Biodistribution of Orally Administered Poly(Lactic-co-Glycolic) Acid Nanoparticles for 7 Days Followed by 21 Day Recovery in F344 Rats

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Abstract

The aim of this research was to assess biodistribution of orally administered poly(lactide-co-glycolide) acid nanoparticles (PLGA NPs) in rats and investigate the excretion of PLGA nanoparticles after administration has ended. The experiment was divided into 2 phases. In phase I, F344 rats were orally administered fluorescently tagged PLGA nanoparticles daily (3 mg/day) for 7 days, followed by a mass balance analysis which was performed on tissues of interest to determine NP biodistribution. In phase II, after 7 days of oral exposure, rats were no longer administered PLGA NPs, and amount of NPs excreted was measured each week for 3 weeks. At day seven, **the last day of the nanoparticle exposure period**, over half of the daily administered PLGA NPs were excreted. Among the nanoparticles recovered from the tissues, the majority was recovered in the intestines (**23.4% daily dose**), followed by the liver (**11.4% daily dose**), kidney (**5.5% daily dose**), spleen (**2.5% daily dose**), lung (**2.0% daily dose**), brain (**1.0% daily dose**), plasma (**0.7% daily dose**), and heart (**0.2% daily dose**), respectively. During phase II, the amount of NPs in the feces declined **from the maximum excretion on day 7 (58.3% daily dose) to the minimum value on day 28 (6.7% daily dose)**, 3 weeks after NP administration ended. **Little change in nanoparticle excretion was observed between day 21 and day 28, indicating the baseline had been reached.** The findings are significant for understanding biodistribution and excretion of orally administered PLGA NPs and are relevant to their application in food, agriculture, and medicine.

Keywords: polymeric nanoparticles, biodistribution, excretion, recovery, mass balance

1. Introduction

Polymeric nanoparticles have been developed as viable drug delivery systems for treatment of diseases ranging from Alzheimer's disease to cancer [1-3]. **Due to their flexibility, biodegradability, and relatively low levels of toxicity, polymeric nanoparticles may be preferred to other types of nanoparticles as drug delivery systems [4].** Knowledge regarding the biodistribution of nanoparticles *in vivo* is critically important for determining their efficacy, as well as toxicity. Biodistribution **of polymeric nanoparticles** has been determined for intravenous (i.v.) [5], oral [6], [7], intraperitoneal [8], topical [9] and intranasal [10] **administration**. The techniques applied for tracking nanoparticles *in vivo* fall mainly

into two categories, namely: fluorescent methods [8, 11] and radioactive labelling [12, 13]. Studies dedicated to tracking nanoparticles *in vivo* after oral delivery are relatively sparse compared to those using intravenous administration [14-19], and many are focused on inorganic nanoparticles, such as zinc oxide [20-22] and silver [23-25]. Studies that analyzed the biodistribution of nanoparticles following intravenous administration showed high concentrations of nanoparticles distributed to reticuloendothelial organs, such as the spleen and bone marrow, as well as the liver, kidneys, and intestines [15-17]. Studies focused on the oral administration of metallic nanoparticles indicate that, while some metallic nanoparticles such as titanium oxide are not well absorbed in the GI tract [20], others such as zinc oxide and silver are well absorbed and tend to concentrate in the intestines, liver, and kidneys [21-25]. While only mild to no toxicity was observed following the oral administration of silver nanoparticles [23, 24], much greater toxicity was observed for zinc oxide [26].

Oral delivery of drugs with polymeric nanoparticles is particularly of interest, because it overcomes limitations such as poor stability, low mucosal permeability, and low solubility of the drugs in gastric fluids [3, 27]. Oral drug delivery is the most preferred method of drug delivery due to patient compliance, cheaper production cost, and the potential for many drugs to be taken at home that are typically administered in a hospital or clinic setting [28]. Furthermore, using nanoparticles to administer drugs orally has the potential to bypass adverse effects associated with IV delivery [29].

