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Synthesis and Characterization of Lipid-Polymer Hybrid Nanoparticles with pH-Triggered PEG Shedding

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Abstract

Novel lipid-polymer hybrid nanoparticles are designed with a poly(ethylene glycol) coating that is shed in response to a low pH trigger. This allows the nanoparticles to be stable during systemic circulation and at neutral pH, but destabilize and fuse with lipid membranes in acidic environments. The hybrid nanoparticles consist of a poly(lactic-co-glycolic acid) core with a lipid and lipid-PEG monolayer shell. To make the hybrid nanoparticles pH sensitive, a lipid-(succinate)-mPEG conjugate is synthesized to provide a hydrolysable PEG stealth layer that is shed off the particle surface at low pH. The pH-sensitivity of the nanoparticles is tunable using the molar concentration of the lipid-(succinate)-mPEG incorporated in the lipid shell of the particles. Possible uses of these pH-sensitive nanoparticles include aggregating in the acidic tumor microenvironments, escaping the acidified endosomes, or aggregating in deep lung tissue for improved inhalation administration.

Keywords

Drug delivery; Lipid-PEG conjugate; Acid-cleavable linker; Nanoparticle destabilization; Fusogenic nanoparticles

Introduction

Nanotechnology is becoming increasingly successful in advancing the state of the art in drug delivery.[1] The ability to deliver drugs more effectively and efficiently to the site of interest translates into less harmful systemic side effects and more beneficial therapeutic action.[2] This ability is particularly useful in the fight against cancer, where harmful side effects limit the tolerable dose of chemotherapeutics.[3] Using polyethylene glycol (PEG) has become a popular strategy to create long-circulating drug delivery nanoparticles by reducing protein adsorption, macrophage uptake, and particle aggregation, thus increasing systemic circulation lifetime.[2, 4, 5] Although useful in increasing circulation half-life, the PEG layer may become a detriment upon reaching the target tissue, hindering the entry of the nanoparticle into the cell or preventing its escape from the endosome after being endocytosed.[6]

A drug delivery vehicle with pH-sensitive PEG shedding would be especially useful in cancer drug delivery by exploiting the slightly acidic extracellular space of tumors (around pH ~ 6.5).[7, 8] Upon arrival at the tumor site, a correctly tuned pH-sensitive particle would be able to shed its PEG coating, thus enabling it to fuse with the cell membrane and be

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internalized.[9] Additionally, nanoparticles taken up via the endosomal pathway can be tuned to lose the protective PEG coating upon acidification of the late endosome or early lysosome[10]. Fusing with the endosomal membrane then becomes possible and escape from the endosome into the cytoplasm can be achieved.[11, 12] Endosomal escape is especially critical for the delivery of degradable payloads like siRNA, proteins, and other biologics that are typically degraded inside the highly acidic lysosome.[13] Previous research has shown PEG shedding to improve intracellular drug delivery using polymersomes[14] and polyplex micelles.[15] Other PEG shedding molecules that rely on the reduction of disulfide bonds have been used in liposomes and lipoplexes to some success.[16] More recently, Gao *et al*. have demonstrated a technique to directly observe PEG shedding using a pair of dye and quencher, confirming the benefits of PEG shedding to intracellular delivery.[17]

Herein we report a novel approach to enable pH-triggered PEG shedding from lipid-polymer hybrid nanoparticles by using a lipid-(succinate)-mPEG conjugate that is highly sensitive to acidic hydrolysis via a di-ester succinate linker between the lipid and PEG moieties. The relatively slow hydrolysis rate of the di-ester bond allows the PEG shedding to be more finely tuned, offering controllable shedding over a wide range of pH values unavailable with other strategies. The shedability of the lipid-(succinate)-mPEG layer was tested using a previously developed drug delivery platform, lipid-polymer hybrid nanoparticles. The hybrid nanoparticles have been demonstrated to be an effective drug delivery vehicle with controllable drug loading and release characteristics,[18] superior *in vitro* and *in vivo* stability,[19–21] and excellent scalability for large scale production.[22]

