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## Controlling Ligand Surface Density Optimizes Nanoparticle Binding to ICAM-1

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### Abstract

During infection, pathogens utilize surface receptors to gain entry into intracellular compartments. Multiple receptor-ligand interactions that lead to pathogen internalization have been identified and the importance of multivalent ligand binding as a means to facilitate internalization has emerged. The effect of ligand density, however; is less well known. In this study, ligand density was examined using poly(DL-lactic-co-glycolic acid) nanoparticles (PLGA NPs). A cyclic peptide, cLABL, was used as a targeting moiety as it is a known ligand for intercellular cell adhesion molecule-1 (ICAM-1). To modulate the number of reactive sites on the surface of PLGA NPs, modified Pluronic<sup>®</sup> with carboxyl groups and Pluronic<sup>®</sup> with hydroxyl groups were combined at different ratios and the particle properties were examined. Utilizing a surfactant mixture directly affected the particle charge and the number of reactive sites for cLABL conjugation. The surface density of cLABL peptide increased as the relative amount of reactive Pluronic<sup>®</sup> was increased. Studies using carcinomic human alveolar basal epithelial cells (A549) showed that cLABL density may be optimized to improve cellular uptake. These results compliment other studies suggesting surface density of the targeting moiety on the NP surface should be considered to enhance the effect of ligands employed for cell targeting.

### Keywords

ligand density; poly(DL-lactic-co-glycolic acid) nanoparticles; cLABL; Pluronic<sup>®</sup>; binding and cellular uptake

### Introduction

The interaction between leukocyte function associated antigen-1 (LFA-1) present on lymphocytes such as T cells and intercellular cell adhesion molecule-1 (ICAM-1) on antigen presenting cells (APC) represents an interesting therapeutic target<sup>1-3</sup>. This interaction is a part of T-cell activation through the immunologic synapse, which forms at the interference

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between T cells and APCs<sup>3-6</sup>. The expression of ICAM-1, which is a ligand for LFA-1, has been investigated in a variety of cells<sup>1,7</sup>. Different cytokines can up-regulate ICAM-1 on the surface of cells<sup>2,7-8</sup>. Reports have also suggested a link between tumor growth attenuation and overexpression of ICAM-1 induced by inflammatory cytokines such as TNF- $\alpha$ <sup>1-2,7-8</sup>. ICAM-1 up-regulation has been observed in many different inflammatory diseases and cancers cells such as melanomas, lymphomas, and lung carcinomas, which makes this receptor an interesting target for therapeutic delivery<sup>1,8</sup>.

Peptides derived from binding domains of LFA-1 and ICAM-1 have been developed as ligands for the opposing receptors. Cyclo(1,12)-PenITDGEATDSGC (cLABL), a cyclic peptide, is derived from I-domain of  $\alpha_L$ -subunit of LFA-1 integrin. This peptide can inhibit homotypic and heterotypic T-cell adhesion to epithelial and endothelial cells by blocking the LFA-1/ICAM-1 interaction<sup>9</sup>. Furthermore, cLABL has been employed as a targeting ligand for intracellular delivery<sup>2,10</sup>. Accordingly, cLABL conjugated to poly(DL-lactic-co-glycolic acid) nanoparticles (PLGA NPs) was designed to target anti-cancer drugs to carcinomic human alveolar basal epithelial cells, A549, via ICAM-1<sup>1</sup>.

Understanding the ligand-receptor interactions between cells or other biological components can be helpful to enhance the efficiency of targeting systems by improving binding avidity and cellular uptake. The structure of viruses such as adenovirus type 2 and the surface density of their ligands have been studied to surmise the importance of multivalent ligands on binding efficiency<sup>11</sup>. Studies showed that penton-base proteins presented on adenovirus type 2 cover the surface of these viruses with regular spacing<sup>11</sup>. The surface density presented by this virus may substantially improve binding avidity to the cell receptors. The simulation of the natural structure of viruses based on their ligand density has been utilized to engineer targeted clustered ligands<sup>11-12</sup>. Studies showed that, the optimum distribution of ligands on the surface of viruses is a key factor for binding and cellular uptake. Control of ligand density on NPs should provide a means to increase receptor binding avidity and cellular uptake.

The effect of ligand density (e.g. peptide conjugated to NPs) on binding and cellular uptake has not been thoroughly explored. Reports have shown that increasing conjugated ligands on particles often increases the cellular uptake<sup>13-15</sup>. In general, these studies showed that ligands must be present on the surface of NPs above a minimum threshold for binding to occur<sup>16</sup>. However, a few studies also showed that dense surface coverage may not offer expected improvements in binding and cellular uptake<sup>13-16</sup>.

In this study, the effect of ligand density on receptor binding and cellular uptake was investigated using surface modified PLGA NPs exhibiting conjugated cLABL peptide. To control the number of reactive sites for conjugation on the surface of NPs, mixtures of two surfactants were used during NP fabrication. Such surface modification provided a controllable number of reactive sites on the surface of the NPs for peptide conjugation, allowing us to modulate the peptide surface density on the NPs as a means to optimize binding and cellular uptake. Binding experiment using A549 cells were utilized to evaluate the effect of ligand density on NP binding to the cells.

## Experimental Procedures

### Materials

Polymers of poly(DL-lactic-co-glycolic acid) (50:50) (PLGA with inherent viscosities of 0.22 dL/g, (Mw~6.7 kDa; carboxyl terminal group) and 1.05 dl/g (Mw~101 kDa) were purchased from LACTEL Absorbable Polymers International (Pelham, AL, USA). Pluronic® F38 (Mw~4700 Da), Pluronic® F68 (Mw~8400 Da), Pluronic® F108 (Mw~14600 Da), and Pluronic® F127 (Mw~12600 Da) were obtained from BASF Corporation (Mount Oliver, NJ, USA). 1-ethyl-3-[3-dimethylaminopropyl]carbodiimidehydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Thermo Scientific (Rockford, IL, USA). Coumarin-6 was purchased from Polysciences, Inc. (Warrington, PA, USA). Tetrahydrofuran (THF), triethylamine, acetone, diethyl ether, 1X Tris/EDTA buffer solution pH 8, sodium hydroxide, and ethyl alcohol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). D-mannitol, (dimethylamino)pyridine (DMAP), succinic anhydride, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclo(1,12)-PenITDGEATDSGC (cLABEL) was synthesized in-house using a reported method<sup>1-2</sup>. Millex™ syringe driven filter unit (MCE 0.45 µm MF) was purchased from Millipor Corporation (Bedford, MA, USA). Dialysis membrane (MWCO 100,000) was obtained from Spectrum Laboratory Products Inc. (Rancho Dominguez, CA, USA). F-12K medium and A549 cell line were purchased from American Type Culture Collection (Manassas, VA, USA). Recombinant, human, tumor necrosis factor-α (TNF- α) was obtained from Promega (Madison, WI, USA). BCA™ protein assay kit was obtained from Thermo Scientific (Rockford, IL, USA). Costar® 3596 and Costar® 3631 were purchased from Corning Incorporated (Corning, NY, USA).

### Conversion of terminal hydroxyl groups to terminal carboxyl groups on Pluronic®

To conjugate cLABEL to Pluronic®, the terminal hydroxyl groups were converted to carboxyl groups on Pluronic® F38, F68, F108, and F127<sup>1-2,17</sup>. First, 12.87 mM of each surfactant was dissolved in 10 mL of tetrahydrofuran (THF). Then, 4-dimethylaminopyridine (DMAP, 24.5 mg), triethylamine (27 µL) and succinic anhydride (200 mg) were added to the solution. The mixture was kept on a stirrer for 48 hours at room temperature in a closed bottle. THF was then removed using a rotary evaporator (Rotovaporator-R, Büchi, Switzerland) and the product was dissolved in about 15 mL of carbon tetrachloride. Filtration was utilized to remove the excess succinic anhydride (0.45 µL cutoff). Each type of Pluronic® with terminal carboxyl groups was purified by precipitation using 25 mL diethylether and recrystallized with 10 mL ethyl alcohol. Rotary evaporation was performed to remove the solvents after precipitation and recrystallization. Finally, the products were kept in a vacuum chamber overnight to remove the solvents. Modified Pluronics® were characterized by <sup>1</sup>H NMR spectroscopy (Bruker AVANCE 400 MHz spectrometer) to confirm the functional group conversion. Chloroform-d (CDCl<sub>3</sub>) was used to dissolve Pluronics® for <sup>1</sup>H NMR sample preparation.

