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Nanospheres formulated from L-tyrosine polyphosphate as a potential intracellular delivery device

Andrew J. Ditto[a], Parth N. Shah[b], Stephanie T. Lopina[b], Yang H. Yun[a,*]

[a] Department of Biomedical Engineering, The University of Akron, Sidney Olson Research Center, Akron, OH 44325, USA
[b] Department of Chemical & Biomolecular Engineering, The University of Akron, Whitby Hall, Akron, OH 44325, USA

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ABSTRACT

Current delivery devices for drugs and genes such as films and microspheres are usually formulated from polymers that degrade over a period of months. In general, these delivery systems are designed to achieve an extracellular release of their encapsulated drugs. For drugs that require interaction with cellular machinery, the efficacies of both macroscopic and microscopic delivery systems are normally low. In contrast, nano-sized drug delivery vehicles could achieve high delivery efficiencies, but they must degrade quickly, and the delivery system itself should be nontoxic to cells. In this aspect, biodegradable nanospheres formulated from L-tyrosine polyphosphate (LTP) have been produced from an emulsion of oil and water for the potential use as an intracellular delivery device. Scanning electron microscopy (SEM) and dynamic laser light scattering (DLS) show that LTP nanospheres possess a diameter range between 100 and 600 nm. SEM reveals nanospheres formulated from LTP are spherical and smooth. Additionally, DLS studies demonstrate that nanospheres degrade hydrolytically in 7 days. Confocal microscopy reveals LTP nanospheres are internalized within human fibroblasts. Finally, the cell viability after exposure to LTP nanospheres and determined with a LIVE/DEAD® Cell Viability Assay is comparable to a buffer control. In conclusion, our nanospheres have been shown to be nontoxic to human cells, possess the appropriate size for endocytosis by human cells, and degrade within 7 days. Therefore LTP nanospheres can be used for a sustained intracellular delivery device.

1. Introduction

In recent years, biodegradable films, scaffolds, microspheres, and nanospheres as delivery devices for drugs and genes have generated a considerable interest (e.g. Murphy et al., 2000; Jang and Shea, 2003; Kim et al., 2003a,b; Luu et al., 2003; Yoon et al., 2003; Cook et al., 2005). A major objective in the design of a drug delivery system is the controlled delivery of a therapeutic agent at an optimal rate and a specific target site of action. These delivery systems aim at improving the access of therapeutic agents to the intended tissue or organ by protecting the therapeutic molecules as well as delivering them at zero order kinetics (e.g. Sarkar et al., 2006). Using various biomaterials ranging from synthetic polymers such as poly [lactide-co-glycolide acid] (PLGA) (McGee et al., 1994; Lee et al., 2007), poly [caprolactone] (PCL) (Cao and Shoichet, 1999; Barbato et al., 2001), and poly [acrylic acid] (PAA) (Ishizu et al., 1997; Wu et al., 2006) to natural polymers such as hyaluronan (Yun et al., 2004), collagen (Kimura et al., 2003; Wu et al., 2004), and alginate (Chan and Heng, 2002; Zielhuis et al., 2007), microspheres and nanospheres have been formulated to encapsulate a host of drugs including DNA.

For many drugs, a direct interaction with cellular machinery would be advantageous (e.g. Astier et al., 1988; Tidefelt et al., 1996; Inoue et al., 1997; Raghunand and Gilles, 2000; Stayton et al., 2000; Varga et al., 2000; Akhtar and Benter, 2007; Borchard, 2001; Lee et al., 2001; Chen et al., 2007; Hallow et al., 2007). Thus, the delivery vehicles must possess the appropriate dimensions for endocytosis (Couvreur et al., 1977; Suh et al., 1998), have rapid degradation rates (Labhasetwar et al., 1999; De and Robinson, 2003), and be nontoxic (Lu et al., 2007; Warheit et al., 2007). Nanospheres are ideal candidates for sustained intracellular delivery drugs, genes, and other therapeutic agents, because their size range is under a micron, and eukaryotic cells are able to uptake these particles (Desai et al., 1997; Van der Lubben et al., 2001). Once the nanospheres are in the cytoplasm, the sustained intracellular release can increase the drug's bioavailability before being cleared from the body (Chu et al., 1990; Panyam and Labhasetwar, 2004; Vasir and Labhasetwar, 2007). Unfortunately, nanospheres derived from most polymers lack the appropriate degradation rate for intracellular delivery (Cai et al., 2003; Tanodekaew et al., 2004; Zweers et al., 2004; Hong

* Corresponding author. Tel.: +1 330 972 6619; fax: +1 330 972 5856.
E-mail address: yy@uakron.edu (Y.H. Yun).

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et al., 2005). PLGA, PAA, and PCL can take months to degrade and release their encapsulated drugs (Nijenhuis et al., 1996; Jeong and Park, 2002; Duvvuri et al., 2005; Chen and Ooi, 2006). These degradation rates exceed the lifetime of most cells (Lodish et al., 2004) and persist in the tissues for extended periods. Additionally, studies have shown that degradation products of polyesters such as PCL and PLGA can lead to a localized drop in pH (Witt et al., 2000; Sen Gupta and Lopina, 2005), and the maintenance of artificial materials in the tissues has been shown to be cytotoxic, inflammatory, and initiate an immune response (Ara et al., 2002; Li and Chang, 2005).