Experimental Section

Materials

Poly(lactic-co-glycolic acid) (PLGA, 50:50, 0.82 dL/g) was purchased from Lactel Absorbable Polymers (Pelham, AL). All lipids including 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dipalmitoyl-sn-glycero-3-phospho (ethylene glycol) (PtdEG), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG carboxylic acid) were purchased from Avanti Polar Lipids (Alabaster, AL). Oleic acid was purchased from Alfa Aesar (Ward Hill, MA). Methoxy poly(ethylene glycol) (M_W = 2000 Da, mPEG-2000) was purchased from Sigma-Aldrich (St. Louis, MO) and modified in our lab to have a carboxyl end group, making mPEG-COOH. *N*,*N*'-diisopropyl carbodiimide (DIPC), *p*-toluenesulfonic acid monohydrate, tetrahydrofuran (THF), and *N*,*N*-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis of lipid-(succinate)-mPEG conjugate

The pH sensitive lipid-(succinate)-mPEG was synthesized by conjugating mPEG-COOH (M_w = 2000 Da) to a PtdEG phospholipid molecule using a dual-ester bond linkage. The synthesis reaction was carried out as follows. First, 4-(*N*,*N*-dimethylamino) pyridinium-4-toluenesulfonate (DPTS) was prepared by mixing equal parts of saturated tetrahydrofuran (THF) solutions of *N*,*N*-dimethylaminopyridine (DMAP) and *p*-toluenesulfonic acid monohydrate at room temperature. The precipitate was filtered, washed three times with THF and dried under vacuum. Next, 5 mg of PtdEG was dissolved in chloroform at 10 mg/mL. mPEG-COOH was also dissolved in chloroform at 10 mg/mL and mixed with the PtdEG at a molar ratio of 2 mPEG-COOH:1 PtdEG. Then DPTS at a molar ratio of 6 DPTS: 1 PtdEG was added to the chloroform mixture and vortexed vigorously for 5 minutes. An equalvalent amount of DIPC as that of DPTS was then slowly added into the solution while stirring. The reaction was allowed to proceed overnight at room temperature while stirring. After the reaction was complete, the volume of chloroform was reduced using a dry argon

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stream and the lipid-(succinate)-mPEG product was precipitated with cold ether. The precipitate was then resuspended in chloroform and washed three times with saturated brine. The chloroform layer was collected and used as a stock solution for subsequent studies. NMR spectroscopy was carried out to characterize the produced lipid-(succinate)-mPEG conjugate. ^{1}H NMR ($CDCl_3$): δ = 0.87 (-CH_3, a), 1.25–1.13 (-CH_2, Lipid, b), 1.65 (-CH_2, Lipid, c), 2.3 (-CH_2-COOR, d), 2.65 (-CH_2-CH_2-, succinate, f), 3.1 (-COH, i), 3.3 (-OCH_3, h), 3.64 (-CH_2-CH_2-, PEG, g), 4.13 (-CH_2-COH-CH_2-, e) ppm.

Synthesis of lipid-polymer hybrid nanoparticles

To prepare lipid-polymer hybrid nanoparticles, DOPE/oleic acid (molar ratio = 4:1, 91.4 μg of DOPE and 8.7 μg of oleic acid to make 1 mg of PLGA particles), or DOPE/oleic acid/lipid-(succinate)-mPEG were dissolved in THF and added to water at desirable molar ratios. The amount of lipid-(succinate)-mPEG was varied and DOPE was reduced by a corresponding molar amount. The lipid solution in H_2O/THF was heated to 68°C while stirring. The PLGA polymer was dissolved in THF at 1 mg/mL and 1mL was added dropwise to the heated lipid solution while stirring. The mixture solution was then vortexed for 3 minutes at high speed and then 1 mL of additional water was added dropwise to the solution. The solution was allowed to stir for 2 hours in a chemical hood to allow the organic solvent to evaporate and the nanoparticles to solidify. The nanoparticles were then washed three times using an Amicon Ultra centrifugal filter (Millipore, Billerica, MA) with a molecular weight cutoff of 10 kDa. The nanoparticles were then collected and suspended to 1 mL of water. The nanoparticle size and surface zeta potential were obtained from three repeat measurements using a dynamic light scattering (Malvern Zetasizer, ZEN 3600) with backscattering angle of 173°.

