Acute and subacute toxicity studies of Pluronic P85/poly (lactic acid) nanoparticles in mice

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The objective of the present reported study was to evaluate the oral toxicity of poly (lactic acid)-b-Pluronic-b-poly (lactic acid) (PLA-P85-PLA) nanoparticles (NPs) so as to demonstrate their applicability for drug delivery applications. In acute oral toxicity studies, the animals were fed with 0.4 ml PLA-P85-PLA NPs solution in different concentrations (low concentration: 20 mg/l, intermediate concentration: 100 mg/l and higher concentration: 500 mg/l) for 14 days; no deaths or treatment related complications were observed even in the higher concentration treatment. In case of the subacute oral toxicity test, the NPs were administered orally to mice for a period of 28 days. At the end of the study, blood samples were collected for biochemical analysis. For histopathological analysis, organs of the animals were weighed and processed. All the animals survived during the study, with no significant changes in clinical signs, body weight, feed consumption, biochemistry parameters, organ weights and histopathological findings. These results demonstrate that PLA-P85-PLA NPs produced no treatment related toxicity in mice following oral administration, thus, they can be exploited for potential therapeutic applications.

1. Introduction: Nanotechnology is developing rapidly and has achieved tremendous progress in the past several decades [1, 2]. The combination of nanotechnology and biomedicine has been studied more and more closely. At nanoscale, materials exhibit unique physicochemical properties such as small size, surface area, chemical composition, surface structure and special shapes [3, 4], which makes nanostructures attractive for a wide range of applications in various fields including drug delivery systems [5, 6], gene engineering [7, 8], cancer therapy [9] and molecular imaging systems [10, 11].

Poly (lactic acid)-b-Pluronic-b-poly (lactic acid) (PLA-P85-PLA) nanoparticles (NPs) representing a novel potential drug carrier in the field of particle-based drug delivery systems [12], have been synthesised in our group. PLA-P85-PLA NPs are prepared from commercial Pluronic P85 and biodegradable poly (lactic acid) (PLA). PLA is a well-known biodegradable and biocompatible polyester, and is currently one of the most popular materials with the brightest development prospects and was considered as a 'green' eco friendly material [13, 14]. Pluronic block copolymers are one of the very few synthetic polymeric materials approved by the U.S. Food and Drug Administration for use as food additives and pharmaceutical ingredients. Pluronic block copolymers are commonly used in pharmaceutical applications because of their amphiphilic properties [12, 15]. Thus, the PLA-P85-PLA NP has been proposed to be a novel optimal candidate for drug delivery systems. Consistent with this hypothesis, a previous study in our group demonstrated that PLA-P85-PLA NPs could be used as a promising polymeric carrier for oral insulin delivery application with sustained and enhanced hypoglycaemic effect [12]. To study in a better manner the possibility of using PLA-P85-PLA NPs in drug delivery systems, it becomes necessary to conduct its toxicity studies. Hence, the present study was undertaken to assess the toxicity of PLA-P85-PLA NPs by carrying out acute and subacute oral toxicity in mice.

2. Materials and methods

2.1. Materials: Pluronic P85 (Mn 4600, 50 wt% PEO) was kindly supplied by the BASF Corporation (Mount Olive, NJ, USA). It was dried overnight under vacuum before use. L-lactide and stannous octoate ($Sn(Oct)_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and recrystallised twice to form ethyl acetate (EtAc). The purified L-lactide was stored at 4–5 °C under argon environments.

2.2. Synthesis and characterisation of PLA-P85-PLA NPs: The PLA-P85-PLA NPs were synthesised in our group as described previously [12, 16]. Briefly, a round-bottom flask with a stopcock was heated under reduced pressure to remove moisture. After cooling to room temperature, argon was introduced into the flask. Following this, appropriate amounts of L-lactide and Pluronic P85 were added and the mixture was heated with continuous stirring to produce a well-mixed molten phase. The mixture was then cooled, and $Sn(Oct)_2$ (0.1 wt% of L-lactide) was added to the flask under argon environment. The mixture was degassed by several vacuum-purge cycles, and then heated to 180 °C. After stirring for 15 h, the content was cooled to room temperature. The product was dissolved in methylene chloride, and precipitated twice in methanol and once in diethyl ether. The polymers were filtered and dried overnight under vacuum. The polymer powder was obtained with a 70% yield. To evaluate the characteristics of the NPs, the particle size distributions were analysed using dynamic light scattering (PSS NICOMP 380, USA). Transmission electron microscopy (TEM) (Pleasanton, CA, USA) was also conducted for observing the surface morphology of the particles.