Poly(Lactic-co-Glycolic) acid (PLGA) is a biodegradable polymer that has seen extensive use in research and medicine due to its biocompatibility [30]. Common applications of PLGA include its use as material for sutures and implantable scaffolds as well as its use as a vehicle for drug delivery. PLGA is of particular interest for drug delivery due to its ability to biodegrade, and its versatility; by adjusting the ratio of its components, lactic acid and glycolic acid, and conjugating it to copolymers and other molecules the adhesion, diffusion, and distribution properties of the delivery systems can be modulated [30]. The available literature on the mass balance of orally administered PLGA nanoparticles is limited, especially for cases where nanoparticle administration is carried out for a certain time followed by a recovery period when no nanoparticles are incorporated into the diet. Instead, most studies focused on polymeric nanoparticles address the efficacy of nanodelivered drugs for the treatment of diabetes, cancer, and other diseases [31, 32], without addressing the biodistribution and elimination of the nanoparticles. In general, the studies that do focus on the biodistribution of polymeric nanoparticles involve a single dose [15] or repeated doses of nanoparticles varying from 7 [11, 33, 34] to 21 days [4]. The recovery time of the tissues to the baseline after a long exposure to a nanoparticle treatment is not currently addressed in the available literature, to the authors' knowledge.

The aim of this study was to quantify the biodistribution and excretion of PLGA nanoparticles after 7 days of oral administration in F344 rats, and to analyze nanoparticle excretion in animals exposed to nanoparticles for 7 days after a recovery period of 7, 14 and 21 days. To achieve this aim, nanoparticles were covalently linked to a fluorophore, namely, tetramethylrhodamine-5-isothiocyanate (TRITC), which allowed nanoparticle tracking *in vivo* in tissues, blood, feces and urine at various time points.

2. Material and methods

2.1 Reagents

Poly(lactide-co-glycolide acid)(PLGA) 50:50, (MW: 30,000 to 70,000 g/mol). poly- vinyl alcohol (PVA) 87-89% hydrolyzed (31,000-50,000 g/mol), -[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU), N-Boc-ethylenediamine, N,N-Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), triethylamine, tetramethylrhodamine-5-isothiocyanate (TRITC), and dichloromethane (DCM) were purchased from Sigma-Aldrich (St. Louis, MO). Acetone, ethanol, ethyl acetate were HPLC grade (Mallinckrodt Baker, Pittsburgh, NJ).

2.2 PLGA-TRITC Nanoparticles synthesis & residual PVA measurement

The attachment of TRITC to PLGA was performed using uronium salt chemistry, following the protocol previously described by Navarro et al [4]. The polymeric nanoparticles were synthesized by an emulsion evaporation technique described by Astete and Sabliov [35] and by Murugesu et al [27]. The purification was performed by dialysis (MWCO 100,000 g/mol) with regenerated cellulose membranes (Spectrum Laboratories, Rancho Dominguez, CA) for a period of 2 days. Then, polymeric nanoparticles were dried using a freeze drier for 2 days (Labconco, Kansas City, MO). The final dry product was stored at 4°C for characterization and animal studies. The quantification of residual PVA was done by a colorimetric method [36], detailed by Navarro et al. [33]. After preparation, the standard curve and samples were read using a spectrophotometer (Genesis 6, Thermo Scientific, Asheville, NC) at 690 nm.

2.3 Morphology, particles size, size distribution, and zeta potential measurements

A method detailed by Navarro et al. [33] was used to determine NP morphology. Transmission electron microscopy (TEM) pictures were taken with a JEOL 1400 (Jeol USA Inc., Peabody, MA). The image was

taken on a sample of nanoparticle powder re-suspended in water, immediately following freeze-drying step. Uranyl acetate (2%) was added to the sample as contrast agent.

Nanoparticles size, distribution and zeta potential were measure with a Malvern Zetasizer Nano ZS (Malvern Instrument Ltda., Worcestershire, UK). Zeta potential was measured using a 10 mM NaCl solution at pH 7.2; both parameters, size and zeta potential measurements were done at a nanoparticle concentration of 200 µg/ml.

2.4 *In vivo* biodistribution of fluorescently tagged PLGA nanoparticles

Male F344 rats (200-300 g) were used for the *in vivo* studies. At the time of the experiment, the average age of the rats was 12 weeks. The animals were acclimated for one week prior to the start of the study. Animals were on a 12-hour light/dark cycle and randomly divided according to the previous study [33]. Rats (n=8) were housed, 2 per cage, and provided feed (Lab Diet 5001, St. Louis, MO) and water *ad libitum*. Four animals were gavaged daily with a solution of fluorescently tagged PLGA nanoparticles (3 mg/ml) suspended in 1 ml PBS (pH 7.4). A typical dose of drug loaded polymeric nanoparticles is 10 mg/kg body weight, which is why 3 mg/ml was chosen as the daily dose for rats. The control group consisted of four rats which were daily gavaged with PBS (pH 7.4). An additional three rats were euthanized after acclimation and used to prepare fluorescent standard curves by measuring fluorescence as a function of nanoparticle concentration, separately, in the tissues of interest: brain, intestine, kidney, liver, and spleen. The tissues recovered from treated animals were stored at -80°C until processing. Each tissue was homogenized in PBS and digested with 1M NaOH, following a protocol described by Yin et al [34]. Finally, samples were centrifuged for 10 minutes and the fluorescence was measured in the collected supernatant following a protocol previously described by Navarro et al [33]. The amount of nanoparticles distributed in each tissue was calculated based on the standard curve for that individual tissue.