pH sensitivity of nanoparticles

In order to test the pH sensitivity of the nanoparticles, particles with lipid-(succinate)-mPEG replacing 15% of DOPE were suspended in either a pH = 7.4 buffer (PBS) or a pH = 5 buffer (potassium hydrogen phthalate/NaOH). As a control, nanoparticles with DSPE-PEG replacing 15% of DOPE were suspended in the same pH = 7.4 and 5 buffers. The DSPE-PEG was chosen as a negative control because the PEG is linked to the lipid via an amide bond, which is more stable than the synthesized ester bond of the lipid-(succinate)-mPEG. The size of the nanoparticles was measured by dynamic light scattering after 1, 3, 6, and 24 hours of incubation to determine the stability of the particles. In addition, nanoparticles with different amounts of lipid-(succinate)-mPEG replacing the DOPE were tested in different pH buffers ranging from pH = 7.4 to pH = 3. The size of the particles in the different buffers was measured after 20 hours of incubation using dynamic light scattering. The morphology and size of the particles were further characterized using scanning electron microscopy (SEM). Samples for SEM were prepared by dropping 5 μL of a dilute nanoparticle solution onto a polished silicon wafer. After drying the droplet at room temperature overnight, the sample was coated with chromium and then imaged by SEM. In order to adjust the pH value of the dilute nanoparticle solution without adding excess salts, 1 mM HCl was titrated in until the solution reached pH = 5.

Results and Discussion

Figure 1 shows a schematic representation of the lipid-polymer hybrid nanoparticle system as designed. The nanoparticle core is made of a hydrophobic polymer and is used to encapsulate poorly water soluble and difficult to deliver therapeutic agents. In this study, poly(lactic-*co*-glycolic acid) (PLGA) was used to form the particle core for its biocompatibility and biodegradability. A lipid monolayer consisting of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and oleic acid is self-assembled onto the surface of

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the polymeric core to solubilize it in aqueous environments and also to serve as a diffusion barrier against encapsulated drugs leaching out. In addition, the DOPE/oleic acid formulation was chosen for its fusogenic properties[23–26], which are revealed upon the shedding of the PEG layer. The lipid-(succinate)-mPEG molecules form a “stealth” corona on the surface of the hybrid nanoparticle with the phospholipid portion of the lipid-(succinate)-mPEG inserting into the DOPE/oleic acid monolayer. It is expected that the resulting nanoparticle is sterically stabilized prior to PEG shedding, while it becomes unstable and fusogenic when the PEG layer comes off at acidic pH values.

Figure 2A shows the scheme for synthesizing the lipid-(succinate)-mPEG molecule by conjugating mPEG-COOH (Mw = 2000 Da) to a 1,2-dipalmitoyl-sn-glycero-3-phospho(ethylene glycol) (PtdEG) phospholipid molecule via a dual-ester bond linkage. Sequential ester bonds in the resulting lipid-(succinate)-mPEG conjugate increase the sensitivity of the conjugate to hydrolysis by offering two hydrolysable ester bonds capable of releasing the PEG from the phospholipid. When one of the ester bonds is activated through protonation, it becomes more electrophilic, which will then catalyze the hydrolysis of the neighboring ester bond. Upon the hydrolysis of either ester bond, the PEG molecule is shed. The production of lipid-(succinate)-mPEG conjugate was first confirmed by ^{1}H NMR spectroscopy with all the characteristic peaks of PtdEG, mPEG and the di-ester bond, respectively, as indicated in Figure 2B.

