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ScienceDirect
International Journal of Pharmaceutics 356 (2008) 267–273
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Histological examination of PLGA nanospheres for intratracheal drug administration

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Received 1 August 2007; received in revised form 4 December 2007; accepted 19 December 2007
Available online 8 January 2008

Abstract

Polylactide–glycolide (PLGA) nanospheres were reported as useful pulmonary drug delivery carriers for improving the pharmacological effect of drug. This paper describes the pathological and histological examinations of tissues after intratracheal instillation of drug encapsulated PLGA nanospheres. After intratracheally introducing FITC encapsulated PLGA nanospheres (dispersed in the 0.5 ml saline followed by mixing with an equal volume of air) to a rat, FITC was found existing in the rat's lungs, liver, kidney, brain, spleen and pancreas as demonstrated by immuno-histo-chemical staining with the dye.

In this study, FITC stayed in alveoli at least for 1.5 h after the intratracheal administration of the PLGA nanospheres, but the FITC almost disappeared 24 h later.

In addition, it was found that the PLGA nanospheres were absorbed in the blood immediately (within 0.25 h after the intratracheal administration) through the type 1 alveolar epithelium cell. Furthermore, the PLGA nanospheres were found resistant to uptake by macrophages such as alveolus macrophages and kupffer cells.

The results showed that the possibility to induce tissue damage caused by the excessive immune response from the deposition of PLGA nanospheres was very low, because the nanospheres were not treated as foreign substances.

Keywords: Drug delivery system; PLGA nanosphere; Intratracheal instillation; Immuno-histo-chemical staining

1. Introduction

In a preclinical study, pulmonary administration has been recognized as a noninvasive alternative to intravenous administration not only for local action drugs delivered to the lung but also for systemic action drugs absorbed via blood vessel such as peptides (Paton, 1996; Owens, 2002; Ralph et al., 2005; Anthony et al., 2006; Okamoto, 2005). We have developed composite particles containing excipient and drug encapsulated polymeric nanospheres for dry powder inhalation applications to treat systemic diseases with improved pharmacological effects. The polymeric nanospheres consisted of bioabsorbable and biodegradable material, e.g. polylactic-*co*-glycolic acid (PLGA), and had an average particle size of ~200 nm. In in vivo study, it was found that the pharmacological effects of applying insulin encapsulated PLGA nanosphere composites, evaluated by the area above the curve (AAC) of blood glucose level after intratracheal administration with rats (Yamamoto et al., 2004), were 1.6 times higher than that of applying intravenous administration of insulin solution. Similarly, 3.5 times higher pharmacological effects were found with beagle dogs (Tsujimoto et al., 2005).

These results suggested that insulin absorption efficiency was enhanced because the nanospheres adhered to the bronchial mucus and lung tissue and sustained releasing insulin at the adsorption site. In addition, the degradation of insulin might be prevented by being encapsulated in the PLGA nanospheres. Drug delivery systems with the nanospheres are expected to

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doi:10.1016/j.ijpharm.2007.12.041

improve the bioavailability of drugs. However, the behavior of PLGA nanospheres after pulmonary administration is not fully examined so far. It is important to evaluate the tissue pathology and histology to insure the safe applications of PLGA nanospheres.

In this study, 0.5 ml of saline containing fluorescein isothiocyanate (FITC) encapsulated PLGA nanospheres simultaneously mixed with an equal volume of air was administered intratracheally to rats and the FITC distributions in several tissues was stained with immuno-histo-chemistry for histological and pathological examinations of PLGA nanospheres.

The experimental results suggested that the PLGA nanospheres were absorbed in the blood immediately (within 0.25 h after the intratracheal administration) through the type 1 alveolar epithelium cell. Furthermore, the PLGA nanospheres were found resistant to uptake by macrophages such as alveolus macrophages and kupffer cells.

2. Materials and methods

2.1. Materials

Co-polymer PLGA with a 75:25 ratio of lactic acid to glycolic acid and an average molecular weight of 20,000 (PLGA7520, Wako Pure Chemical Industries, Japan) was used in the experiments. Polyvinyl alcohol (PVA403, Kuraray, Japan) was used as the dispersant for the production of PLGA nanospheres. The model drug encapsulated in the PLGA nanospheres as labeling agent was fluorescein-4-isothiocyanate (FITC-1, Dojindo laboratories, Japan).