2.3. Animals: Six- to seven-week-old Chinese Kunming (KM) mice (30 ± 2 g) were obtained from the Experimental Animal Center of Jiangxi Province, China. The animals were housed at 24 ± 2 °C, 55 ± 5% humidity and for 12 h light/dark cycles with free access to water and food. All the animals were given one week to adapt to the new environment prior to the initiation of the treatment. The care and use of the animals and the experimental protocol of this study were approved by the Institutional Care and Use Committee of our university.

2.3.1 Experimental design: All the animals were divided into four groups containing ten mice per group for acute and subacute studies. The animals were administered orally at 0.4 ml at prefixed times daily throughout the whole experimental process. Group A served as the control (vehicle distilled water). NPs groups were Group B 20 mg/l, Group C 100 mg/l and Group D 500 mg/l.

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2.3.2 Clinical observation: Observations were made at least twice daily for signs of toxicity. The effect of treatment on the general health of the animals, their body weight, behaviour, skin and hair were noted [17].

2.3.3 Body weight and food intake: Before administration of the PLA-P85-PLA NPs, all the animals were weighed using a calibrated balance. For recording the food and water consumption, 100 g of standard food pellets and 200 ml of water were placed in the food tray of the cage, the unconsumed pellets were weighed and surplus water was recorded every day, then replaced with fresh 100 g of pellets and 200 ml of water in each cage, the time of filling the food tray was noted down and kept constant throughout the study.

2.3.4 Biochemical parameters: The animals were anaesthetised 24 hours after the last dose of control and PLA-P85-PLA NPs. Blood samples were collected into 1.5 ml EP tubes and kept aside for 30–60 min at 4 °C, then centrifuged for 15 min in 3000 r/min at 4 °C. Finally, liquid supernatants (blood serum) were collected into EP tubes for the next test. In this study, the following biochemical parameters were evaluated in the collected blood serum: alanine aminotransferase (ALT), aspartate aminotransferase (AST), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC). Creatinine, urea and uric acid were determined by standard procedures (blood samples were analysed using a microplate reader (MK3, Thermo), an ultraviolet spectrophotometer (UV) and kits specific to the test (all the kits were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.3.5 Histopathological parameters: The animals were sacrificed by an overdose of anaesthetic ether. During autopsy, all pathlogical changes to the organs were observed macroscopically. Organs, namely heart, liver and kidney, were dissected quickly and washed in sterile phosphate buffered saline, dehydrated on a filter paper and weighed carefully on an analytical balance. The isolated organs were trimmed into small pieces and subsequently preserved into 4% paraformaldehyde for 24 h. The specimens were subjected to dehydration with strengths of 70, 80 and 100% each for 1 h. The specimens were cut into slices 8 µm in thickness by a freezing microtome, then the slices were stained by haematoxylin-eosin (H&E) stain.

2.4. Statistical analysis: The results were expressed as means ± SD, and the statistical significance of differences between the groups was analysed by the independent Student's *t*-test for paired samples. A level of confidence of $P < 0.05$ was employed for the statistical significance.

3. Results and discussion: Polymer nanomaterials have huge potential in the fields of medical diagnostic and therapeutic applications and are reported to improve the quality and performance of many products. This will increase the exposure of polymer nanomaterials to the public, hence it becomes necessary to test the effect of these NPs on humans and the environment [18, 19]. In the present study, the acute and subacute toxicity PLA-P85-PLA NPs were studied in mice.

3.1. Characterisation of PLA-P85-PLA NPs: The biocompatible PLA-P85-PLA NPs were synthesised by ring opening polymerisation of the monomer L-lactide using Pluronic copolymer P85 as the initiator and stannous octoate ($Sn(Oct)_2$) as the catalyst in our group [12]. Figure 1*a* shows the particle size distribution of the PLA-P85-PLA NP by using dynamic light scattering; the size of the NP did not show evidence of a multimodal distribution, suggesting that the NPs reflect a main single type with a mean diameter of about 170 nm. The TEM was performed to check the morphology and size of the formed NPs [17]. The TEM images recorded indicated the presence of