### Preparation of PLGA nanoparticles with a mixture of two surfactants

A solvent displacement method was employed to prepare PLGA NPs<sup>1-2</sup>. To obtain different degrees of reactive sites for peptide conjugation (via terminal Pluronic® carboxyl groups),

the following method was employed. Briefly, PLGA (inherent viscosity 0.22 dL/g or 1.05 dL/g) was dissolved in acetone (15 mg/mL). For nanoparticle labeling, Coumarin-6 was also dissolved in acetone (2 mg/mL). The mixture of 1425  $\mu$ L of PLGA solution, 75  $\mu$ L 1X Tris/EDTA buffer solution, and 20  $\mu$ L Coumarin-6 solution was injected into a 15 mL water phase containing 0.1% w/v Pluronic<sup>®</sup> using a syringe pump at 10 mL/hr while stirring (1000 rpm). In order to prepare NPs without labeling, a mixture of 1425  $\mu$ L of PLGA solution and 75  $\mu$ L of 1X Tris/EDTA buffer solution was injected into the water phase. Aqueous mixtures of surfactants were made by dissolving different types of Pluronic<sup>®</sup> with different functional groups (hydroxyl or carboxyl groups) (0.1 % w/v). To fabricate NPs for cLABEL conjugation, several surfactant ratios of Pluronic<sup>®</sup> F108-COOH and Pluronic<sup>®</sup> F68-OH (100:0, 75:25, 50:50, 25:75, and 0:100 ratios (v:v)) were made by combining these surfactants before transferring the PLGA solution to the surfactant solution. The PLGA NPs spontaneously formed as a result of the rapid diffusion of acetone that occurred when the organic solution was injected into the water solution. Stirring was continued for 1.5 hours. The excess surfactant was removed by dialysis against a 0.2% D-mannitol aqueous solution.

### cLABEL peptide conjugation to PLGA nanoparticles

For each conjugation reaction, 1 mL of an aqueous suspension of PLGA NPs (~1.5 mg/mL) coated with the different Pluronic<sup>®</sup> F108-COOH and Pluronic<sup>®</sup> F68-OH ratios was buffered at pH 6.5 using 100  $\mu$ L 2-(*N*-morpholino)ethanesulfonic acid (MES, 0.1 mM). NPs encapsulating Coumarin-6 and NPs without Coumarin-6 were used to make NPs with conjugated cLABEL (PLGA NPs+Coumarin-6+cLABEL) or unlabeled NPs conjugated with cLABEL (PLGA NPs+cLABEL), respectively (Table 2). The NP suspension was then incubated with 150 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimidehydrochloride (EDC) and 50 mM *N*-hydroxysulfosuccinimide (sulfo-NHS) for 15 minutes with mild agitation<sup>1,18-19</sup>. Before starting the conjugation process, NP suspensions were washed three times with distilled water to remove excess EDC and sulfo-NHS using centrifugation (15,000 rpm, 15 min, 4°C) and sonication to aid resuspension of the centrifuged pellet (Branson 2510 ultrasonic cleaner, USA). Then, NP suspensions were buffered with 100  $\mu$ L of phosphate buffered saline (PBS). The carboxyl terminal groups of Pluronic<sup>®</sup> F108-COOH were activated to conjugate the amino terminus of cLABEL peptide. After activation of the carboxyl groups of Pluronic<sup>®</sup> F108-COOH, 100  $\mu$ L of cLABEL aqueous solution (1 mg/mL) was added to NP suspensions react the N-terminus of cLABEL peptide to the activated carboxyl group on Pluronic<sup>®</sup> F108-COOH on the NPs. The conjugation was performed overnight at room temperature. After conjugation, NPs were separated from the excess peptide using centrifugation (15,000 rpm, 15 min, 4°C) and washed three times with distilled water. Then, the particles were dispersed in water by placing the vials in a sonication bath (Branson 2510 ultrasonic cleaner, USA) (Figure 1).

### Particle size and zeta potential characterization

Particle size and zeta potential were measured using a ZetaPALS dynamic light scattering instrument (Brookhaven, USA). A transmission electron microscope (FEI field emission transmission electron microscope, Tecnai G<sup>2</sup> at 200 kV) was employed for morphological characterization. TEM samples were prepared by depositing a drop of NP solution on a copper carbon grid and allowing it to dry in a dessicator overnight.

## Evaluation of cLABL peptide surface density using HPLC

The amount of cLABL added to the NP suspension before conjugation and the unconjugated recovered peptide after conjugation reaction was quantified by gradient reversed-phase HPLC using a Vydac® HPLC column (protein and peptide C<sub>18</sub>). The HPLC system consists of Shimadzu SCL-10A system controller, SIL-10A XL with sample collector (50 µL injection volume at 4°C), a SPD-10A UV-Vis detector (220 nm wavelength of detection), LC-10AT VP solvent delivery pumps, and a DGU-14A degasser. Data acquisition was performed by Shimadzu class VP software (version 4.2). Mobile phase A was acetonitrile/water (5/95% v/v) with 0.1% trifluoroacetic acid (TFA). Mobile phase B was acetonitrile/water (90/10% v/v) with 0.1% TFA. Gradient elution was performed at a constant flow of 1 mL/min, from 100% A to 0% A (corresponding to 0% B to 100% B) for 15 min, maintained at 100% B for 3 min, followed by a return to 100% A and subsequent column re-equilibration for 13 minutes. The peptide density on the surface of NPs was calculated by subtracting the amount of recovered peptide after conjugation from the amount of peptide added to the NP suspension divided by the total surface area assuming a normal Gaussian particle size distribution. A standard curve was based on several dilutions of cLABL in water and used to calculate the cLABL concentration for each sample. NP suspension buffered with MES and PBS without EDC/Sulfo-NHS was used as negative control. The cLABL was added to the negative control at the same concentration as normal samples in which carboxyl groups on NPs were activated with EDC/Sulfo-NHS.

## Nanoparticle binding to ICAM-1 and cellular uptake

Carcinomic human alveolar basal epithelial cells, A549, were cultured in F-12K medium containing 10% FBS and 0.1% penicillin-streptomycin for 48 hours (37°C, 5% CO<sub>2</sub>). Cell density of 8×10<sup>4</sup> cells/mL was maintained and 100 µL of cell suspension was seeded in each well of a Costar® 3596 96-well plate, and incubated for 12 hours. The medium was then replaced by fresh medium containing TNF-α (1,000 U/mL) for 48 hours to up-regulate ICAM-1 receptor expression<sup>7-8</sup>. F-12K medium was then used to wash the cells three times and the cells were incubated with a fresh serum-free medium containing 0.3 mg/mL of labeled cLABL-NPs (NPs encapsulating Coumarin-6). NPs encapsulating Coumarin-6 but without conjugated cLABL were used as negative controls. The cells were then incubated with NPs for different periods of time (5, 10, 15, 30, and 60 minutes) to evaluate the binding and uptake of cLABL-NPs. Cold PBS was used to wash the cells three times after incubating cells with NPs and then the cells were lysed with 0.2 M NaOH with 0.5% Triton X-100<sup>1</sup>. A fluorescence plate reader (Spectramax M5; ex: 450 nm, em: 500 nm) was employed to quantify fluorescence resulting from NPs incubated with cells<sup>1</sup>. The measured fluorescence intensity was normalized by protein concentration that was evaluated using a BCA™ protein assay kit according to the manufacturer's instructions.

## Fluorescence microscopy of cLABL-NPs with A549 cells

To compare the binding and uptake of NPs with different peptide densities, fluorescence microscopy was utilized to evaluate NP interactions with A549 cells. A549 cells (300 µL, 8×10<sup>4</sup> cells/mL) were seeded on an 8-well plate. Then, the cells were activated by using fresh medium (200 µL) containing 1000 U/mL of TNF-α for 24 hours. Cells were then

washed with F-12K medium three times and incubated with labeled cLABL-NPs (0.5 mg/mL in fresh serum free medium) (100:0, and 50:50 surfactant ratios). Labeled NPs without conjugated cLABL were used as a control. Different time points (0, 10, 30, and 60 minutes) were selected to evaluate NP binding and uptake. After 0, 10, 30, and 60 minutes, cells were washed three times using cold PBS to remove unbound NPs. Finally, the cells were fixed using 4% paraformaldehyde. Fluorescence micrographs were acquired using the FITC filter set of a Nikon Eclipse 80i microscope equipped for epifluorescence. Micrographs were captured using an Orca ER camera (Hamamatsu, Inc., Bridgewater, NJ) and analyzed by Metamorph, version 6.2 (Universal Imaging Corp., West Chester, PA).

### Statistical analysis

Statistical analysis was performed by analysis variance (one-way ANOVA). To assess the significance of differences, Newman-Keuls was used as a post hoc test. The t-test was utilized to compare the significance of the difference between the means of two groups. A value of  $p < 0.05$  was accepted as significant in all cases.

## Results and discussion

### Conversion of terminal hydroxyl groups to terminal carboxyl groups on Pluronic®

In order to utilize Pluronic® for conjugation to the terminal amine of the cLABL peptide, the hydroxyl groups of Pluronic® were converted to carboxyl groups. Pluronic® with carboxyl groups were prepared by the reaction of Pluronic® with succinic anhydride. The reaction was performed in 10 mL THF. To confirm this conversion, <sup>1</sup>H NMR spectra before and after conversion were compared. After conversion, the signal corresponding to the hydroxyl group ( $\delta = 4.3$ ) disappeared whereas peaks due to the protons adjacent to the succinic moiety and the proton of the carboxylic acid could be detected at  $\delta = 4.1$  ppm and  $\delta = 11.95$  ppm. <sup>1</sup>H NMR validated functional group conversion (Supplementary figure 1)<sup>1,17</sup>.