Therefore, nanospheres designed for intracellular delivery need to be formulated from a polymer that biodegrades within an order of days instead of months. We have developed such a polymer by modifying L-tyrosine (Sen Gupta and Lopina, 2004). Naturally, amino acids are the building blocks of proteins and are polymerized using peptide bonds. While cells are able to fold poly [amino acids] into secondary and tertiary structures with relatively ease, this technique offers limited options for preparing drug loaded devices such as microspheres or nanospheres. In general, poly [amino acids] such as proteins and peptides have found limited applications in biomedical device development due to problems associated with their processing (Sen Gupta and Lopina, 2005).

We have overcome these processing limitations by chemically modifying the structure of poly [amino acids]. Specifically, the structure of L-tyrosine (Fig. 1) has been modified with two coupling agents. Desaminotyrosine, a molecule that resembles the structure of L-Dopa, is linked to the amine group of L-tyrosine through a peptide bond. Phosphate is coupled to the R group of the amino acid. In addition, a hexyl ester bond protects the carboxyl terminal of L-tyrosine in order to prevent branched growth and undesired byproducts (Sen Gupta and Lopina, 2004). The polymerization of repeating units results in L-tyrosine polyphosphate (LTP) with a molecular weight of 8–10 kDa (Sen Gupta and Lopina, 2004) and is classified as a 'pseudo' poly [amino acid]. LTP is soluble in common organic solvents (Sen Gupta and Lopina, 2004); thereby, it is easily processed into nanospheres. Previous studies show LTP films to fully degrade in 7 days (Sen Gupta and Lopina, 2005) due to the presence of hydrolytically unstable phosphoester linkages in LTP's polymeric backbone. The degradation rate makes LTP an ideal candidate for nanosphere formulation and for intracellular delivery. In addition, the possible degradation products arising from LTP degradation are L-tyrosine based derivatives, alcohols, and phosphate ions. Furthermore, the degradation products of LTP do not alter the local pH (Sen Gupta and Lopina, 2005).

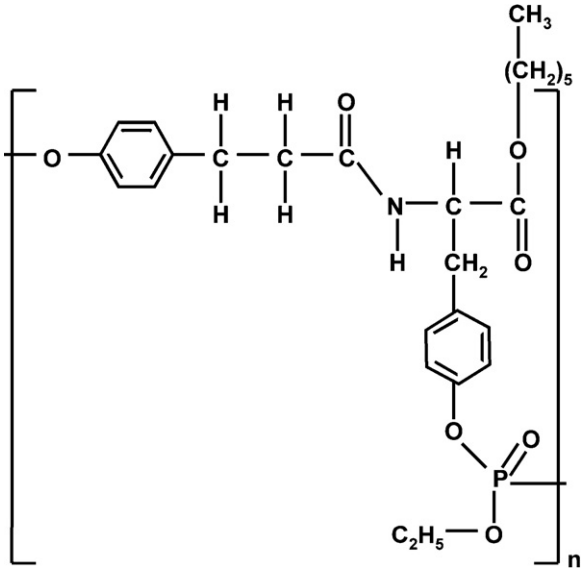

Fig. 1. Chemical structure of LTP (Sen Gupta and Lopina, 2005).

In this study, we show a method of producing nanospheres using LTP for the potential use as an intracellular delivery device. The resulting nanospheres are characterized for their morphology, size distribution, and degradation rate. In addition, we investigate the cytotoxicity of LTP nanospheres and the ability of fibroblasts to uptake these nanospheres. These nanospheres are designed to be non-cytotoxic, to have the appropriate size for endocytosis by human cells, and to be degraded within the lifetime of most human cells.

2. Methods

2.1. Chemicals

Linear polyethylenimine (LPEI) from PolyScience Inc. with a molecular weight of 25,000 Da was dissolved in autoclaved (American Standard 25X-1 Autoclave) distilled and deionzied water (DH_2O, Barnstead NanoPure II Deionizer) at 70 °C at a concentration of 1 mg/ml. Polyethylene glycol grafted to chitosan (PEG-g-CHN) obtained from CarboMer Inc. (80% acetylation) was dissolved at a concentration of 3.33 mg/ml in 0.1N acetic acid for 48 h at 37 °C under rotation. The surfactant, 5% polyvinylpyrrolidone (PVP) from Sigma–Aldrich, was prepared in DH_2O. PLGA (inherent viscosity = 0.59 dl/g in hexafluoroisopropanol at 30 °C, LACTEL Absorbable Polymers International) was dissolved in dichloromethane (DCM) obtained from Emanuel Merck Darmstadt at a concentration of 100 mg/ml. Fluorescein 5-isothiocyanate (FITC, Invitrogen) was dissolved at a concentration of 3 mg/ml in dimethyl sulfoxide (DMSO, FisherChemical). Rhodamine Phalloidin stock solution (Molecular Probes) was diluted from 6.600 to 0.165 μM in phosphate buffer saline (PBS).