The resulting lipid-(succinate)-mPEG was then used to replace a molar percentage of DOPE in the DOPE/oleic acid monolayer to prepare lipid-polymer hybrid nanoparticles following a previously described method that takes advantage of nanoprecipitation and lipid self-assembly in order to create a simple and scalable manufacturing process.[22, 27, 28]. Figure 3 summarizes the measured hydrodynamic size of the hybrid nanoparticles with various amounts of lipid-(succinate)-mPEG incorporated into the lipid shell. By simply varying the amount of lipid-(succinate)-mPEG, we are able to synthesize particles within the desired size range of 50~150 nm. Particle size predictably decreases with increasing amounts of the stabilizing lipid-(succinate)-mPEG, reaching a minimum size of 64 nm when 50 mol% lipid-(succinate)-mPEG is used. When only 15 mol% lipid-(succinate)-mPEG is used the size is 123 nm. Note that extensive studies have been reported that particles with a hydrodynamic size around 100 nm or less are desirable for systemic drug delivery because of their favorable *in vivo* pharmacokinetics and tumor accumulation.[29–31] In this study, the threshold of stability for the lipid-(succinate)-mPEG incorporated nanoparticle formulation was determined to be 10% lipid-(succinate)-mPEG incorporation. Having 10% lipid-(succinate)-mPEG incorporation resulted in unstable particles, even at neutral pH (data not shown) while more than 10% lipid-(succinate)-mPEG resulted in a stable formulation (Figure 3). It is assumed that at 10% incorporation or less, the steric stabilization is not sufficient with so few PEG molecules on the surface. This stability threshold can be used in the subsequent studies to estimate the amount of dissociated PEG by observing when the particles became unstable. It is assumed that the point of destabilization reflects the point at which the particles pass through the stability threshold of approximately 10% lipid-(succinate)-mPEG still present on the nanoparticle surface.

The pH-sensitive stability of the synthesized hybrid nanoparticles was first examined by scanning electron microscopy (SEM) to image the size and morphology of the particles at different pH values. As shown in figure 4A, the particles with 15 mol% lipid-(succinate)-mPEG at pH = 7.4 show individually dispersed particles that are spherical in shape with little to no aggregation. The morphology of the particles appears smooth. In contrast, when the particles were titrated with HCl to pH = 5, they appear clumped and aggregated indicative of a destabilized lipid shell. As shown in figure 4B, the majority of the particles have aggregated into clumps at least several hundred nanometers in size with only a few

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individually dispersed particles visible. Individual spherical particles can be seen to make up the aggregates; however, the overall shape and morphology is flat and lumpy. Once the linking ester bond is hydrolyzed and the stabilizing PEG coating attached to the lipid shell is released, the particles are free to contact other particles and the lipid layers can fuse, causing aggregation. The resulting structure consists of multiple PLGA particles clumped and surrounded by a most likely multi-lamellar lipid layer. This result suggests the fusogenic properties of the hybrid nanoparticles after the PEG layer is shed.

Next, we examined the time-dependent particle aggregation of the 15 mol% lipid-(succinate)-mPEG nanoparticles at pH=7.4 and 5 over 24 hours. As shown in figure 5, the hydrolysis of the ester bonds is shown to proceed over the course of several hours. The particles with 15 mol% lipid-(succinate)-mPEG show only a slight size increase from 107 nm to 135 nm at pH = 7.4 over the 24 hour period. This confirms the slow rate of hydrolysis expected at neutral pH. At pH = 5, the 15% lipid-(succinate)-mPEG particles show a dramatic and rapid increase in size due to aggregation. After 1 hour the size had already increased to 905 nm and the size continues to increase over 6 hours. For the control samples, instead of using lipid-(succinate)-mPEG, 15 mol% DSPE-PEG was used to replace the corresponding amount of DOPE in the DOPE/oleic acid lipid shell. It was found that the size of these control particles remained constant at 65 nm at both pH =7.4 and 5 conditions for the duration of the 24 hour test. The lack of size increase in the control particles which have PEG linked to the lipid shell via an amide bond rather than the more easily hydrolysable ester bond indicates that the mechanism triggering the aggregation is the depletion of the PEG layer due to ester hydrolysis.