On day 7, blood was collected as was previously described [33] and centrifuged to separate the plasma fraction at 2,800g for 10 minutes at room temperature using a Sorvall Legend Mach 1.6R centrifuge (Thermo Scientific, Waltham, MA). The plasma samples were kept at -80°C until use. The collected plasma was used to quantify the fluorescent PLGA NPs. The amount of fluorescence was determined in plasma based on the standard curves previously prepared. The nanoparticle concentration in plasma was expressed as % dose based on the assumption that an adult male rat of 250g contains 6.4ml of blood and 4.2 ml of plasma for every 100g of body weight [37]. The amount of

fluorescent nanoparticles excreted in urine and feces on day 7 was also determined for the biodistribution study in rats (n=4).

2.5 Excretion of fluorescently tagged PLGA NPs

Feces and urine were collected from F344 rats kept in metabolic cages (1 rat/cage). 50ml tubes were attached to the cages and samples were collected daily for the 7 days during nanoparticle administration. Feces and urine were also collected during the recovery time (7, 14 and 21 days with regular food after 7 days of PLGA treatment).

The experiment was divided into two phases (Figure 1). During phase I, 12 rats were fed daily with 3mg of PLGA nanoparticles for 7 days, and control rats for this group (n=4) were gavaged with PBS. During phase II, no particles were administered and the animals were fed with a regular lab diet for the following 7, 14 and 21 days (n=4 for each time point). At each time point, the animals were euthanized by CO₂ asphyxiation 24 hours after their last fed for sample collection. Blood was collected first, followed by gross necropsies where heart, spleen, kidney, liver, lung, intestine, and brain were excised for further analysis.

2.6 Statistical Analysis

The statistical analysis was performed in SAS (Cary, NC), and the mixed procedure with Tukey adjustment and the macro by Arnold Saxton was used for post hoc adjustment were applied to detect differences between treatment and control. Data were expressed as means $\pm$ S.D. All data was analyzed to compare treated rats versus control animals (n=4). A significant difference was declared at p values < 0.05.

Figure 1. Summary of recovery study: Rats were fed for 7 days with PLGA nanoparticles (Phase I) followed by a recovery period of 7, 14 and 21 days (Phase II). Feces and urine were recovered daily from rats housed in metabolic cages.

3. Results

3.1 Particle characterization

Dynamic light scattering measurement of the PLGA NPs showed that the particles measured in average $112 \pm 9\text{nm}$ in diameter. The amount of PVA ($23.7 \pm 1.2\%$) was determined relative to the initial amount used in the polymeric nanoparticles synthesis ($n=3$ for all measurements). The polydispersity index (PDI) of the NPs was 0.112 ± 0.017 and zeta potential was $-2.1 \pm 0.8\text{mV}$. The particles were spherical in shape and displayed little aggregation (Figure 2). These results are similar to previous studies using PLGA nanoparticles [4, 33].

Figure 2. TEM pictures of PLGA nanoparticles: Spherical particles measured in average $112 \pm 9\text{nm}$ in diameter (based on DLS measurement) and displayed little aggregation. The voltage is 120kV and magnification is 30x.

3.2 Nanoparticles biodistribution

The presence of fluorescently-tagged PLGA nanoparticles was assessed in tissues of interest (intestine, liver, kidneys, spleen, lung, heart and brain) and blood in accordance with the standard curves acquired for each tissue individually (Figure 3). Overall, the percentage of PLGA nanoparticles recovered in all tested tissues after daily gavage with 3mg for 7 days was 46.1% daily dose. Relative to other analyzed tissues, the highest nanoparticle concentration was detected in the intestine (23.4%), followed by the liver (11.4%), kidney (5.5%), spleen (2.5%), lung (2.03%), brain (1.01%) and heart (0.1%). Statistical significance was found in the intestines and liver, which had significantly more nanoparticles than the other tissues, while the feces had significantly more nanoparticles than each tissue (Figure 3). The total mass of PLGA recovered in the tissues after 7 days of treatment was 1.4 mg of the 21 mg administered over the 7 days (3 mg/day for 7 days). The remaining 19.6 mg of administered PLGA nanoparticles was either excreted in the feces and urine or metabolized into water and carbon dioxide.