2.2. Polymer synthesis

LTP with a molecular weight of 8000–10,000 Da was synthesized according to the protocol developed by Sen Gupta and Lopina (2004). Briefly, the synthesis protocol for LTP was as follows: a diphenolic monomeric molecule, desaminotyrosyl-tyrosine-hexyl ester (DTH), was produced from the natural amino acid L-tyrosine using carbodiimide mediated solid phase synthesis techniques. The monomeric molecule of DTH was polymerized by reaction with equimolar proportions of ethyl dichlorophosphate to yield LTP (Sen Gupta and Lopina, 2004). LTP was stored in a desiccator at room temperature.

2.3. Synthesis of LTP nanospheres

Nanospheres formulated with LTP were prepared using an emulsion of oil-in-water (o/w) by sonication and solvent evaporation technique. For a typical nanosphere formulation, the emulsion consisted of 300.0 mg of LTP dissolved in 3.0 ml of chloroform (100 mg/ml), 3.0 mg of PEG-g-CHN dissolved in 0.9 ml of 0.1N acetic acid, 100.0 ml of 5% PVP, and 3.0 mg of LPEI in 1 ml of DH_2O. The emulsion was sonicated for 1 min, and the chloroform was allowed to evaporate for 5 h. Nanospheres were collected and washed with DH_2O by centrifugation at 15,000 × *g* for 10 min. Finally, nanospheres were shell frozen in 10 ml of DH_2O, lyophilized (Labconco Freezone 4.5) for 72 h, and stored in a desiccator. PLGA nanospheres were produced similar to the preceding protocol except 3 ml of 100 mg/ml PLGA in DCM was used in place of the LTP, PEG-g-CHN, and LPEI.

2.4. Characterization of shape, size, and morphology of nanospheres

Scanning electron microscopy (SEM) (Hitachi S2150) was used to qualitatively evaluate the size and shape, and morphology of the nanospheres. The SEM samples were prepared by suspending 1 mg of nanospheres in 1 ml of DH_2O. Then, 200 μl of the suspended nanospheres were pipetted onto a stub, dehydrated, sputter coated with silver/palladium, and examined at an accelerating voltage of 25 kV.

2.5. Characterization of size and degradation of nanospheres

Dynamic laser light scattering (DLS) was used to quantify the size of the nanospheres and also their degradation. The nanosphere sample was prepared by suspending 1 mg of nanospheres in 10 ml of 1× PBS that had been passed through a 0.2 μm filter. The suspended nanospheres were centrifuged for 10 s at 1000 × *g* to remove any large aggregates. Then, the sample was decanted into a glass scintillation vial. The DLS system (Brookhaven Instruments BI-200SM) calculated the diameters by the Regularized Non-negatively Constrained Least Squares (CONTIN) method. The range of nanosphere sizes was reported as differential distribution values. The differential distribution value varied from 0 to 100. The highest peak or modal value was assigned to the number 100 and reported as relative amounts. In order to determine the degradation of the nanospheres in vitro, the nanospheres were incubated at 37 °C under constant rotation for 7 days, and the DLS measurements were performed on days 0, 1, 2, 3, 4, and 7. The mean diameter of nanospheres was calculated by the Brookhaven software and reported for each day. The mean diameter of the LTP nanospheres was calculated according to the weighted average based upon the differential distribution value.

2.6. Nanosphere cellular viability

The cell viability of primary human dermal fibroblasts after exposure to nanospheres was determined using a LIVE/DEAD® Cell Viability Assay (Invitrogen). Primary human dermal fibroblasts (passage 6, a gift from Judy Fulton at the Kenneth Calhoun Research Center, Akron General Medical Center) were seeded onto 24 well tissue culture plates (Falcon) at a density of 25,000 cells/well and maintained overnight at 37 °C with fibroblast feeding media (90% Dulbecco's Modified Eagle Medium and 10% fetal calf serum containing 1% antimycotic). The next day, the fibroblast feeding media was replaced. Fibroblasts in 500 μl of feeding media were exposed to 400 μg of LTP nanospheres suspended in 200 μl of feeding media. After 1, 3, 7, and 11 days a LIVE/DEAD® Cell Viability Assay was performed according to the manufacturer's instructions. Fibroblast viability was visualized using fluorescent microscopy (Axiovert 200, Carl Zeiss) and imaged with a CCD camera (AxioCam HRm, Carl Zeiss). Controls were 200 μl of TE buffer, PLGA nanospheres (same concentration as LTP nanospheres), and 2 mM H_2O_2.

2.7. Cellular uptake of nanospheres

In order to determine if the uptake of the LTP nanospheres, primary human fibroblasts were exposed to FITC encapsulated nanospheres and visualized with confocal microscopy (Olympus Fluoview). FITC (1%) loaded nanospheres were produced according to the aforementioned nanosphere synthesis procedure in Section 2.3 except 3 mg/ml FITC in DMSO was added to the emulsion. Next, primary human dermal fibroblasts were seeded onto sterile German glass cell culture cover slips (Fisher Sci) in 24 well tissue culture plates at a density of 25,000 cells/well and maintained overnight at 37 °C with fibroblast feeding media. The next day, the fibroblast feeding media was replaced. Then, 1 mg of FITC labeled nanospheres was suspended in 1 ml of DH_2O, and 80 μl of the suspended nanospheres was added to the feeding medium of each well. Human dermal fibroblast cells without any exposure to FITC loaded nanospheres were seeded onto German glass cover slips and used as negative controls. After 24 h of incubation, the fibroblasts were washed with PBS and fixed with 1% formaldehyde (Fisher Scientific) in PBS for 10 min. After fixation, the cells were washed with PBS, and then 100 μl of 0.165 μM Rhodamine Phalloidin in PBS was added onto each cover slip. After 20 min of incubation at room temperature, the cells were washed with PBS. The cover slips were then mounted to glass slides with Vectashield mounting media containing DAPI (Vector Laboratories), and the cellular uptake was visualized with confocal microscopy at Northeastern Ohio Universities Colleges of Medicine (NEOUCOM).