### Effect of Pluronic® structure on particle size

PLGA of two different inherent viscosities (0.22 dL/g and 1.05 dL/g) were used to evaluate the effects of different types of Pluronic® on particle size. Pluronic®, a triblock copolymer composed of a central hydrophobic chain of poly(propylene oxide) flanked by two hydrophilic chains of poly(ethylene oxide) was used as a surfactant in the processes of NP fabrication and surface functionalization<sup>20–22</sup>. The central hydrophobic part of Pluronic®, poly(propylene oxide), can embed within PLGA during NP fabrication, linking Pluronic® to the NP<sup>1</sup>. The hydrophobic chain must be long enough to hold Pluronic® to the surface of PLGA NPs. These polymeric chains also provide a hydrophilic shell for NPs, which can stabilize the colloid and limit opsonization, potentially extending the *in vivo* half-life to prolong the therapeutic effect<sup>23–24</sup>.

Polymeric chains extending from the NP surface can also make targeting moieties more accessible to cellular receptors. Poly(ethylene glycol) (PEG) has been used in many studies to conjugate ligands to the surface of polymeric NPs and liposomes<sup>3,14–15,25–27</sup>. These studies have shown binding to cellular receptors and uptake by the cell are dependent on the length of the PEG utilized<sup>3,25</sup>. Targeting moieties are usually conjugated to an intermediate

length polymeric chain (~2–15kDa) to make a targeted NP system<sup>18–19,28–29</sup>. According to some studies, PEG with higher molecular weight (~10–15 kDa) may improve ligand accessibility to the receptor<sup>3,25,30</sup>, but the optimum reported length has varied depending on the NP system. To attach targeting moieties to the polymer chains, polymer chains must have suitable reactive sites<sup>22,31</sup>. In this study, poly(ethylene oxide) chains on Pluronic<sup>®</sup> have terminal hydroxyl groups that can be converted to terminal carboxyl groups prior to NP formation to enable facile peptide conjugation<sup>1,17</sup>.

Pluronic<sup>®</sup> has a variety of structures with differing molecular weight and number of repeating units in the blocks<sup>20–21</sup>. Unmodified Pluronic<sup>®</sup> F38, F68, F108, and F127 (Table 1) were selected since each type provides a different coating thickness (e.g. hydrophilic block length) to NPs, and also provides different ligand accessibility to the receptors<sup>21</sup>. This study showed that increasing PLGA inherent viscosity reduced the particle size by about 50% in each formulation. The molecular weight of surfactants resulted in differences in the size of NPs based on the type of Pluronic<sup>®</sup>. The result suggested that increasing surfactant molecular weight caused slight increase in particle size (Figure 2). Pluronic<sup>®</sup> F38 was not used in the final formulation for peptide conjugation due to concerns that it may detach from the particle surface as a result of the small hydrophobic block. Instead, Pluronic<sup>®</sup> F68 and Pluronic<sup>®</sup> F108 were selected to design a formulation for peptide conjugation.

### Preparation of PLGA nanoparticles using a mixture of two Pluronic<sup>®</sup> surfactants

NPs fabricated using unmodified Pluronic<sup>®</sup> (terminal hydroxyl groups) and carboxyl-modified Pluronic<sup>®</sup> were compared to determine the effect of terminal group conversion on PLGA particle size. For all four types of Pluronic<sup>®</sup>s employed in the preparation process, no significant change in particle size was observed as a result of converting the termini (Figure 3). This effect was observed in all four formulations independent of the molecular weight of surfactant.

To control the number of reactive sites (carboxyl groups) on the NP surface for peptide conjugation, NPs were prepared using a mixture of unmodified Pluronic<sup>®</sup> with hydroxyl terminal groups and low molecular weight (Pluronic<sup>®</sup> F68-OH) and modified Pluronic<sup>®</sup> with carboxyl terminal groups and high molecular weight (Pluronic<sup>®</sup> F108-COOH). This mixture was chosen to keep particle size smaller than 200 nm and increase ligand accessibility as a result of the longer hydrophilic block on the reactive Pluronic<sup>®</sup>. Particles smaller than 200 nm were desired since this size has been reported to evade clearance by macrophages<sup>23</sup>.

Unmodified Pluronic<sup>®</sup> F68 (Pluronic<sup>®</sup> F68-OH) and modified Pluronic<sup>®</sup> F108 (Pluronic<sup>®</sup> F108-COOH) were utilized to make a 1% w/v aqueous solution for preparing PLGA NPs. The ratios of Pluronic<sup>®</sup> F108-COOH and F68-OH were 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100 (v:v). PLGAs with two different inherent viscosities were again studied for each surfactant ratio (0.22 dL/g and 1.05 dL/g). Comparing this result with Figure 2 shows that increasing the PLGA molecular weight decreased PLGA particle size approximately 1.5-fold for each sample; moreover, an increase in the relative amount of Pluronic<sup>®</sup> F68-OH decreased particle size slightly (Figure 4).

Reducing the amount of Pluronic<sup>®</sup> F108-COOH used to prepare surfactant mixture reduces the number of reactive sites on PLGA NPs for peptide conjugation. The amount of peptide conjugated to the surface of NPs was, therefore, modulated by controlling the number of carboxyl groups (reactive sites) on the surface of NPs (i.e. changing the surfactant ratio). Changes in the relative amount of Pluronic<sup>®</sup> F108-COOH on the NP surface were supported by changes in the zeta potential of samples (Figure 5). Terminal carboxyl groups carry more negative charge at neutral pH than hydroxyl groups, so zeta potential should become more negative as the number of carboxyl groups increases. Reducing the amount of Pluronic<sup>®</sup> F108-COOH in the formulation tended to increase particle zeta potential (Figure 5). The relative increase in zeta potential by reducing carboxyl groups on the surface of NPs was approximately the same for different types of PLGA.

### Effect of cLABL peptide conjugation on particle size and charge

cLABL was conjugated to NPs by reacting the amine terminus (N-terminus) of the peptide to carboxyl groups on the NP surface. NPs for peptide conjugation were prepared using the ratios of Pluronic<sup>®</sup> F108-COOH and F68-OH 100:0, 75:25, 50:50, 25:75, and 0:100 (v:v). PLGA with an inherent viscosity of 1.05 dL/g was used to achieve the small particle size observed in preliminary studies.

The conjugation reaction increased the particle size in all of the samples (Figure 6A). The size increase was slightly higher for NPs that had 100% Pluronic<sup>®</sup> F108-COOH. A very small increase in particle hydrodynamic radius may be attributed to the attachment of cLABL with the reported molecular weight of 1,170 Da<sup>32</sup>. However, the sample with 100% unreactive Pluronic<sup>®</sup> F68-OH had no reactive sites, and the particle size also increased after conjugation. A significant cause of the size increase may be the activation procedure, which uses a hydrophobic intermediate. It is more likely that the purification process for NPs, which used centrifugation and resuspension cycles, caused increasing particle size. This particle size increase was also observed in the negative control after the conjugation reaction ( $104 \pm 1.5$  nm), even though this simulated reaction did not link the peptide to the NPs. The negative control was NPs with 100:0 surfactant ratio incubated with cLABL after incubation with EDC/Sulfo-NHS. Transmission electron micrographs of PLGA NPs (50:50 ratio of Pluronic<sup>®</sup> F108-COOH and Pluronic<sup>®</sup> F68-OH) from NPs before and after conjugation suggested particle size increased after conjugation. This result supported the dynamic light scattering data for particle size increase after conjugation. The images taken after conjugation suggested particle aggregation as a probable source of the particle size increase (Figure 6B, C). Conjugation of the cLABL peptide also increased the charge of NPs (Figure 7). The relative charge increase was similar for all of the formulations. Since increase in charge occurred for all ratios (even 0:100) without apparent relation to the amount of cLABL conjugated to the particle, the conjugation process using EDC/ Sulfo -NHS might be the reason for this increase. Zeta potential for NPs with 100:0 surfactant ratio incubated with cLABL after incubation with EDC/Sulfo-NHS was unchanged ( $-16.4 \pm 1.2$  mV) compared to the zeta potential of NPs with 100:0 surfactant ratio before conjugation ( $-16.98 \pm 0.61$  mV) (Table 2).