Finally, the stability of the hybrid nanoparticles at different pH values was correlated to the amount of lipid-(succinate)-mPEG incorporated into the lipid shell of the particles. Hydrolysis of the di-ester bond that links the mPEG to the PtdEG lipid occurs under either acidic or basic conditions but very slowly at neutral pH. Figure 6 shows the size of particle aggregates with varying amounts of pH-sensitive lipid-(succinate)-mPEG conjugate incorporated into the lipid shell incubated for 20 hours over a range of pH values. The greater the amount of lipid-(succinate)-mPEG incorporated into the particle's lipid shell, the more stable the particles become, even at low pH, thus, tuning the pH sensitivity of the particles. With only 15 mol% lipid-(succinate)-mPEG, the particles completely destabilize when the pH is lowered to 6, resulting in large micron sized aggregates (average size 1370 nm). The 20 mol% lipid-(succinate)-mPEG particles begin to destabilize at pH 6 forming dimers and trimers (average size 263 nm) but do not fully aggregate until the pH is lowered to 5. The 20 mol% lipid-(succinate)-mPEG particles are said to be fully sensitive to acidic environments at or below pH = 5. The 30 mol% lipid-(succinate)-mPEG particles show full destabilization at pH = 4. Particles with 40 mol% lipid-(succinate)-mPEG begin to destabilize at pH = 3 increasing in size from 97 nm to 175 nm but do not show full destabilization. The 50 mol% lipid-(succinate)-mPEG particles do not show any measurable destabilization over the pH range tested. These results demonstrate that the more lipid-(succinate)-mPEG on the surface of the particles, the more ester bonds must be hydrolyzed before the PEG layer becomes sufficiently depleted to allow the aggregation of particles. Moreover, it is worth noting that the di-ester bond used in the present study produces a wider range of pH values for which the nanoparticles can be destabilized, ranging from even smaller changes in pH than those observed with diorthoester linkers[32] down to very acidic pH values of 3 or lower. This allows the nanoparticles to be tuned specifically for the application and gives the drug delivery designer greater flexibility.

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Conclusions

We synthesized a novel lipid-(succniate)-mPEG conjugate, of which the PEG moiety can be shed readily at acidic conditions. By incorporating lipid-(succinate)-mPEG onto the surface of lipid-polymer hybrid nanoparticles, the particles are made fusogenic, triggered by a reduction in pH value. The amount of lipid-(succinate)-mPEG incorporated into the lipid shell of the hybrid nanoparticles determines the pH value at which the particles destabilize and begin to aggregate. We found that the higher the amount of lipid-(succinate)-mPEG incorporated into the particle's lipid shell, the more stable the particles become, even at low pH. The wide range of pH values for which the particles can be tuned to shed the PEG coating adds potential functionality to the nanoparticle drug delivery toolkit. The aggregation of the lipid-coated polymeric nanoparticles indicates a promising method of fusing nanoparticles with cellular or endosomal membranes, making drug delivery more efficient and effective. Moreover, the novel lipid-(succinate)-mPEG conjugate may be used to make a range of other drug delivery vehicles, including polymeric micelles, liposomes, lipoplexes, and nanoemulsions, environmentally sensitive.

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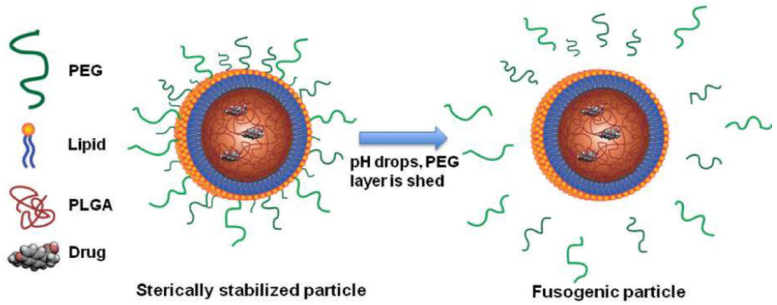

Figure 1.
Schematic of a lipid-polymer hybrid nanoparticle with pH-triggered PEG shedding. The hybrid nanoparticle consists of a drug-loaded PLGA polymeric core, a fusogenic DOPE/oleic acid monolayer shell, and a sterically stabilizing PEG corona. The PEG layer comes off in response to environmental acidity, making the particle fusogenic toward lipid membranes.