2.8. Statistics

All quantitative studies were performed in six replicates determined by power analysis with $\alpha = 0.05$. The Shapiro–Wilk test for normality was performed to determine if each sample group was normally distributed (Shapiro and Wilk, 1965). Samples were considered normally distributed when $p \leq 0.05$. Sample data was considered continuous since they were calculated mean values (Sokal and Rohlf, 2003). Tukey's analysis of variance was then performed among the normally distributed sample groups. All results were considered significant if $p \leq 0.05$.

3. Results

3.1. Scanning electron microscopy of nanospheres

SEM has been utilized to examine the nanospheres' morphology, size, and shape. The images obtained by SEM reveal a smooth surface morphology of nanospheres formulated from LTP (Fig. 2). The diameter range of the nanospheres is between 100 and 500 nm (Fig. 2). Nanospheres with diameters of 100 nm or less are difficult to visualize due to the limitations of the SEM. The shape of the nanospheres is spherical (Fig. 2). Nanospheres aggregates are also seen in the SEM images (Fig. 2).

3.2. Laser light scattering of nanospheres

DLS has been used to quantitatively measure the nanosphere diameter range. The diameters of the LTP nanospheres are between 156 and 562 nm (Figs. 3 and 4). Larger particles ranging from 7

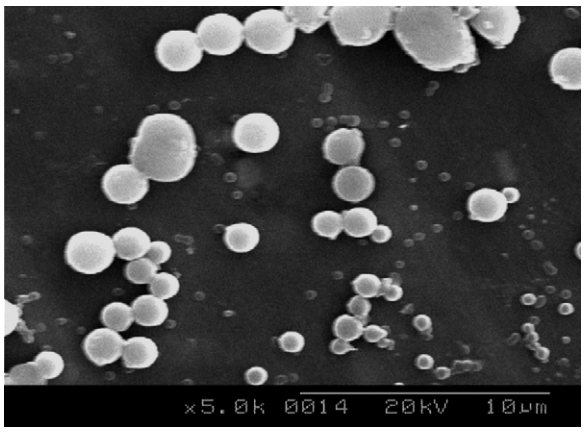

Fig. 2. SEM (5000×) of nanospheres synthesized from LTP, LPEI, and PEG-g-CHN.

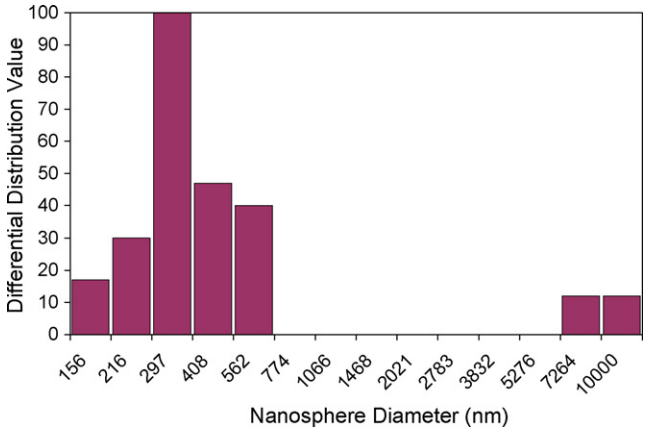

Fig. 3. Representative size distribution of LTP nanospheres. Determined using Regularized Non-negatively Constrained Least Squares (CONTIN).

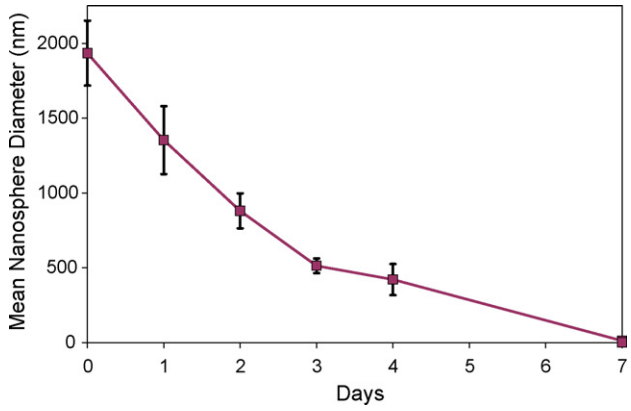

Fig. 4. Degradation of LTP nanosphere mean diameter. Determined using Regularized Non-negatively Constrained Least Squares (CONTIN).