### Calculating cLABEL peptide density on the surface of nanoparticles

Reversed phase HPLC was used to determine the efficiency of the coupling reaction between the amino group of cLABEL and the carboxyl group of Pluronic<sup>®</sup> F108-COOH. Unreacted peptide was collected, quantified and used to calculate the amount of peptide conjugated to the NPs. Blank NPs and empty vials were used as controls to ensure that peptide was not being adsorbed to surfaces non-specifically. The peptide density on the surface of NPs was calculated based on the total NP surface area, assuming a normal Gaussian particle size distribution (Figure 8). Peptide density was calculated for all five samples that were prepared using different surfactant mixture ratios and using PLGA with inherent viscosity of 1.05 dL/g. The calculation showed a significantly higher density of cLABEL conjugated to the sample with 100% Pluronic<sup>®</sup> F108-COOH in the formulation. Reducing reactive sites by reducing the Pluronic<sup>®</sup> F108-COOH and increasing the Pluronic<sup>®</sup> F68-OH correspondingly decreased peptide density on the surface of NPs (Figure 8). Although no carboxyl group was available on NPs with the 0:100 surfactant ratios, a small amount of cLABEL was found, which may be the result of a small amount of peptide adsorption on the surface of NPs.

### Nanoparticle binding to ICAM-1 and cellular uptake

NP binding to ICAM-1 and cellular uptake was studied using cLABEL-NPs and the A549 cell line. PLGA NPs encapsulating coumarin-6 were prepared using analogous surfactant mixture ratios to yield an incrementally increasing peptide density on the surface of PLGA NPs (Table 2). Results from this bioassay suggested that peptide density could be optimized for cellular uptake. Over the time course studied, maximum uptake was observed for the PLGA NPs when the surfactant ratios of 50:50 or 25:75 were used (Group A). Interestingly, the minimum uptake for PLGA NPs occurred with the surfactant ratio of 100: 0 and 75:25 (Group B) where the maximum peptide density was observed (Figures 9). High peptide density is assumed to increase NP binding to cell receptors; however, it seems that the density of the peptide on the surface of NPs must be at an optimum level to facilitate binding to the receptors<sup>16</sup>. In this case, the suspected optimum may be about 2–4 pmol/cm<sup>2</sup>. NPs with the surfactant ratio of 0:100 behaved in the same manner as control NPs (Group C). In each group, two samples with similar peptide density exhibited similar binding behavior. Although NPs with the surfactant ratio of 0:100 contained 100% Pluronic<sup>®</sup> F68-OH incubated with EDC/sulfo-NHS, the results confirmed that conjugation likely did not occur. A small amount of cLABEL was observed for these NPs, but again this amount was probably adsorbed non-specifically.

### Fluorescence microscopy of cLABEL-NPs with A549 cells

After incubation of labeled NPs with cells for different periods of times, fluorescence micrographs were acquired. Labeled NPs without cLABEL conjugation were utilized as a negative control. Samples with 50:50 and 100:0 ratios were also employed to evaluate the effect of peptide density on cell binding and uptake. The NPs encapsulated Caumarin-6 and were readily detected with the fluorescence microscope. The micrographs for the negative control showed that localization of NPs to the cells was much less than NPs conjugated with cLABEL. NPs with a 50:50 surfactant ratio also showed the highest localization of NPs

around the cells during incubation. Over time, more green color was evident in the images of cells and this increase seemed to be maximal in the NPs with the 50:50 surfactant ratio.

The reason for improved receptor binding at moderate ligand density may be due to the behavior of receptors during the binding process. ICAM-1 is a transmembrane receptor with a molecular weight of 80–114 kDa that is connected to the cytoskeleton and is able to move laterally in the lipid membrane<sup>33</sup>. ICAM-1 mobility on the cell surface leads to a phenomenon called “clustering” during ligand binding<sup>34</sup>. The size of ICAM-1 and the steric hindrance effect are two significant factors in receptor clustering during ligand binding to ICAM-1, dictating that the receptors stay at an optimum distance from each other<sup>35–36</sup>. The density of ligands on the surface of NPs may perturb optimal clustering. At one extreme, the binding affinity may not be sufficient, thus, requiring an increase in ligand density. On the other hand, lowering the ligand density to a level that can facilitate clustering without steric hindrance may also result in an increase in receptor binding<sup>16</sup>. The previous simulations of virus structure suggest an optimum ligand density on targeting systems can be helpful to increase the avidity of receptor binding and cellular uptake<sup>11</sup>. As a result, ligand spacing on engineered targeted nanoparticle systems will be a design consideration in the future.

In this study, a NP system was used to evaluate the effect of ligand density when targeting (ICAM-1). Previous studies have demonstrated that cLABEL-NPs can be used to target ICAM-1 overexpressed on pertinent endothelial and epithelial cell lines<sup>1–2</sup>. The targeting of cLABEL-NPs was improved by varying the amount of conjugated cLABEL peptide using a technique to control the number of reactive sites on the surface of PLGA NPs. After characterizing the effect of process parameters on particle size and zeta potential, methods were identified to produce small particles with a controlled reactive site density for peptide conjugation. Increasing the number of reactive sites directly increased the cLABEL peptide density on NPs. A cell-based assay and fluorescence imaging showed that decreasing surface density to an optimum level enhanced the interaction of cLABEL on PLGA NPs with ICAM-1 on A549. Results suggested that ICAM-1-targeted PLGA NPs with an optimum ligand surface density may be preferred for therapeutic and diagnostic purposes; thus, compelling further research on ligand density effects. Data also showed that the ligand density proved to be a key factor in defining receptor binding and that decreasing ligand surface density on NPs can actually improve binding and cellular uptake of cLABEL-NPs into A549 cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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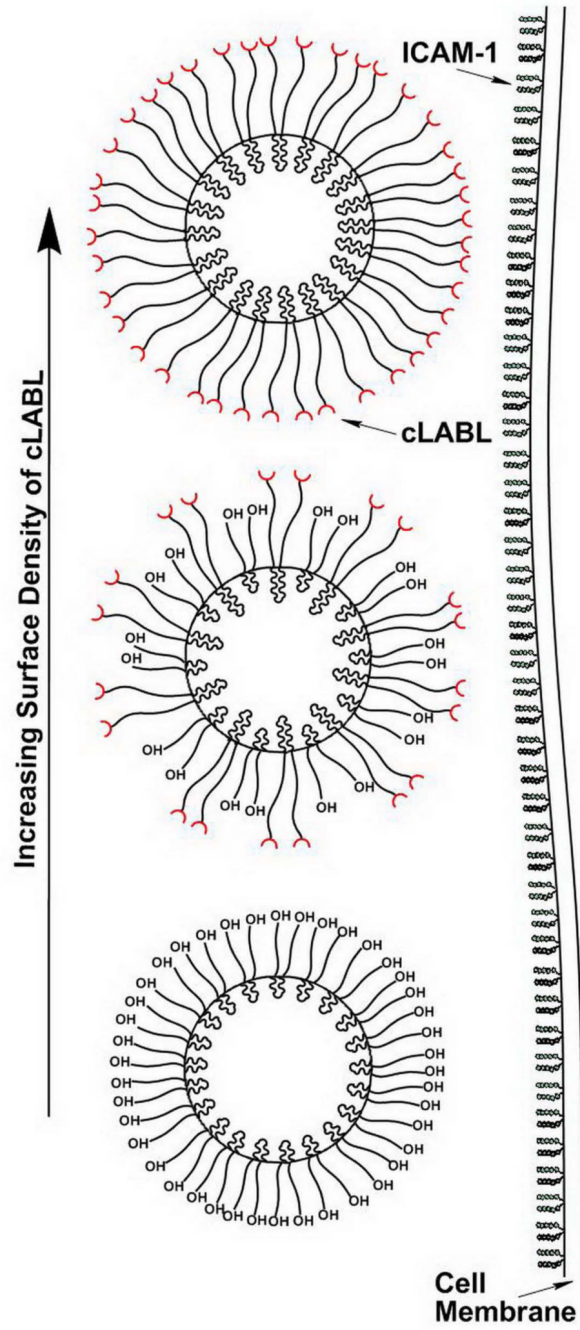


Figure 1.