2.2. Preparation of FITC encapsulated PLGA nanospheres

FITC was encapsulated in the PLGA nanospheres by emulsion solvent diffusion (ESD) method (Kawashima et al., 1982, 1998). After dissolving 2 g of PLGA and 0.1 g of FITC in 40 ml of acetone, 20 ml of ethanol was added to make a polymer solution. The solution was then dropped into 50 ml of 2% PVA aqueous solution stirred at 400 rpm and 40 °C. At this point, nanodroplets were formed in the emulsion by the self-emulsification at the droplet interface due to rapid diffusion and counter diffusion of the organic solvents.

The solvents were then removed by vacuum evaporation at 40 °C with a stirrer speed of 100 rpm. The PLGA nanosphere suspension thus obtained was centrifuged (at 41,000 × *g* and −20 °C for 20 min using CR20G, Hitachi High-Technologies, Japan) to cause sedimentation of the nanospheres (Kawashima et al., 1982, 1998). After removing the supernatant, purified water was added to mix with the sediment. The wet mixture was then centrifuged again to remove the excess PVA that could not adsorb on the surfaces of nanospheres. The water-washing and centrifugation cycle was repeated twice. The wet FITC encapsulated PLGA nanospheres were then pre-freezed at −45 °C (using PFR-1000, Tokyo Rikakikai, Japan) and followed by freeze drying (using FDU-2100, Tokyo Rikakikai, Japan) to produce dry powders. No cryoprotectant was used in the freeze-drying process, because all the test materials, including FITC, were stable in the lyophilization.

2.3. Physical evaluation of FITC encapsulated PLGA nanospheres

The shape of freeze-dried nanospheres was analyzed by field emission scanning electron microscopy (FE-SEM, JSM-7401F, JEOL DATUM, Japan). The particle size distribution of the nanospheres dispersed in the distilled water was measured by a dynamic light scattering instrument (MICROTRAC UPA 150, Nikkiso Co., Japan). The zeta potential of the freeze-dried nanospheres dispersed in the pure water was measured by M3PALS method (zetasizer nanoZ, Malven, England). The encapsulation ratios of FITC in the PLGA nanospheres were analyzed by using high-speed liquid chromatography at 254 nm (UV detector SPD-20A, Shimadzu, Japan) with the following procedures. After dissolving 20 mg of nanospheres in 5 ml of acetone, 5 ml of distilled water was added to separate PLGA. The suspension was then centrifuged at 41,000 × *g* and −20 °C for 20 min to remove the PLGA. Thus, FITC dissolving in the supernatant could be quantified with Inertsil ODS-3 column (GL science, Japan, acetonitrile/phosphate buffer = 60/40 mobile phase (pH 2.7) at 40 °C).

2.4. Method for intratracheal instillation of FITC encapsulated PLGA nanospheres

Male SD rats aged 8 weeks (Nihon SLC Inc., $n = 4$) were used. The animal's mouth was kept open by hanging the upper incisor teeth on wire hook as soon as anesthesia was accomplished with diethyl ether. An assistant held the rat and pulled its neck to the back to help the operator to detect the epiglottis and vocal cord. A syringe was pushed against the soft plate to enter the trachea following the vocal cord. When definite bumping against a tracheal cartilage ring was detected, the needle was inserted almost to the bottom of the trachea and the PBS solution containing FITC encapsulated PLGA nanospheres (20 mg/0.5 ml) was gently injected with 0.5 ml of air, as shown in Fig. 1.

This method was proprietary for intratracheally instillating materials to the entire lung of rats developed by the author (Oka et al., 2006), which administering materials solution with an equal volume of air. This procedure enabled the exposure of a sample to the entire lung from the main bronchi to the periphery and allowed repeated examinations of drug administration, because it was very simple and did not have to open and close bronchi with special equipment for inhalation tests.

If excessive amounts of FITC encapsulated PLGA nanospheres were administered in the experiment, the nanospheres might agglomerate in the lung. Because the agglomerates were recognized as extraneous substances by alveolar macrophages and causing abnormal immune response, the dynamic state of the PLGA nanospheres in rats might be different from that in the clinical test. Therefore, we decided to apply minimum dose of PLGA nanospheres, enough to detect and identify the cells in lung without forming agglomerates through the preparative experiment. We administrated the sus-