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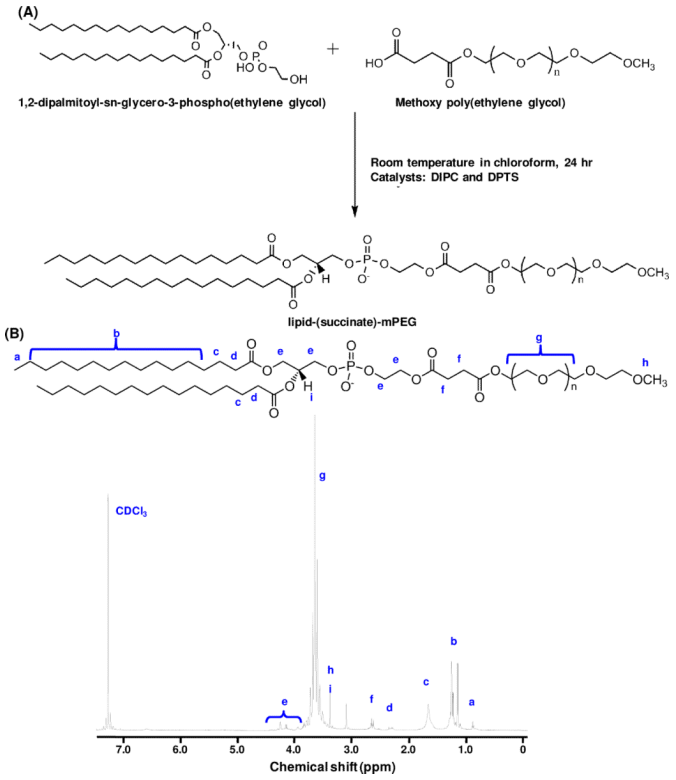

Figure 2.

Synthesis and characterization of lipid-(succinate)-mPEG conjugate. (A) Schematic description of the synthesis of lipid-(succinate)-mPEG conjugate that is sensitive to acidic pH. (B) ^{1}H NMR spectrum of the synthesized lipid-(succinate)-mPEG conjugate.

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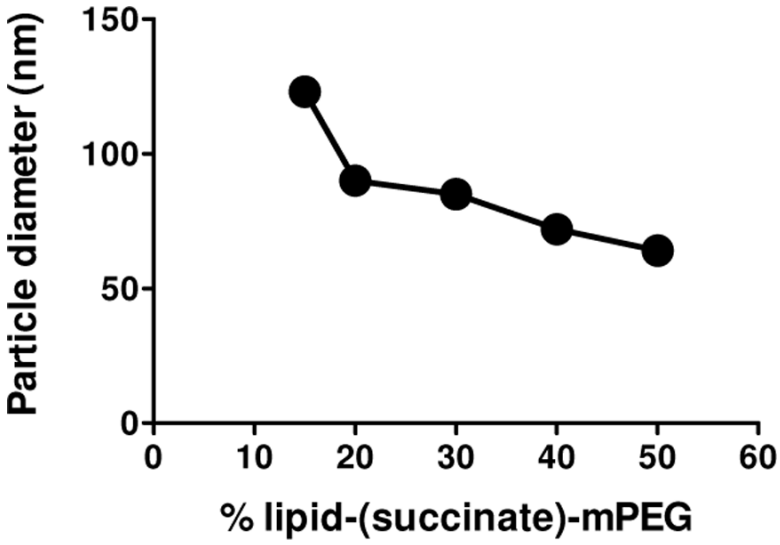

Figure 3.
Hydrodynamic size (diameter, nm) of the lipid-polymer hybrid nanoparticles synthesized with various amounts of lipid-(succinate)-mPEG replacing a molar percentage of DOPE lipid in the lipid monolayer shell. All samples are at pH = 7.4.

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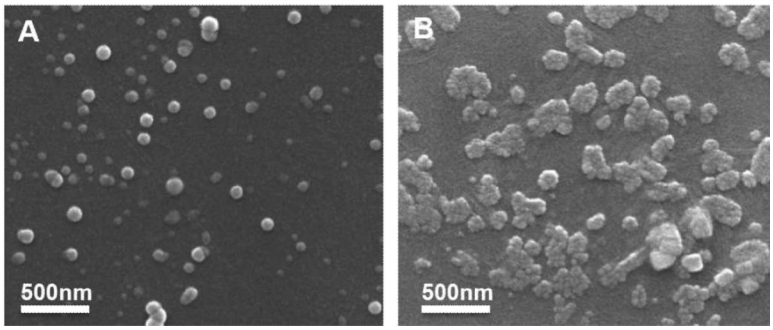

Figure 4.
Scanning electron microscopy (SEM) images of lipid-polymer hybrid nanoparticles, in which lipid-(succinate)-mPEG makes up 15 mol% of the lipid monolayer shell. (A) At pH = 7.4, isolated particles show spherical morphology and little aggregation. (B) At pH = 5, particles have aggregated and show irregular morphology.