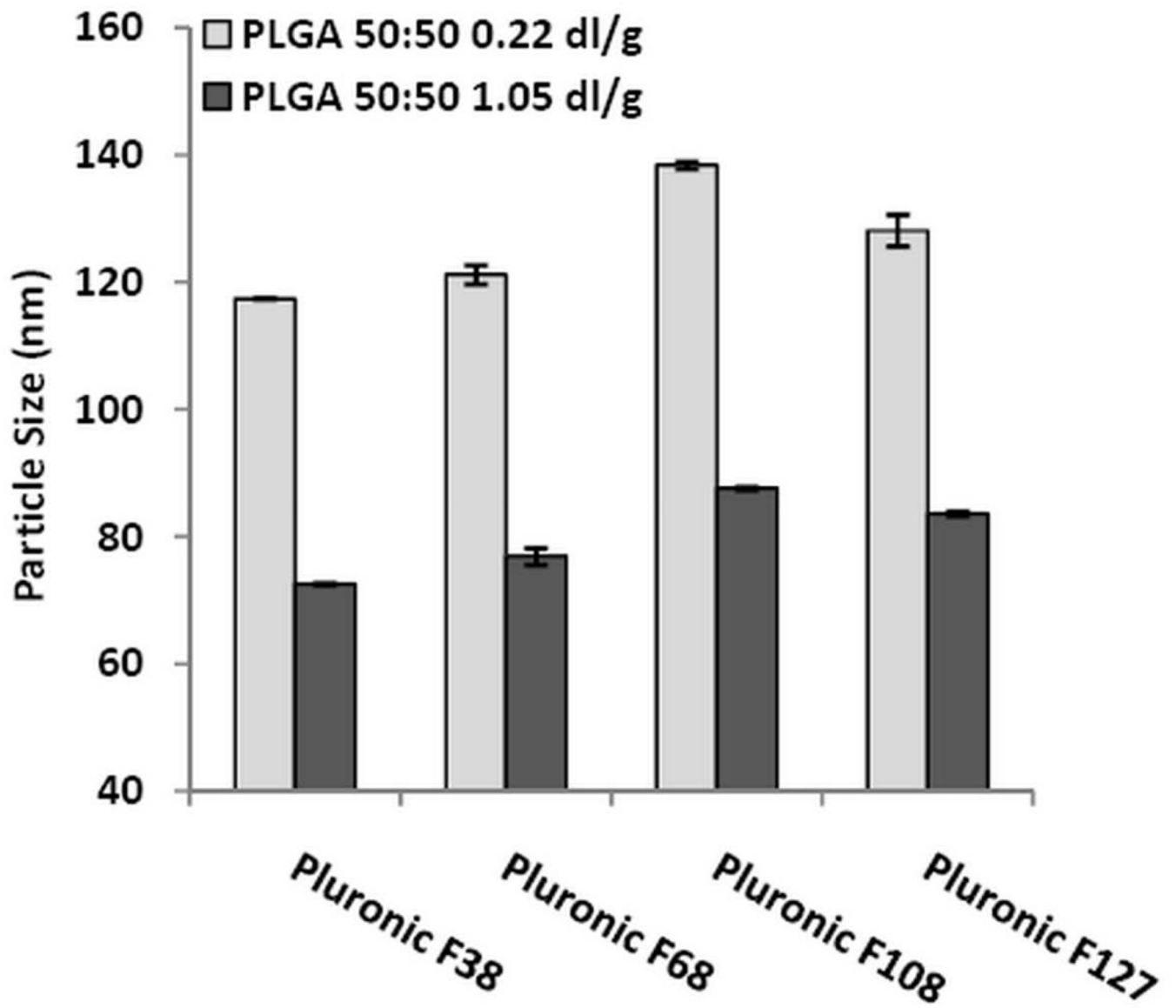


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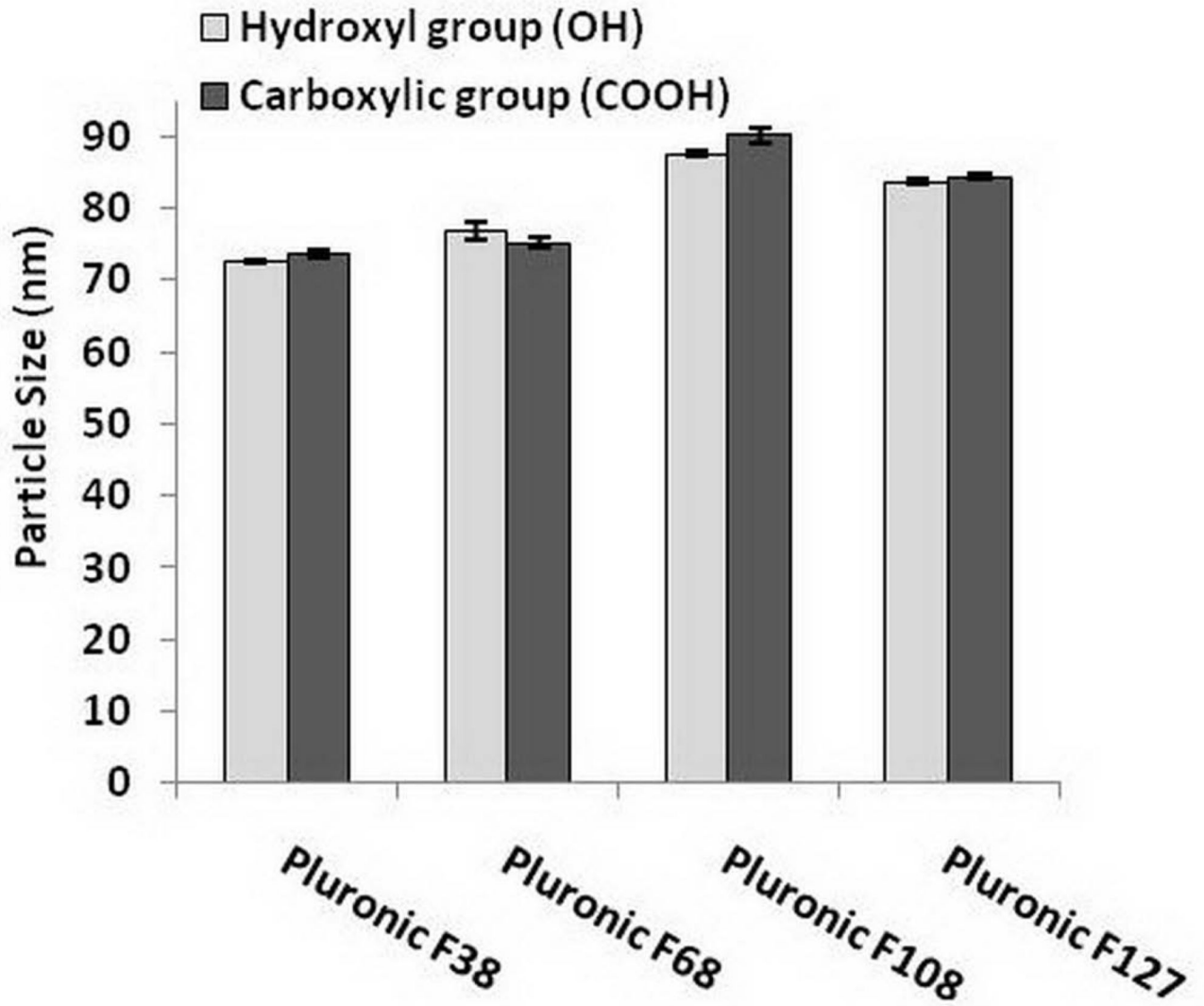


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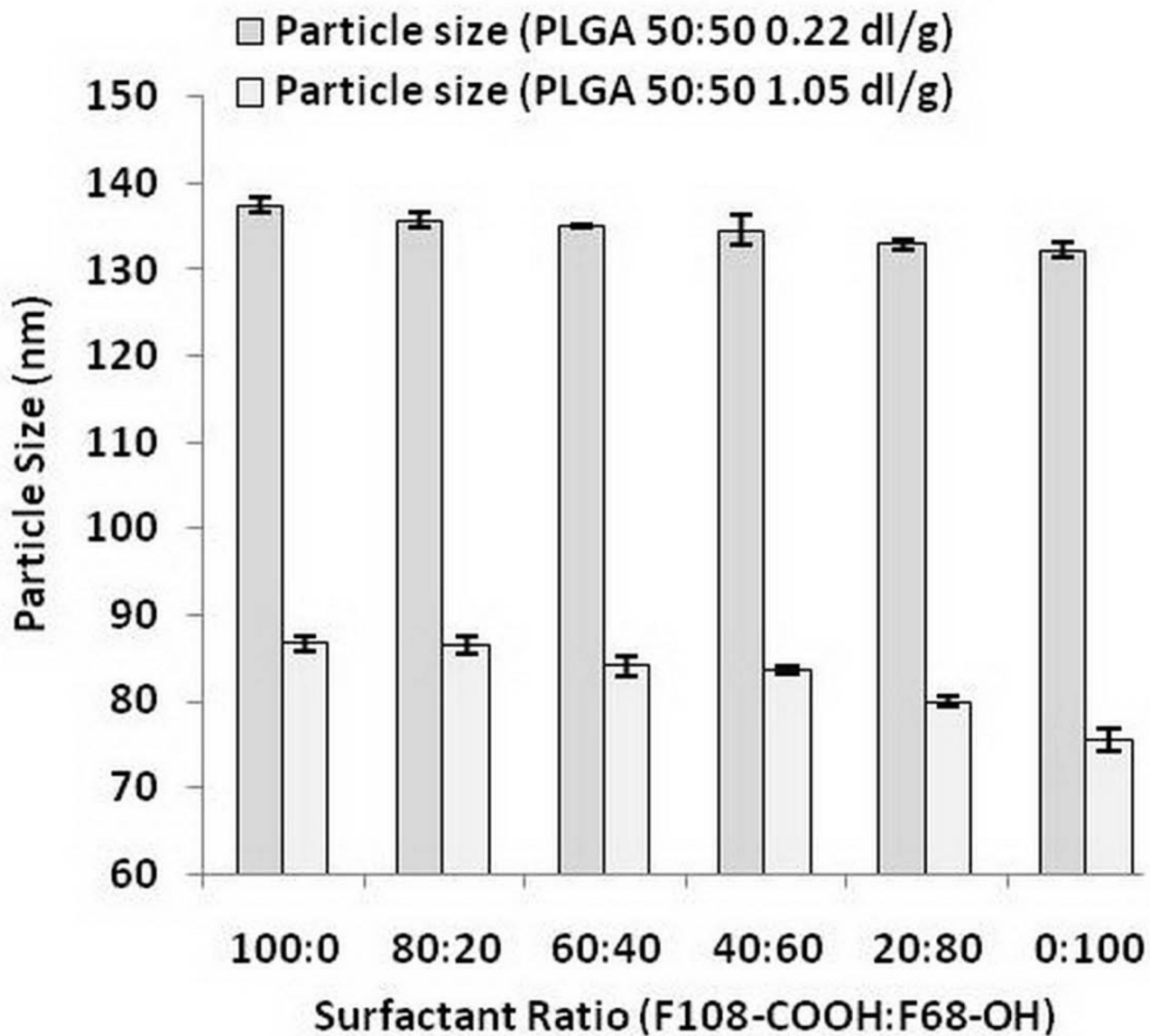