to 10 μm are also observed. These larger particles could be either aggregates of nanospheres or actual microspheres. DLS has been further utilized to characterize the degradation of the nanospheres. Nanospheres are found to completely degrade in PBS at 37 °C after 7 days (Fig. 4), which mirrors the degradation of LTP films (Sen Gupta and Lopina, 2005). The nanospheres lose nearly 75% of its diameter after 3 days. After 3 days, the mean particle diameter decreased from 1.9 μm to 500 nm. Since the mean diameter has been calculated by weighted averages, the value of 1.9 μm shown in Fig. 4 appears larger than the diameter range of the LTP nanospheres shown in Fig. 3 (156–562 nm). However, these two results are consistent with each other since the calculation of the mean diameter is influenced by the aggregated nanospheres ranging from 7 to 10 μm. Finally, the mean diameter is 2 nm after 7 days of degradation (Fig. 4).

3.3. Cell viability after exposure to nanospheres

The viability of human dermal fibroblasts after 7 days of exposure to nanospheres has been determined using a LIVE/DEAD® Cell Viability Assay. Cell viability is recorded as the percentage of live cells. In this assay, metabolically active cells reduce C_{12}-resazurin to red fluorescent C_{12}-resorufin. Dead or dying cells fluoresce green since these cells lose the ability to reject Sytox green. Nanospheres, which fluoresced green with the same filter as dead cell nuclei, are distinguished by their smaller diameters and more spherical shape as opposed to the typical shape of normal nuclei. For positive control, fibroblasts that have been exposed to 2 mM H_2O for 3 h have 0% viability (Fig. 5). In Fig. 5, the dead cells are circled in yellow to improve their visualization. The addition of the negative control buffer to fibroblasts results in cell viability of 92% ± 1% after 7 days (Fig. 6). In the fluorescence images, few green nuclei are seen, but red fluorescent resorufin, which indicates metabolically active cells, are seen in the majority of the fibroblasts (Fig. 5). Fibroblasts exposed to the nanospheres formulated with LTP have a cellular viability of 86% ± 3% (Fig. 6). Viabilities of 92% ± 5% are seen for PLGA nanospheres after 7 days, a time period that does not appreciably degrade this polymer. After 1, 3, and 7 days, Tukey's comparison of means shows the viability of cells exposed to LTP nanospheres is not significantly different from TE buffer ($p = 0.9897$, 0.7030, 0.7417, respectively) or PLGA nanospheres ($p = 1.000$, 0.9881, 0.7922, respectively). Each group is normally distributed ($p \leq 0.05$) as shown by the Shapiro–Wilk test.

3.4. Cellular uptake of LTP nanospheres

Cellular uptake of nanospheres formulated from LTP is confirmed with confocal microscopy of human dermal fibroblasts

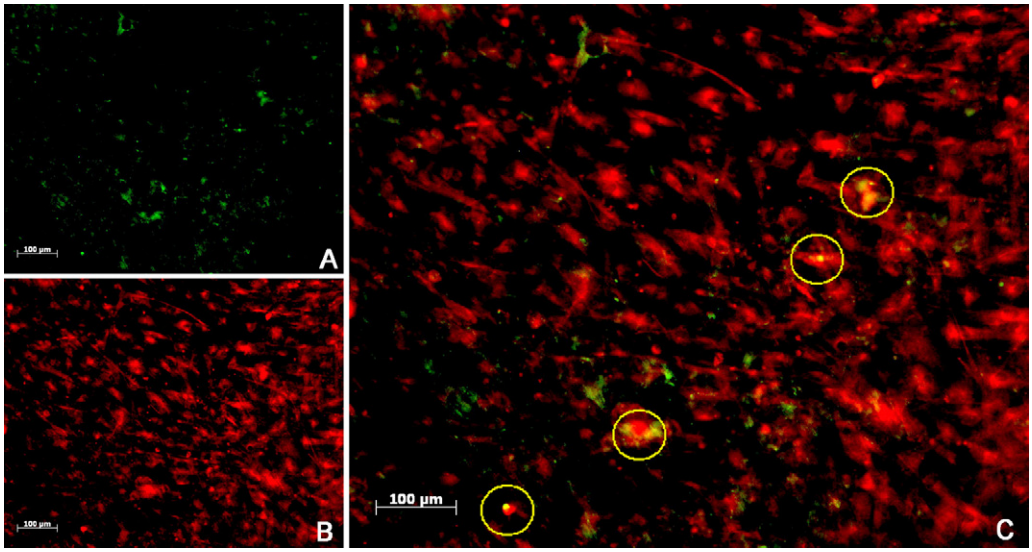

Fig. 5. LIVE/DEAD® Cell Assay on day 7 of human dermal fibroblasts exposed to 400 μg of LTP nanospheres. (A) Dead cells (green), (B) live and metabolically active cells (red), (C) combined fluorescence channels with dead cells circled in yellow.