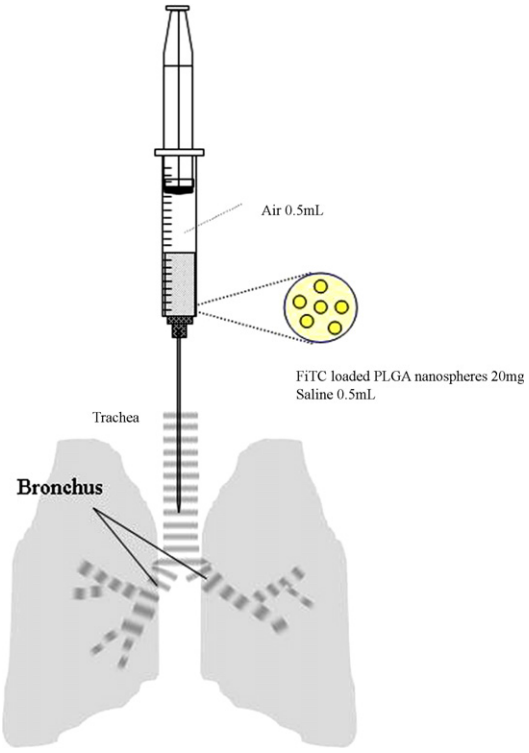


Fig. 1. Schematics of the intratracheal instillation of FITC encapsulated PLGA nanospheres.

pension of PLGA nanospheres by intratracheal instillation and the rats were sacrificed at 0.25, 0.5, 1, 1.5, 24 h later. Their lungs, liver, kidney, brain, spleen and pancreas were excised, rinsed and fixed in the neutral buffered formalin fixative. Each organ was embedded with paraffin and sliced samples with a thickness of 3 μm were prepared to observe the distribution of FITC using fluorescence Microtome. Furthermore, after the samples were deparaffinized, they were stained immuno-histo-chemically to examine the pathological and histological distributions of FITC with normal light microscope. Anti-FITC monoclonal antibody (American Research Products, Inc.) diluted to 1 μg/ml was used as the first antibody; Envion polymer reagent (DakoCytomation) was used as the second antibody; and, 3,3′-diaminobenzidine tetrahydrochloride was used as coloring substrate. In addition, hematoxylin was chosen for nucleus staining comparison.

3. Results

3.1. The characterictics of FITC encapsulated PLGA nanospheres

Fig. 2 showed the FE-SEM (field emission scanning electron microscope) photos of FITC encapsulated PLGA nanospheres prepared in this study, and Fig. 3 showed their particle size distribution as dispersed in water. All particles were in spherical shape and had an average particles size of 241.6 nm (D_{10} = 147.2 nm, D_{90} = 284.1 nm) with a narrow size distribution. Particle surface charge was −34.9 mV. The amount of FITC encapsulated inside the PLGA nanospheres was 3.18 wt%.

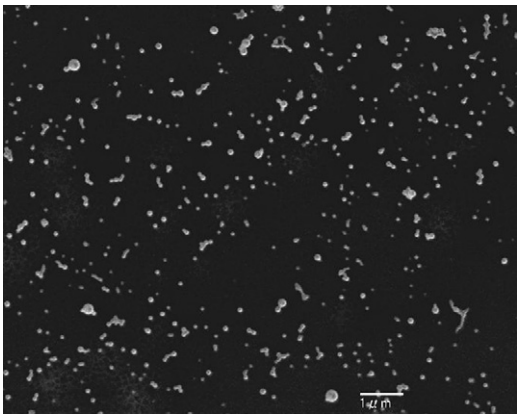


Fig. 2. Field emission scanning electron microscope (FE-SEM) photograph of FITC encapsulated PLGA nanospheres.

3.2. Histological evaluation of FITC in organs

In Fig. 4(a–c) showed the micrograms of cross-section of alveoli at 0.25, 1.5 and 24 h after intratracheal drug administration, and (d) and (e) showed corresponding magnified scale photo macrophotographs of (a) and (c). In the immuno-histochemical staining sample, the existence of FITC was stained by brown color. The distributions of FITC at 0.5 and 1 h were no different from that at 1.5 h (data not shown). In this study, FITC was found in alveoli at least for 1.5 h after intratracheally administering PBS solution containing FITC encapsulated PLGA nanospheres, but the FITC was found almost disappeared 24 h later. Also, when examining macrophotography (Fig. 4 (d)), FITC was found in type 1 alveolar epithelium cell, alveolar space and blood basement membrane, which mainly were concerned of gas exchange. On the contrary, FITC did not distribute as such in type 2 alveolar epithelium cell and alveolar macrophages, which played an important role in immunity and the defense of foreign substances coming to lung. However, when we administered 0.5 ml of saline containing 4.5 mg of FITC (Paul et al., 1999) and observed the rat's lung using fluorescence microscope 3 weeks after the drug administration, it was found that FITC presented in the blood vessel wall, alveolar space and bronchial space, as shown in Fig. 4(f). This result was completely different