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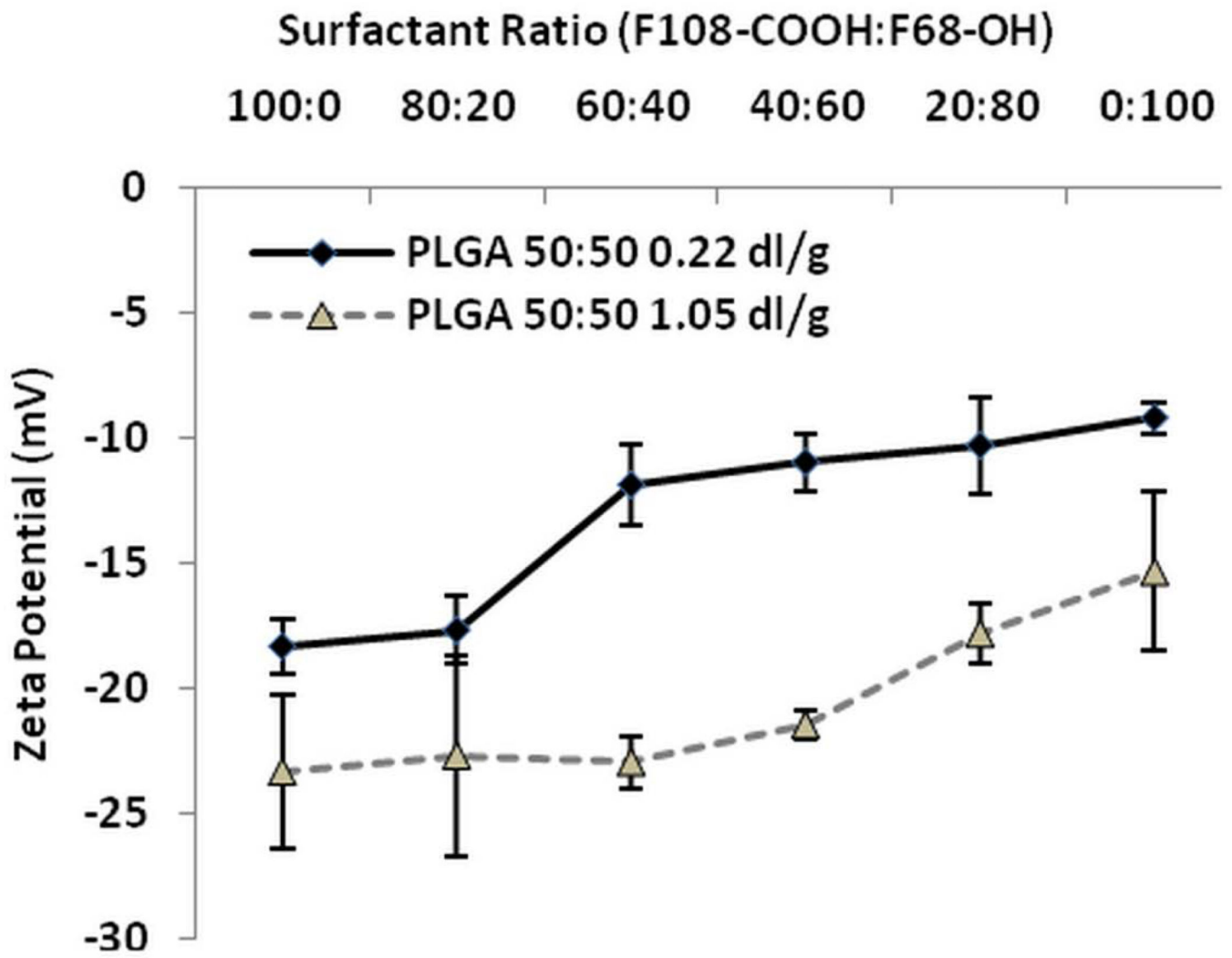
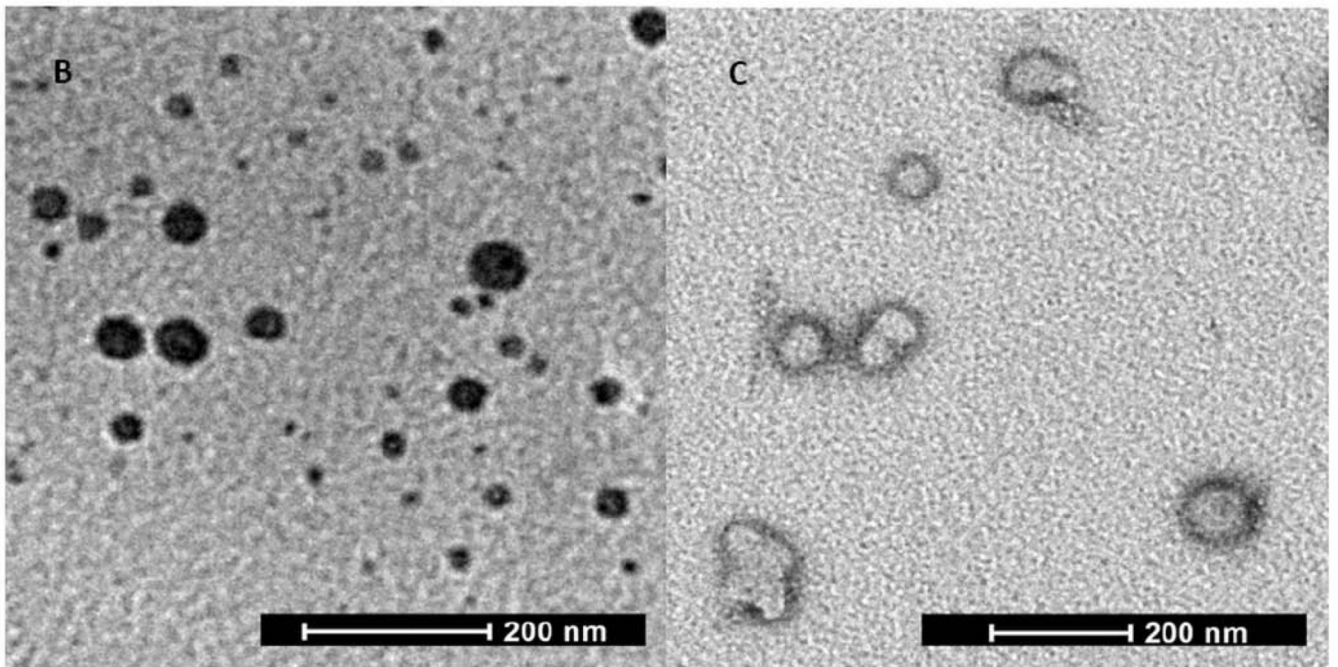
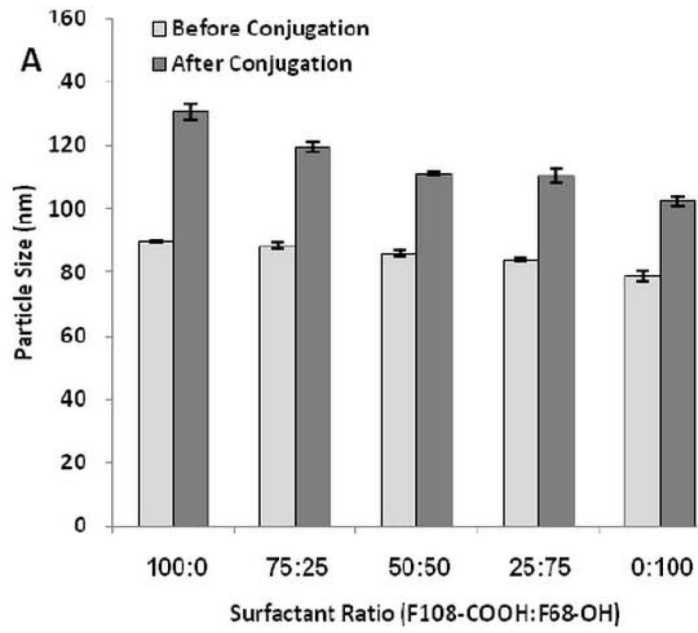


Figure 5.