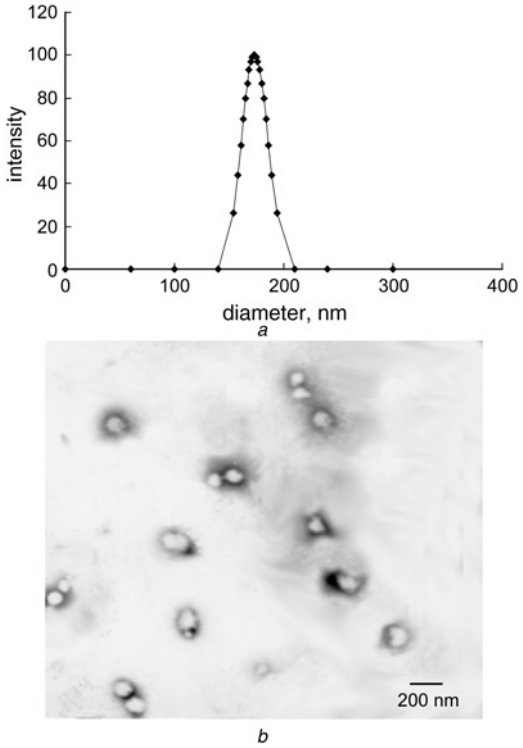

Figure 1 *Characterisation of the PLA-P85-PLA NPs*
a Size distributions for the PLA-P85-PLA NPs
b TEM micrograph of the PLA-P85-PLA NPs in water

sphere-shaped and well dispersed NPs (Figure 1*b*). The results in Figure 1 authenticate the complete formation of NPs synthesised stably and free from any aggregation.

3.2. Oral acute toxicity: The initial study was designed to determine the acute oral toxicity study of the PLA-P85-PLA NPs. During the 14-day experiment, no death, toxic signs and negative symptoms were observed in any animal. No differences were detected in the general health of the animals, including behaviour, skin, hair, diet, urination and defecation as well as the body weight (see Table 1) between the groups.

3.3. Subacute toxicity: During the 28-day period of treatment, animals from the higher concentration dose group (500 mg/l), intermediate concentration dose group (200 mg/l) and low concentration dose group (50 mg/l) were studied. The results obtained are shown in the tabulated results. It was observed that the animals in all the groups were in good condition, no unusual changes in behaviour or in locomotor activity were observed, no overt toxic effects were observed and no mortality occurred during the 28-day study period. All the animals showed normal

Table 1 Effect of PLA-P85-PLA NPs on body weight (g) in mice in the 14-day oral acute toxicity study

Group	Body weight, g		
	0 day	7 day	14 day
Control	30.65 ± 3.57	32.19 ± 3.75	34.15 ± 3.81
20 mg/l NPs	30.24 ± 3.21	32.51 ± 3.96	34.18 ± 3.53
100 mg/l NPs	30.21 ± 3.42	31.73 ± 4.13	35.24 ± 4.66
500 mg/l NPs	30.38 ± 3.59	31.82 ± 2.09	33.56 ± 2.80

Values are expressed as mean ± SD. ($N = 10$ mice per group)

Table 2 Effect of PLA-P85-PLA NPs on body weight (g) in mice in the 28-day oral subacute toxicity study

Group	Body weight, g				
	0 day	7 day	14 day	21 day	28 day
Control	30.86 ± 1.19	32.94 ± 1.04	35.54 ± 1.55	38.69 ± 2.80	41.07 ± 1.23
20 mg/l NPs	30.74 ± 0.92	32.49 ± 0.53	34.24 ± 0.59	38.89 ± 1.72	42.87 ± 1.80
100 mg/l NPs	30.68 ± 1.19	32.00 ± 1.46	34.43 ± 2.06	37.25 ± 2.46	41.89 ± 2.69
500 mg/l NPs	30.82 ± 1.04	31.74 ± 0.79	35.44 ± 0.80	37.52 ± 1.07	40.60 ± 1.41

Values are expressed as mean ± SD. Comparisons were made between the control group and the experimental group. ($N = 10$ mice per group)

Table 3 Effect of PLA-P85-PLA NPs on food consumption in the 28-day oral subacute toxicity study

Group	Food consumption (g/100 g of animal)				
	1st day	7th day	14th day	21st day	28th day
Control	12.50 ± 3.87	13.02 ± 2.01	13.17 ± 1.89	12.66 ± 1.81	13.43 ± 2.67
20 mg/l NPs	12.77 ± 2.03	12.76 ± 1.62	12.85 ± 2.90	12.25 ± 3.08	13.08 ± 1.99
100 mg/l NPs	11.54 ± 2.25	12.35 ± 1.93	12.60 ± 2.05	12.72 ± 1.79	13.15 ± 2.02
500 mg/l NPs	11.81 ± 1.95	12.73 ± 2.08	13.57 ± 1.82	12.75 ± 1.27	12.58 ± 1.55