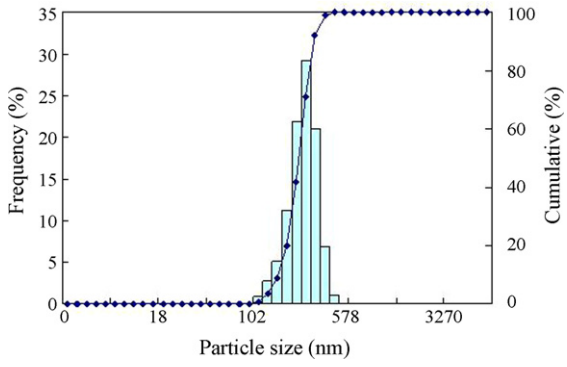
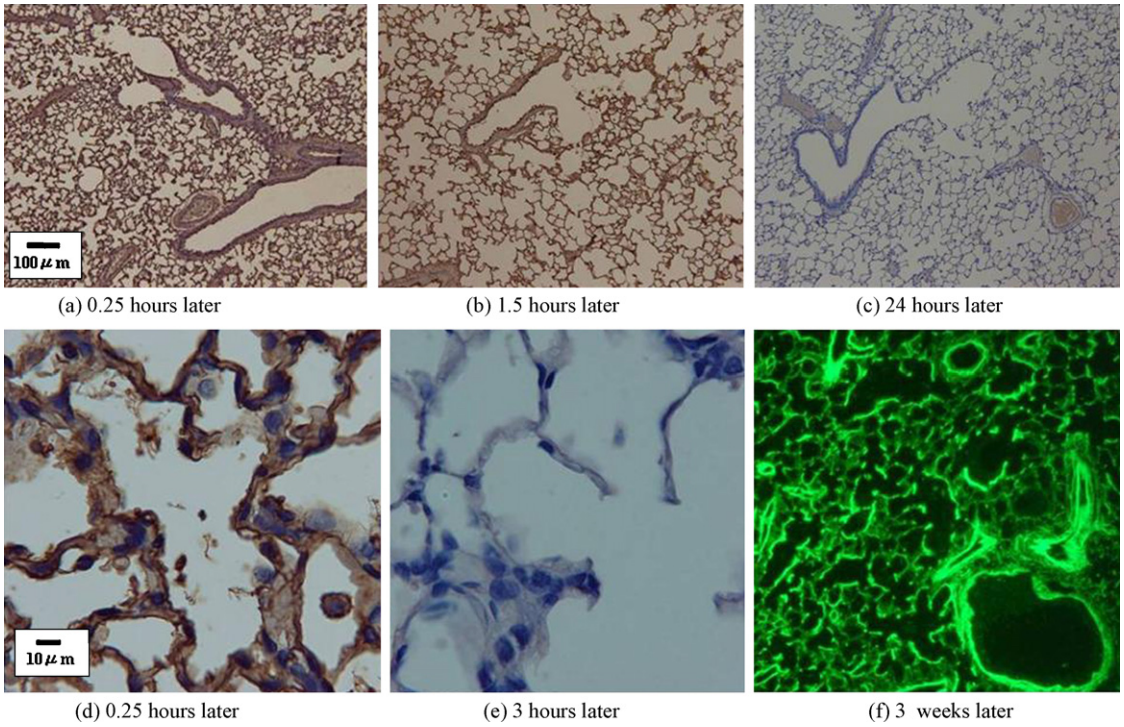


Fig. 3. Particle size distribution of FITC encapsulated PLGA nanospheres in water.



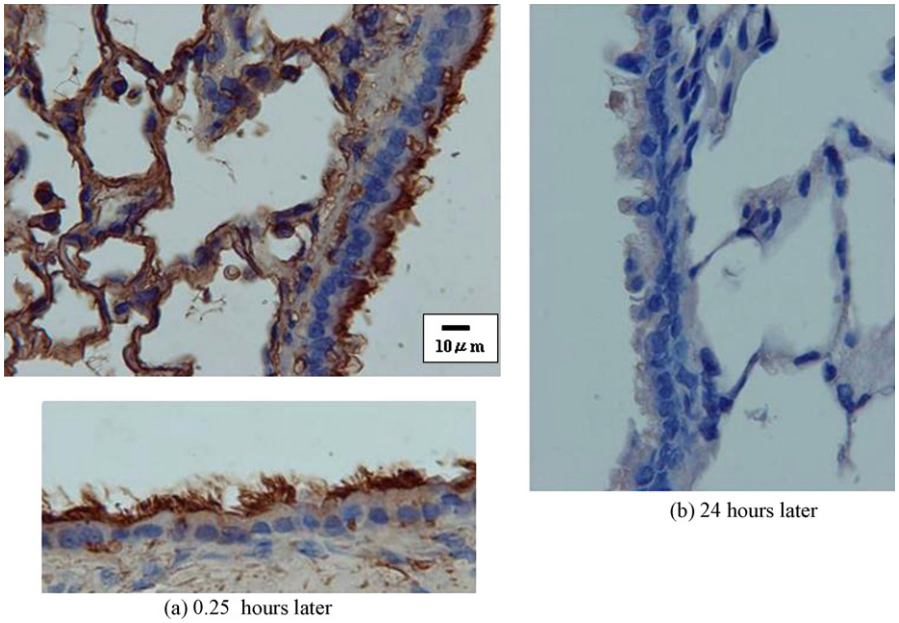
(a) 0.25 hours later (b) 1.5 hours later (c) 24 hours later