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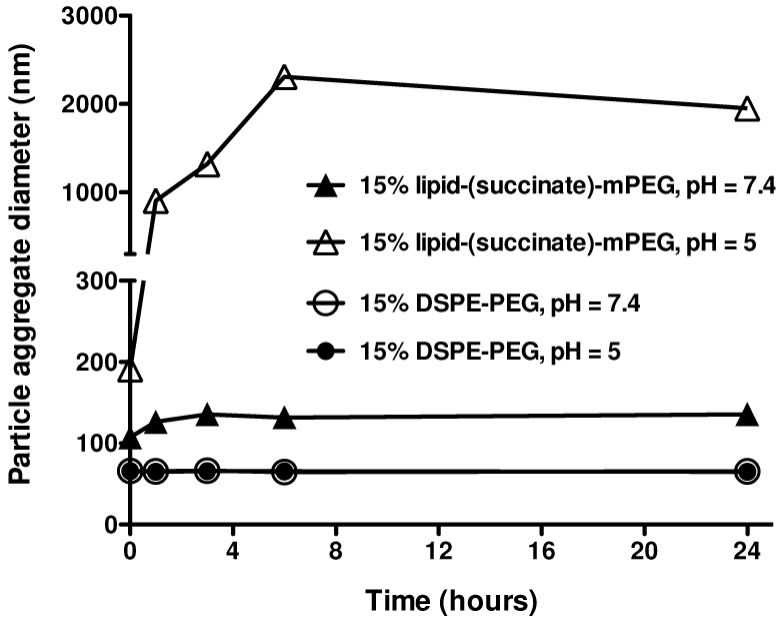

Figure 5.

Time dependent particle aggregation at pH = 7.4 and pH = 5 over 24 hours. Lipid-(succinate)-mPEG was incorporated into the lipid monolayer shell at a molar ratio of 15%. Nonhydrolyzable DSPE-PEG was used in the same ratio as a negative control. At pH = 7.4, both formulations are stable over the 24 hours, with the lipid-(succinate)-mPEG particles increasing in size by only 28 nm (from 107 nm to 135 nm). At pH = 5, the DSPE-PEG particles remain stable while the lipid-(succinate)-mPEG particles begin to aggregate immediately and continue for 6 hours before plateauing at around 2000 nm.

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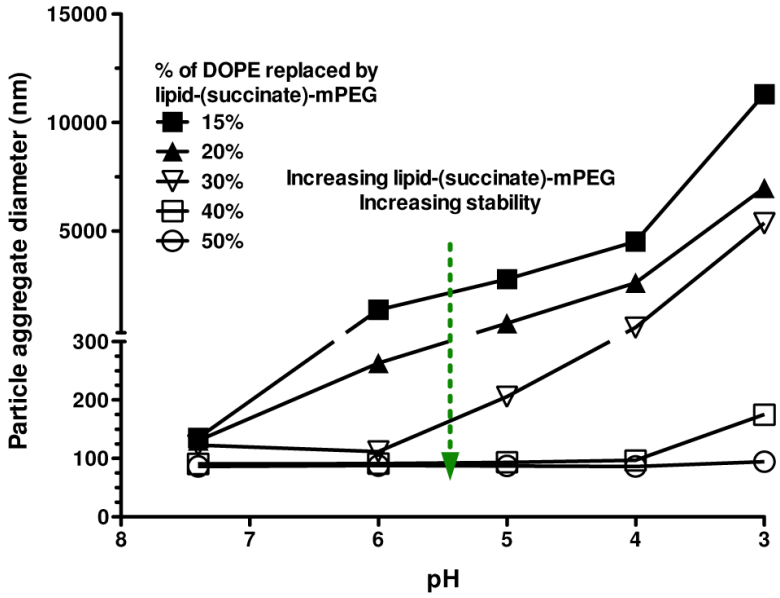

Figure 6.

Particle aggregate size (diameter, nm) at varying pH values after 20 hours incubation. Lipid-polymer hybrid nanoparticles show increasing stability with increasing amounts of lipid-(succinate)-mPEG incorporated to the lipid monolayer shell. With 15 mol%, complete aggregation occurs at pH = 6. With 20 mol%, 30 mol% and 40 mol% lipid-(succinate)-mPEG, aggregating begins at pH = 6, 5, and 3, respectively. With 50 mol% lipid-(succinate)-mPEG, aggregation is not observed over the range of pH values tested.