The nanoparticle concentration in the blood was also analyzed after 7 days of PLGA NP treatment. The concentration was expressed as % daily dose based on the assumption that an adult male rat of 250g contains 6.4ml of blood and 4.22 ml of plasma for every 100g of body weight [37]. The amount of nanoparticles recovered in the blood was 0.7% of daily dose. PLGA nanoparticle excretion in urine and feces was evaluated in successive samples collected from animals (n=4/time point) kept in metabolic cages. After 7 days of daily feeding with PLGA NPs, 58% daily dose was excreted through the feces (Figure 3).

Tissue	NP (mg)	% Daily Dose
Feces	1.75 ± 1.42	58.3 ± 47.4 ^a
Intestine	0.70 ± 0.62	23.4 ± 20.6 ^b
Liver	0.34 ± 0.36	11.4 ± 12.0 ^b
Kidney	0.17 ± 0.11	5.5 ± 3.7 ^c
Spleen	0.08 ± 0.05	2.5 ± 1.6 ^c
Lung	0.06 ± 0.07	2.0 ± 2.2 ^c
Urine	0.05 ± 0.04	1.6 ± 1.2 ^c
Brain	0.03 ± 0.04	1.0 ± 1.3 ^c
Plasma	0.02 ± 0.01	0.7 ± 0.4 ^c
Heart	0.01 ± 0.01	0.2 ± 0.4 ^c
Total	3.21±1.6	106.6±53.3

Figure 3. Biodistribution of PLGA NPs in F344 rats (n=4) at day 7: NP amount was quantified at day 7 after daily gavage with 3 mg NPs in the intestine, liver, kidney, spleen lung, urine, brain, and heart, as well as urine and feces using tissue-specific standard curves and triplicate analysis. Statistical significance is indicated by letters “a,” “b,” and “c.”

3.3 Nanoparticle excretion

During phase I, the percentage of PLGA NPs recovered in feces increased from 16.7% daily dose at 12 hours to 58.3% daily dose at day 7 (Figure 4). The amounts of particles then decreased during phase II (animals fed with regular food) and reached the minimum value on day 28 (7% daily dose). Urine results showed an increasing trend after 12 hours (0.2% daily dose) up to 14 days after treatment (1.8% daily dose); then, the amount of recovered nanoparticles decreased during the phase II until it reached 0.5% daily dose on day 28. Little change was observed between day 21 and day 28, indicating the animals reached the baseline two weeks after they were not exposed to the nanoparticles (Figure 4).

Figure 4. NP excretion in feces and urine over time: NP excretion was measured after exposure to NPs for 7 days (Phase I) and recovery for 1-3 weeks after exposure (Phase II).

4. Discussions

This study describes the absorption, distribution and excretion of PLGA nanoparticles orally administered to F344 rats for 7 days. In addition, the clearance time post treatment was tested through the analysis of feces and urine after 7, 14 and 21 days following the end of the treatment with PLGA NPs. The biodistribution of the polymeric nanoparticles was analyzed by tracking the fluorescent dye (TRITC) covalently linked to the PLGA nanoparticles. The fluorescence was measured in the blood, feces, urine, and every organ of interest collected during necropsy.

The results showed that after daily oral administration of 3mg PLGA NPs of ~100 nm in diameter (Figure 2), a portion of this dose was absorbed and translocated to other organs, but the majority of the nanoparticles were excreted through feces (Figure 3). After PLGA NPs are ingested, NPs not excreted in the feces are absorbed. Absorbed nanoparticles are transported to the liver where they will be metabolized or distributed to other tissues via the bloodstream. PLGA NP metabolites may be excreted in the **urine and bile** or cleared via respiration in the lungs [38]. The total amount of PLGA NPs recovered in tissues after daily gavage with 3mg for 7 days was 46.1% of daily dose (1.4 mg of PLGA). The intestines contained the largest amount of nanoparticles, as expected for an oral delivery system, followed by the liver, kidney, spleen, lung, brain and heart. [Previous studies on the biodistribution of orally administered PLGA nanoparticles have shown greater concentrations in the spleen and kidneys \[4, 33\]. However,](#)