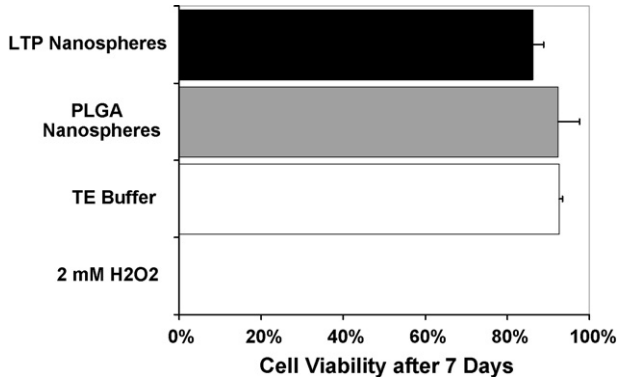

Fig. 6. Cellular viability of various materials after 7 days determined by LIVE/DEAD® Cell Viability Assay.

exposed to 100 μg of FITC labeled nanospheres. Confocal microscopy with 0.5 μm slices reveal nanospheres at various depths within the fibroblasts' cytoskeleton and are circled (Fig. 7). Slice depths are reported in the upper left hand corner of each image in Fig. 7. At the depth 1 μm, the cytoskeleton is visible, but few nanospheres are seen. At 2 and 3 μm, a number of nanospheres appear (Fig. 7) and disappear at depths of 3.5 and 4.0 μm. The appearance and disappearance of the nanospheres within the cytoskeleton verify their uptake in the fibroblasts. The scale of the FITC loaded nanospheres is visualized as well (Fig. 7) and is several hundred nanometers. Furthermore, the normal morphologies of the fibroblasts as seen in Fig. 7 suggest that cellular toxicity has not been induced by free FITC released from the nanospheres (Hara et al., 2008).

4. Discussion

LTP is a biodegradable polymer that promises to be useful for an intracellular deliver system (Sen Gupta and Lopina, 2005), and we have developed a method of producing nanospheres for the first time using LTP. Until now, films of LTP have been produced (Sen Gupta and Lopina, 2005), but the formulation of nanospheres has been elusive. Initial attempts have failed to yield nanospheres using an o/w emulsion technique with LTP polymer dissolved in chloroform and PVP as the external phase. However, the addition of two amphiphilic polymers, PEG-g-CHN and LPEI, at equal ratios has made nanosphere formulation possible. The addition of only one of the amphiphilic polymers has also failed to produce any nanospheres. Thus, we have successfully made nanospheres from LTP using the o/w emulsion technique as seen in Fig. 2.

We hypothesize that the addition of PEG-g-CHN and LPEI stabilizes the LTP emulsion. Similarly, surfactants such as polyglycerol monolaurate (Shima et al., 2004) and amphiphilic copolymers such as poly(ethylene glycol) ethyl ether methacrylate (PEG-EEM) (Tuncel and Serpen, 2001; You et al., 2001) have been used to stabilize oil and water emulsions. During nanosphere fabrication, PEG-g-CHN and LPEI collects at the water and oil interface due to their amphiphilic nature (Lazaridis et al., 1999). The hydrophobic chitosan segment is likely to interact with the oil phase containing the LTP dissolved in chloroform. Meanwhile, hydrophilic segments of LPEI and the PEG-g-CHN are likely to associate in the water phase. The presence of PEG-g-CHN and LPEI at the oil and water interface stabilizes the emulsion by preventing coalescence of the nanospheres due to their steric stabilization properties (Chern and Lin, 1997; Ouchi et al., 1998).

Furthermore, the addition of PEG-g-CHN has produced nanospheres with enhanced biocompatibility. During solvent evaporation, PEG is hypothesized to enrich at the nanospheres surface (Jiang et al., 2004). PEG on the surface of the nanospheres is ideal, since PEG prevents plasma protein adsorption, inhibits platelet adhesion, avoids thrombus formation, and increases circulation time by the steric repulsion mechanism (Lin et al., 1994; Amiji, 1997; Hawley et al., 1997). Essentially, PEG enrichment at the surface could potentially provide a means for our nanospheres to elude the immune system.

We can also postulate that the addition of LPEI assists in the nanospheres escape from endosomal entrapment and lysosomal degradation (Kichler et al., 2001). LPEI has been documented to provide a means of escape from endosomes after internalizations by cells (Behr, 1997; Leong et al., 1998; Itaka et al., 2004; Akinc et al., 2005). Studies by Leong show that internalized LPEI nanoparticles can be found in endosomes (Leong et al., 1998), but LPEI has been shown to provide escape from endosomal entrapment by the proton sponge theory. The proton sponge theory states that the amino groups in the backbone structure of LPEI have a low p*K*a and buffer non-physiological pH (Itaka et al., 2004; Akinc et al., 2005). Thus, LPEI can increase the ion osmotic pressure, cause endosomal swelling, and bursting of the vesicles (Behr, 1997). Consequently, endocytosed nanospheres containing LPEI are likely to be released into the cytoplasm where they can further degrade and release their drug.