**Figure 6.**

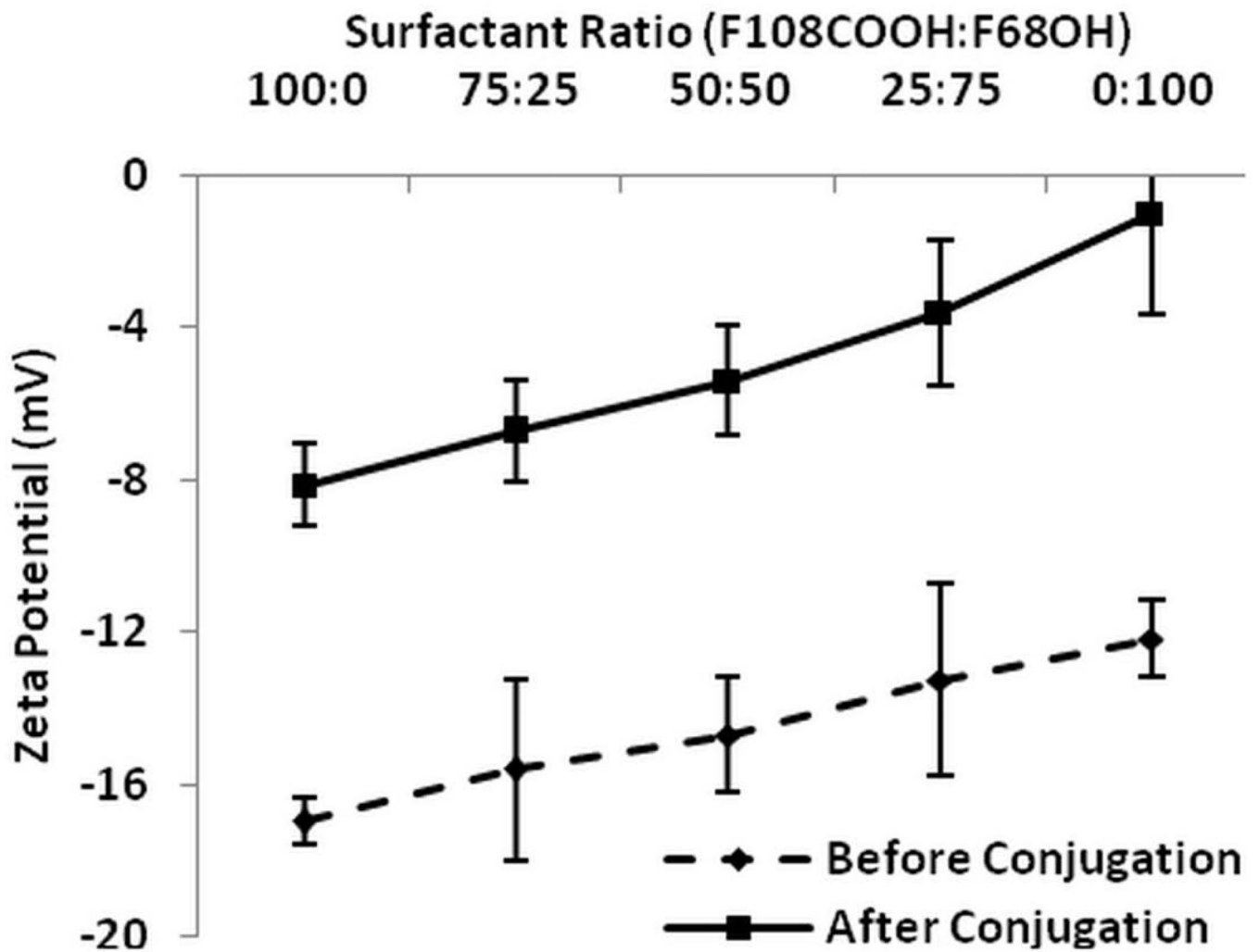


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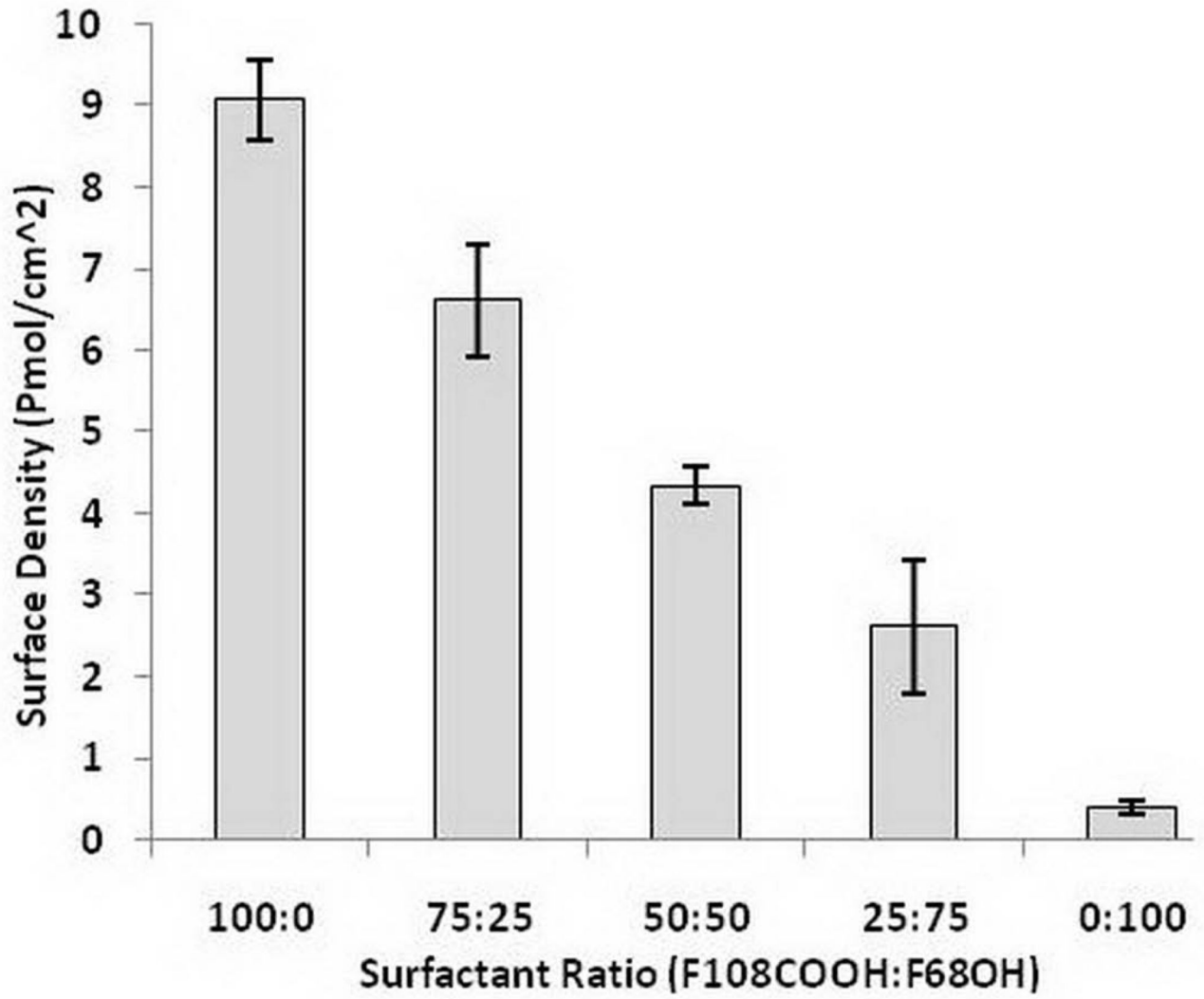