these studies measured the distribution of nanoparticles relative to the organ size. While much of the nanoparticles were distributed to the intestines and liver in this study, these are relatively large organs and are not necessarily more concentrated with nanoparticles than smaller organs such as the spleen and kidneys. Meanwhile, the amount of nanoparticles collected from urine and blood were very low. The percentage of nanoparticles recovered in feces was high, indicating that more than the half of the nanoparticles (relative to the daily dose) were excreted at day 7. A study by Navarro et al [4] examined the toxicity of orally administered PLGA nanoparticles (3 mg/ml daily) in rats for 7, 14, and 21 days. The authors noted minimal histological changes in the liver and intestines and slightly elevated serum alanine transaminase (ALT), indicating minimal intestine and liver toxicity, while no toxicity was observed in any other organs analyzed [4].

These results are consistent with previous studies regarding excretion of orally administered PLGA NPs [15] [33]. A study conducted in 1994 by Leray et al. [15] showed that recovery in feces and urine were $65.5\% \pm 32.5\%$ and $0.3\% \pm 0.2\%$, respectively, 24 hours after one dose of PLGA NPs in rats. The excretion rates of metallic nanoparticles [25] demonstrated that the recovery of orally delivered silver nanoparticles was $63 \pm 23\%$ in the feces and $0.005 \pm 0.003\%$ in urine. Another study done with orally administered zinc oxide nanoparticles recovered 58.67% in feces and 1.47% in urine [20], all very consistent with our findings.

PLGA NPs excretion in feces and urine over time after a 7-day daily PLGA treatment has stopped, indicated that between day 7 and day 14, the daily excreted PLGA NPs in feces dropped from 58% to 39% (Figure 4). This was followed by an even larger drop in daily excreted NPs at days 21, when only 9% daily dose were recovered in the feces. Little change was noted between the amount of PLGA NPs excreted at 21 days and 28 days, indicating that the rats were returning to the baseline. NPs still detected in the feces during phase II were likely the result of unabsorbed NPs built up in the GI tract and NP metabolites secreted back into the intestines in bile. It is unclear what percentages of NP detected in the feces are the result of buildup or bile excretions. While much less PLGA NPs are excreted in the urine compared to the feces when administered orally, the PLGA NPs excreted in the urine were still characterized by a large drop ($0.5\% \pm 0.9\%$ daily dose) at 28 days compared to 7 days ($1.6\% \pm 1.2\%$ daily dose) (Figure 4), further indicating a return of the animals to the baseline, two weeks after feeding with PLGA NPs had stopped. Our results support that PLGA nanoparticles are a safer choice for drug delivery relative to other nanodelivery systems because, in addition to being biocompatible, biodegradable, and highly modifiable, PLGA nanoparticles are quickly cleared from the body following the end of its administration, such as in a pharmaceutical treatment regimen.

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5. Conclusions

After 7 days of administering fluorescently tagged PLGA NPs (3 mg/day) orally, it was found that over half of the administered daily dose of the PLGA was being excreted in the feces, while only 1.6% of the daily dose was being excreted in the urine. Among the PLGA nanoparticles recovered in the tissues, most was recovered in the intestine, followed by the liver, kidney, spleen, lung, brain, plasma, and heart, respectively. The results suggest that orally administered PLGA nanoparticles may be effective vehicles for drug delivery to multiple organs, especially the intestines and liver. However, the low concentration of nanoparticles recovered in the brain and heart indicate they are not ideal targets for PLGA nanoparticle delivered drugs when administered orally. When rats were no longer administered PLGA nanoparticles, the total amount of PLGA NPs detected in the feces and urine declined each week and began to level off between day 21 and day 28, indicating a return to the baseline. The findings are significant for understanding biodistribution and excretion *in vivo* of biodegradable polymeric nanoparticles to all applications of nanoparticles as oral drug delivery systems ranging from food, agriculture, and medicine.

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Research compliance

This study was approved by the Louisiana State University Institutional Animal Care and Use Committee, following the guidelines of the National Institutes of Health's (NIH) Guide. The protocol complied with the provisions of the School of Veterinary Medicine, Division of Laboratory Animal Medicine (DLAM), requirements for the proper care and treatment of all vertebrate laboratory animals.

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Phase I: Biodistribution

Phase II: Recovery