Values are expressed as mean ± SD. Comparisons were made between the control group and the experimental group. ($N = 10$ mice per group)

Table 4 Effect of PLA-P85-PLA NPs on water consumption in the 28-day oral subacute toxicity study

Group	Water consumption (ml/day of mice)				
	1st day	7th day	14th day	21st day	28th day
Control	5.63 ± 1.06	6.88 ± 1.27	6.88 ± 1.61	7.50 ± 1.04	6.88 ± 1.39
20 mg/l NPs	6.25 ± 2.01	5.63 ± 1.99	6.25 ± 1.75	7.5 ± 1.82	7.5 ± 1.90
100 mg/l NPs	6.88 ± 1.67	6.25 ± 2.02	5.63 ± 1.93	6.88 ± 1.78	6.88 ± 1.83
500 mg/l NPs	6.25 ± 2.11	5.00 ± 2.27	6.25 ± 2.01	5.63 ± 1.97	6.88 ± 1.08

Values are expressed as mean ± SD. Comparisons were made between the control group and the experimental group. ($N = 10$ mice per group)

Table 5 Subacute toxicity effects of PLA-P85-PLA NPs on biochemical values in the 28-day study

Parameters	Control	20 mg/l NPs	100 mg/l NPs	500 mg/l NPs
ALT, U/L	78.03 ± 4.18	70.25 ± 13.52	66.28 ± 10.55	68.31 ± 11.50
AST, U/L	28.15 ± 1.63	24.95 ± 4.98	27.93 ± 5.90	27.37 ± 1.91
HDL-C, mmol/l	1.45 ± 0.13	1.47 ± 0.12	1.54 ± 0.21	1.36 ± 0.28
TC, mmol/l	3.32 ± 0.54	3.82 ± 0.48	3.44 ± 0.59	3.45 ± 0.35
urea, mg/l	150.49 ± 14.73	165.01 ± 30.69	147.39 ± 14.14	170.41 ± 21.16
uric acid, mg/l	36.40 ± 7.16	36.97 ± 6.76	32.01 ± 4.45	37.25 ± 4.25
creatinine, mg/l	0.55 ± 0.10	0.50 ± 0.14	0.54 ± 0.48	0.46 ± 0.16

Note: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol
Values are expressed as mean ± SD. Comparisons were made between the control group and the experimental group. ($N = 10$ mice per group)

weight gains during the study period in body weight between the control group and the experimental group ($P > 0.05$) (Table 2), indicating no effect of the PLA-P85-PLA NPs on the weight of the animals. The PLA-P85-PLA NPs of various concentrations did not have any effect on consumption of food and water. The food and water consumption of the animals in the control and experimental groups were similar (Tables 3 and 4). The consumption of food and water by the animals followed a similar pattern indicating a normal metabolism of the animals and the feed intake of the animals was not affected because of intake of the PLA-P85-PLA NPs.

The biochemical tests are used to diagnose diseases of the heart, liver, kidney and so on. They are also widely used in monitoring the response of the animals to toxic materials [17, 20]. If the organs do not function properly, there will be an increase in the enzyme levels. The elevated levels indicate the occurrence of liver damage, ischaemic heart disease, acute coronary syndromes and so on. In the present study, administration of the PLA-P85-PLA NPs (50, 200 and 500 mg/l) did not cause any significant difference in the control group and the experimental group (Table 5). There was no significant difference ($P > 0.05$) in ALT and AST. The level of HDL-C and TC showed no significant difference ($P > 0.05$) between the control and the PLA-P85-PLA NPs treated groups. To study kidney function, blood urea, uric acid and creatinine levels were determined. It was observed that there was no significant difference ($P > 0.05$) between the control and the experimental

