction provided by the phosphorylcholine groups on their surfaces (Alexis *et al.* 2010, Fadeel and Garcia-Bennett 2010, Ishihara *et al.* 1998, Kim *et al*. 2011, Moro *et al.* 2004, Sengupta *et al.* 2005). Therefore, the surface modification of the PLA nanoparticles with a phospholipid stabilizer reduced their cytotoxicity, possibly by enabling them to escape the trigger point of the inflammatory cascades in the mononuclear phagocyte system.

5. Conclusions

In this study, we obtained both PLA and PLA/PMB30W nanoparticles with similar shape and size distribution using a precipitation method. This preparation method enabled us to understand the influence of surface properties on the cytotoxicity of PLA nanoparticles. A high-content screening *in vitro* assay demonstrated that the surface modification of PLA nanoparticles with PMB30W drastically reduced their cytotoxicity. The presented preparation technique and *in vitro* findings contribute to the understanding of the toxic effects of PLA nanoparticles with different physicochemical properties and enable prediction of the toxic effects of polymer nanoparticles.

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