The formulation of nanospheres composed of LTP, PEG-g-CHN, and LPEI yields nanoscale particles. The nanospheres' size plays a major role in their ability to be internalized by cells, and previous studies show that cellular internalization of particles is size dependent (Monkkonen and Urtti, 1998). Typically, eukaryotic cells are able to internalize nanoparticles with diameters ranging from 50 nm to 1 μm (Crotts and Park, 1995). SEM and DLS results show that LTP nanospheres possess the appropriate size (150–600 nm) for cellular uptake; however, nanosphere aggregates of 1–10 μm particles are also observed (Figs. 2 and 3). Confocal fluorescent microscopy has verified uptake by primary human dermal fibroblasts during 24 h exposure to our nanospheres. From Fig. 7, fluorescence particles can be seen appearing and disappearing between 1 and 4 μm confocal slices. The sizes of these particles, the normal morphology of the fibroblasts, and the 24 h uptake duration suggest that the LTP nanoparticles have been endocytosed. FITC molecules released from the nanospheres are also present in the cell culture medium due to the rapid degradation rate of LTP, but free FITC has been shown to be markedly cytotoxic as compared to nanospheres loaded with FITC (Hara et al., 2008). The lack of toxicity observed in our uptake study (Fig. 7) suggests that free FITC in the cell culture medium has not been metabolized by the fibroblasts. The 24 h time frame also provides the appropriate duration for the endosomal escape and intracellular release by LTP nanospheres since they completely degrades in 7 days.

Nanospheres must be formulated from polymers that degrade in an optimum time frame in order to achieve a sustained intracellular release (Gorner et al., 1999; Kamiya et al., 2001; Shah and Amiji, 2006). Recently, degradable nanospheres made from PLGA and poly-L-lactide (PLLA) using the emulsion method can take several months to degrade (Cai et al., 2003; Tanodekaew et al., 2004; Zweers et al., 2004; Hong et al., 2005). However, the life span of mammalian cells can range from 1 day to years (Lodish et al., 2004). Thus, PLGA or PLLA nanospheres do not degrade fast enough to deliver all their contents with the life span of most cells. However, the DLS data for LTP nanospheres shows they are fully degraded after 7 days. This degradation profile is comparable to LTP films incubated in PBS (Sen Gupta and Lopina, 2005), which is expected since the nanospheres are approximately 98% LTP. In addition, the LTP nanospheres are approximately 75% degraded after 3 days (Fig. 4), which is also comparable to LTP films incubated in