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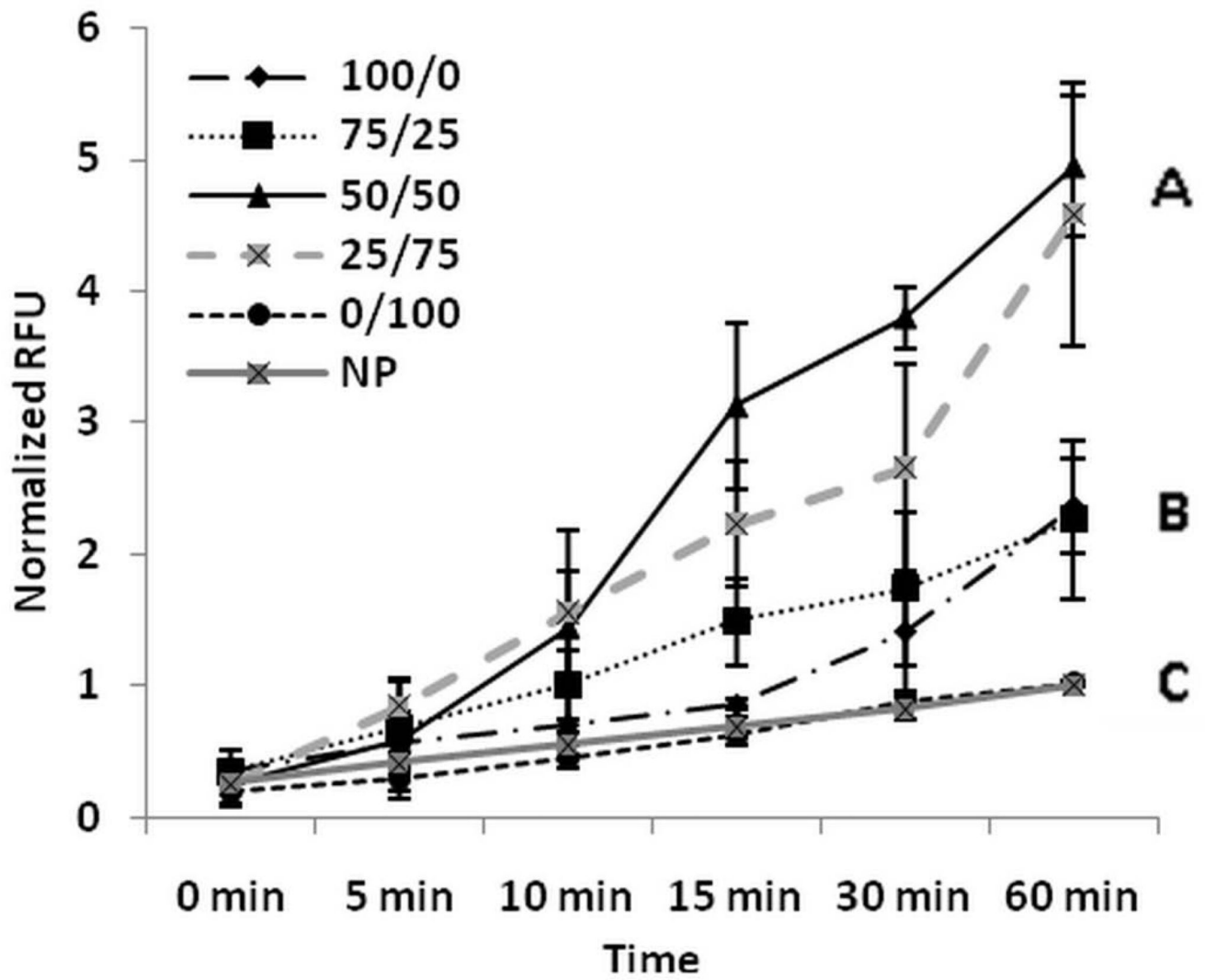


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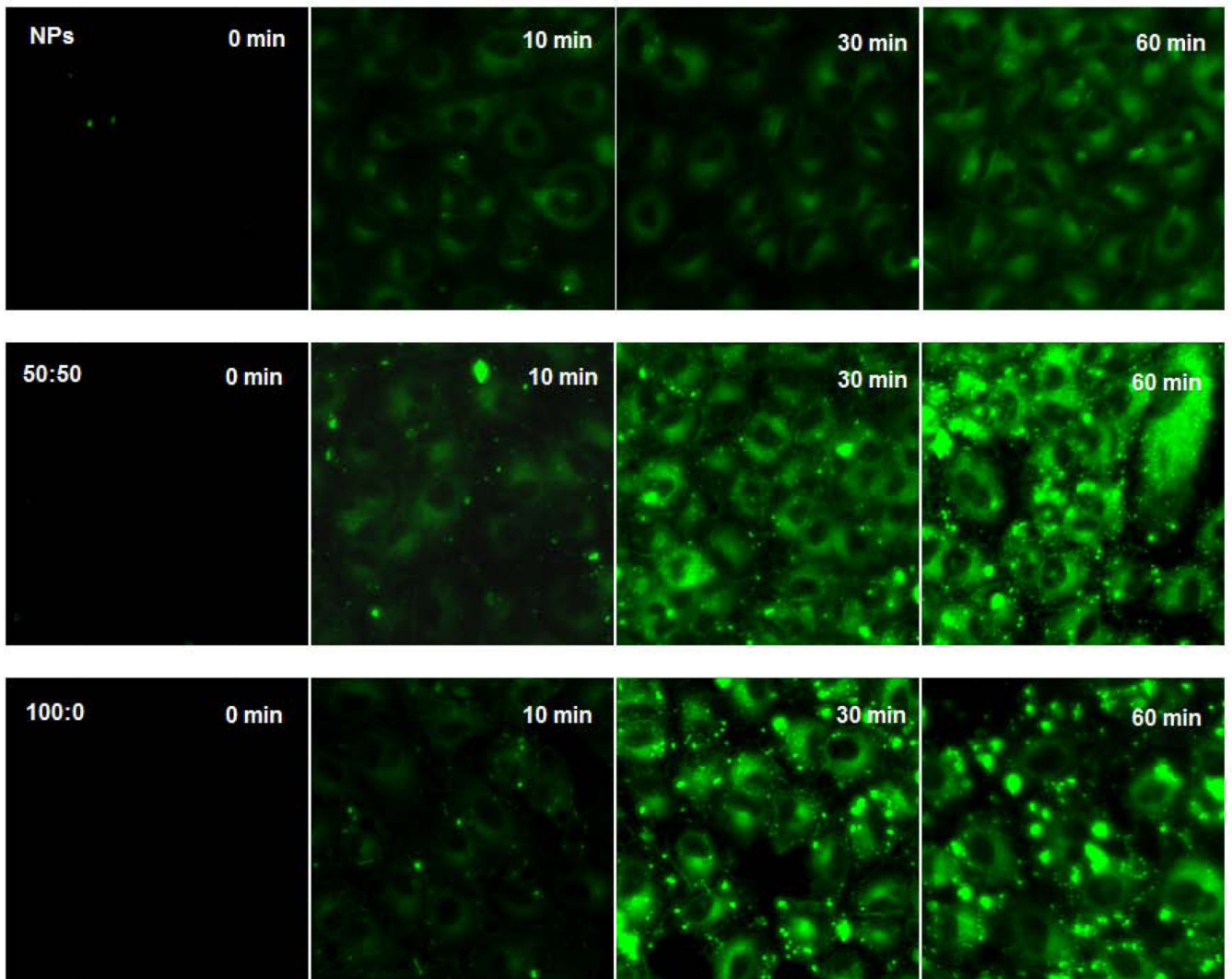


Figure 10.

**Table1**

Summary of the different types of Pluronic® used to make PLGA nanoparticles<sup>19,20</sup>.

Pluronic®	Repeats (A-B-A)*	Approximate M <sub>w</sub>	HLB**	Coating Layer (Å)
F38	46-16-46	5000	30.5	58
F68	75-30-75	8350	29	76
F108	128-54-128	14000	27	154
F127	98-67-98	11500	22	119

\* A is the units of poly(ethylene oxide) and B is the units of poly(propylene oxide) in Pluronic structure

\*\* HLB: Hydrophilic lipophilic balance of the surfactant

Table 2

Particle size and zeta potential of NP formulations. The table compares particle size and zeta potential for PLGA NPs before conjugation, NPs conjugated with cLABL, NPs with Coumarin-6, and NPs with Coumarin-6 conjugated with cLABL for all surfactant ratios

Pluronic® F108COOH: Pluronic® F68OH Ratio	PLGA NPs		NPs+cLABL		NPs+Coumarin-6		NPs+Coumarin-6 +cLABL	
	Size (nm)	Zeta Potential (mV)	Size (nm)	Zeta Potential (mV)	Size (nm)	Zeta Potential (mV)	Size (nm)	Zeta Potential (mV)
100:0	90±0.2	-16.98±0.61	130.7±2.7	-8.12±1.05	127.1±1.1	-16.16±1.36	158.7±4.1	-6.97±2.10
75:25	88.3±1.2	-15.61±2.37	119.6±1.8	-6.73±1.33	117.3±2.3	-15.84±1.11	141.34±2.5	-4.17±2.28
50:50	86±0.8	-14.69±1.53	111.2±0.8	-5.38±1.45	110.8±1.7	-14.53±2.16	136.3±3.1	-3.20±1.55
25:75	84±0.4	-13.26±2.54	110.4±2.3	-3.58±1.92	96.8±2.6	-13.78±0.81	132.8±4.4	-2.82±1.03
0:100	78.7±1.5	-12.16±1.02	102.3±1.5	-0.98±2.67	90.3±1.2	-11.27±2.59	129.5±3.3	-1.02±2.11

Values represented mean±S.D (n=3). Polydispersity < 0.14 for NPs, < 0.20 for NPs+cLABL, < 0.20 for NPs+Coumarin-6, < 0.23 for NPs+Coumarin-6+cLABL.