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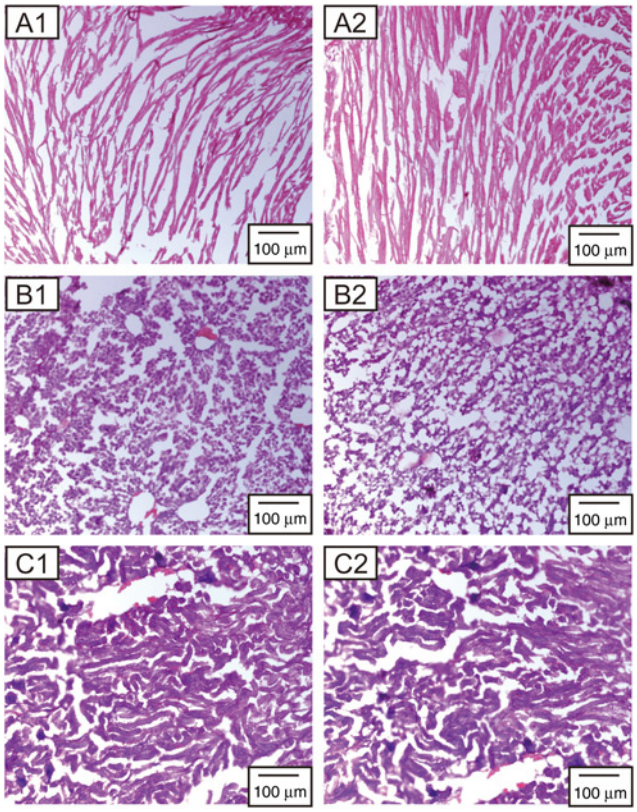

Figure 2 *Light photomicrographs of organ tissues after 28-days administration of PLA-P85-PLA NPs*
(A1), (B1), (C1), tissues of heart, liver and kidney, respectively, of control groups, (A2), (B2), (C2), tissues of heart, liver and kidney, respectively, of NPs groups at higher concentration dose 500 mg/l

Table 6 Effect of PLA-P85-PLA NPs on organ weight of mice after 28-days subacute treatment

Group	Organs weight (g)/100 g body weight		
	heart	liver	kidney
Control	0.48 ± 0.05	4.90 ± 0.45	1.27 ± 0.04
20 mg/l NPs	0.44 ± 0.03	5.22 ± 0.28	1.24 ± 0.10
100 mg/l NPs	0.46 ± 0.02	4.96 ± 0.29	1.26 ± 0.10
500 mg/l NPs	0.47 ± 0.02	5.26 ± 0.29	1.25 ± 0.09

Values are expressed as mean ± SD. Comparisons were made between the control group and the experimental group. ($N = 10$ mice per group)

groups of mice. Mean biochemical values for the control and the treated groups are given in Table 5. Feeding animals with different concentration of PLA-P85-PLA NPs did not alter the functioning of the animal, indicating normal metabolism of the animals with respect to the biochemical tests.

Finally, histopathological studies were conducted to further determine the effect of the PLA-P85-PLA NPs on the vital organs (Figure 2). The sections of the organs were studied by light microscopy. All the histological sections were normal, with no significant difference ($P > 0.05$) between the organ weights of the control and the treated groups (Table 6). In the case of the heart, the myocardium was normal and no pigment deposits were seen in any group. Unremarkable renal glomeruli and tubules were observed in all groups; the tubular epithelial cells showed focal dilatation and in the case of the kidneys also no pigment deposits in the tubules in the treated groups were observed. The kidney function test was not altered and the kidney architecture was normal. In the case of the liver, the hepatocytes and the portal tracts were found to be normal in all the treated groups and no inflammation was observed. There was no histopathological difference between the control and the NP administered groups. All the results suggested that the PLA-P85-PLA NP did not have any toxic effect on the animals when administered for 28 days.

4. Conclusion: PLA-P85-PLA NPs are increasingly recognised as a novel potential drug carrier in drug delivery systems. However, so far, there are very few in vivo studies on these NPs. A previous study in our group has suggested that PLA-P85-PLA NPs have a very good biocompatibility with no cytotoxicity in in vitro studies [12], indicating that PLA-P85-PLA NPs could be used as an optimal drug carrier. Therefore, in order to determine in a better manner the possible use of PLA-P85-PLA NPs as carriers for drug delivery, we have evaluated the acute and subacute oral toxicity of these NPs in vivo. In acute oral toxicity, the animals were fed with 0.4 ml PLA-P85-PLA NPs solution in different concentrations (20, 100 and 500 mg/l) for 14 days. No deaths occurred and no treatment related complications were observed even in the high concentration treatment. In the case of the subacute 28-day oral toxicity study, all the animals showed normal weight gain and feed consumption during the study period. Administration of the PLA-P85-PLA NPs did not cause any significant difference in the biochemical parameters of the control and the experimental groups. Histopathological studies were conducted to record the effect of the PLA-P85-PLA NPs on the vital organs. There was no histopathological difference between the control and the NP administered groups. Our findings indicate that the PLA-P85-PLA NPs did not cause any toxicity and were well tolerated for the 28-day study period. Therefore PLA-P85-PLA NPs are non-toxic and have potential for safe use as drug delivery systems.

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