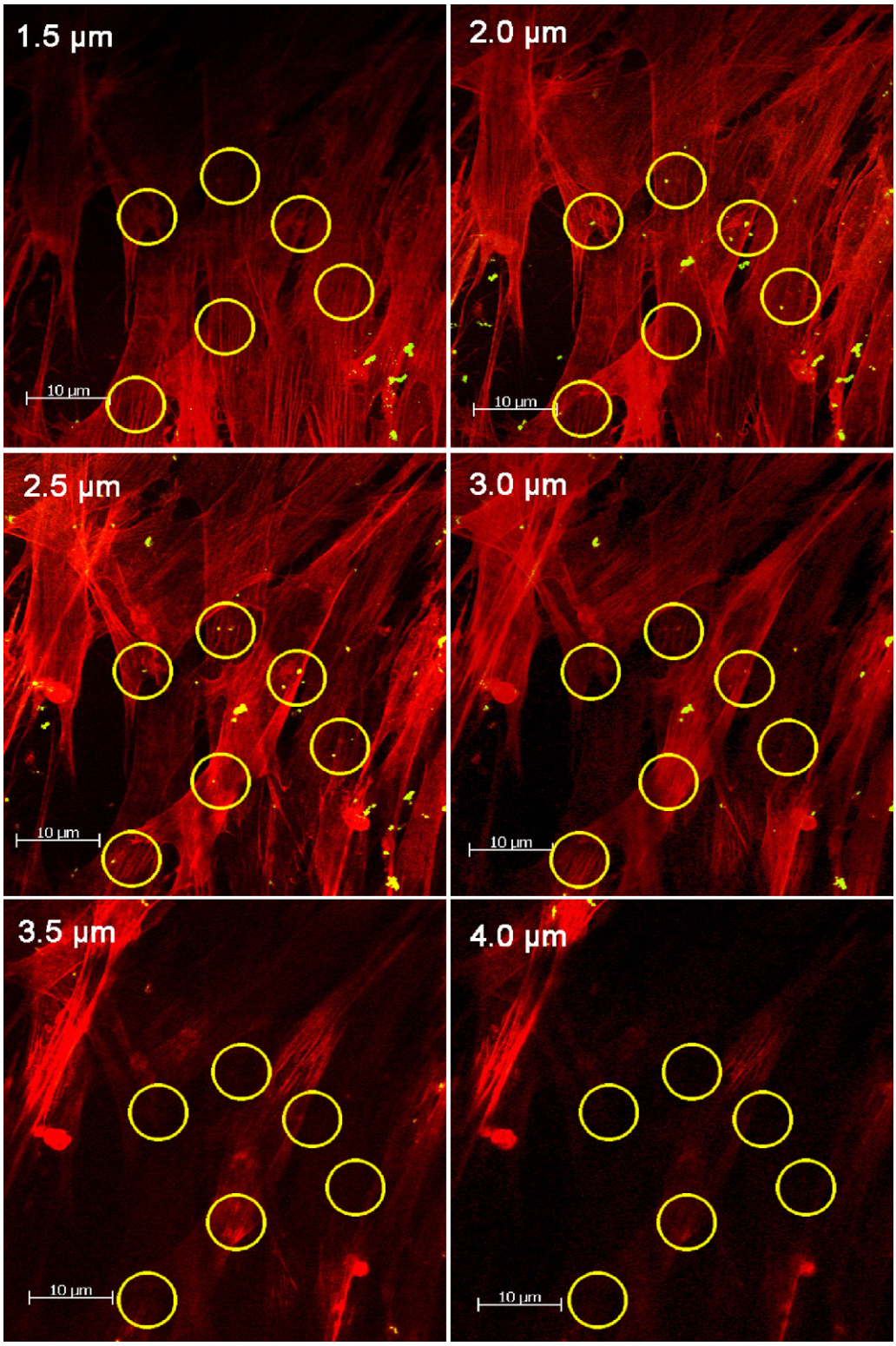

Fig. 7. Confocal microscopy of fibroblast uptake of FITC loaded LTP nanospheres.

PBS (Sen Gupta and Lopina, 2005) undergoing hydrolytic degradation. Therefore, the degradation rate of LTP nanospheres is more suitable for an intracellular delivery than nanospheres formulated from other polymers that degrade in months (Dunne et al., 2000).

The use of degradable nanospheres as an intracellular delivery device cannot be implemented unless nanospheres and degradation products are proven to be non-cytotoxic (Kim et al., 2003a,b; Ameller et al., 2004; Weissenbock et al., 2004). Previous studies have found that as PLGA and PLLA nanospheres drop the local pH below 4.0 (Sen Gupta and Lopina, 2005). This acidic environment is cytotoxic and induces inflammatory responses (Ara et al., 2002; Li and Chang, 2005). The LIVE/DEAD® Cell Viability Assay demonstrates human dermal fibroblast viability over a course of 7 days is comparable when exposed to LTP nanospheres, buffers, and PLGA

nanospheres. Previous studies have shown LPEI to be toxic (Itaka et al., 2004), but the controlled release of LPEI minimizes this effect. High cell viability can also attributed to the nontoxic properties of LTP. Our polymer is synthesized from the amino acid L-tyrosine and phosphates, which are both found naturally in the body. In addition, the coupler desaminotyrosine has the chemical structure similar to L-DOPA. PEG and chitosan have also been shown to be nontoxic as well (Park et al., 2001). The only possible degradation product that could be non-biocompatible is hexanol, but the local concentration does not reach toxic levels within 7 days of nanosphere degradation as seen in Fig. 6. This covalent ester bond breaks due to hydrolysis but does not occur as readily as the hydrolysis of the phosphoester bond in the backbone, which leads to the primary degradation of the polymer (Sen Gupta and Lopina, 2005). The live/dead studies confirm that LTP nanospheres (1 mg/ml) and their degradation products are safe to use in vitro with fibroblasts at their effective concentrations, since they have comparable viabilities over 7 days to buffers and PLGA nanospheres.

The high cell viability of our nanospheres may result from their favorable biocompatible properties under in vitro conditions. Key properties such as surface morphology and composition play a major role in the nanospheres' in vitro biocompatibility (Wang et al., 2004). Previous studies have found that fibrous and irregular particles have shown to stress cells and elicit immune responses (Rejman et al., 2004). The SEM images of LTP nanospheres show smooth surfaces (Fig. 2), which theoretically indicate desirable surface biocompatibility. Furthermore, past data shows nanospheres with spherical and smooth morphologies, which demonstrate higher loading efficiencies than irregular particles (Tsuda et al., 1999).

In the past, many drug and gene delivery devices have accomplished an extracellular release of their constituents, which results in low efficacy for drugs that need interaction with cellular machinery (Suh et al., 1998; De and Robinson, 2003). Intracellular delivery systems such as our LTP nanospheres have the potential to achieve a higher efficiency (Kamiya et al., 2001) since they can be endocytosed and their constituents can interact with cellular machinery. In this study, the biodegradable polymer LTP has been formulated into nanospheres that degrade within the lifetime of cells, have appropriate size for cellular uptake, and are non-cytotoxic to human dermal fibroblasts.

5. Conclusions

Nanospheres have been formulated from LTP for the first time. These nanospheres composed of LTP, PEG-g-CHN, and LPEI show the required attributes for use as a sustained intracellular delivery device. An o/w emulsion produced by sonication and solvent evaporation proves to be an effective means to create nanospheres. The addition of PEG-g-CHN into the nanospheres stabilizes the emulsion and potentially enhances biocompatibility of LTP nanospheres (Amiji, 1997). The addition of the cationic polymer, LPEI, provides escape from endosomal entrapment and lysosomal degradation (Kichler et al., 2001) as well as emulsion stability. Our fabrication method produces nanospheres that are spherical, smooth, and the appropriate sizes for endocytosis. Internalization of our nanospheres by human fibroblasts is achieved in 24 h, which allows time for an intracellular delivery of their contents. In addition, these nanospheres degrade in an appropriate time frame for intracellular release due to LTP's rapid degradation rate. Furthermore, our nanospheres are shown to be nontoxic to human fibroblasts in vitro, which could be a favorable indicator of in vivo biocompatibility. Therefore, the LTP nanospheres could be a valuable drug delivery device that provides a rapid but a controlled release of the encapsulated drug or gene.

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