(d) 0.25 hours later (e) 3 hours later (f) 3 weeks later

Fig. 4. Microscopic features of alveoli (a–e): 0.5 ml of saline containing FITC encapsulated PLGA nanospheres (20 mg) and (f) 0.5 ml of saline containing FITC encapsulated PLGA nanospheres (4.5 mg).

from those of the test group administering the PBS solution containing 20 mg of FITC encapsulated PLGA nanospheres. When foreign substances such as agglomerates having sizes over several micrometers or submicron particles infiltrate into alveoli, it often causes tissue damage from the excessive immune response and phagocytosis by alveolar macrophages. It appeared that the FITC encapsulated PLGA nanospheres could be better dispersed in the low concentration, which reduced the chance of being phagocytosed by alveolar macrophages and inducing tissue damage.

Fig. 5(a) and (b) showed the representative micrograms of cross-section of bronchiole at 0.25 and 24 h after the drug administration. It was appeared that FITC did not distribute in the clara cells, which were concerned of foreign substance metabolism by alveoli macrophages. On the other hand, the surface of bronchial ciliated epithelium cells had very strong stain showing the exis-



(a) 0.25 hours later (b) 24 hours later

Fig. 5. Microscopic features of bronchi.

tence of FITC. This result indicated that PLGA nanospheres could easily adhere to the mucus layer of the cilia surface due to their high specific surface areas.

Fig. 6(a) and (b) showed representative microscopic photos of cross section of liver, mainly central vein, portal vein and biliary, and Fig. 7(a) and (b) showed representative microscopic photos of cross section of kidney, mainly glomerulus, convoluted tubule and collecting duct.

Both liver and kidney were stained 0.25 h after the drug administration. It appeared that FITC either encapsulated in the PLGA nanospheres or released from the nanospheres passed through the type 1 alveolar epithelium cell immediately after the drug administration and made its transition to the blood circulation. The delivery mechanisms of FITC are not clear at the moment. In the case of FITC encapsulated PLGA nanospheres, the main route of drug transfer is possible not paracellular pathway but rather transcellular pathway by endocytosis of the type 1 alveolar epithelium cell, because the gaps between the cells are very tight and the possibility for substances to penetrate is extremely low.

The distributions of FITC in each tissue were further examined in detail.

In the liver, most of the FITC was never distributed to the kupffer cells which had phagocytic function for removing the foreign substances. It is found that PLGA nanospheres were hardly recognized as foreign substances similar to that in the alveoli. In addition, comparing central vein area with portal vein area, FITC accumulated around central vein area with much more drug-metabolizing enzyme for 24 h. It suggested that this area was excretion route of FITC.

On the other hand, FITC disappeared 24 h later from where it has been 0.25 h after the drug administration in the bile duct epithelial cell. This suggested that the metabolic pathway was different between the PLGA nanospheres containing FITC and the released FITC.

As to the kidney, the capillary of the glomerulus, which filtered the blood, was first examined. FITC was found 0.25 h after the intratracheal instillation, but completely disappeared 24 h later. It suggested that the released FITC or FITC encapsulated PLGA nanospheres almost did not present in the blood 24 h after the drug administration.

In addition, it seemed that no FITC has been over the mesangial matrix, which played a role on adjusting the glomerulus filtration by controlling the diameter and pressure of the capillary of the glomerulus. Therefore, it is unlikely to contract the intractable glomerulonephritis by using PLGA nanospheres alone.

Regarding the collecting duct, similarly, FITC was found at 0.25 h, but disappeared 24 h later. On the other hand, the distribution of FITC was found increased in the distal convoluted tubule over 24 h. This difference suggested that the metabolic pathway was different between the PLGA nanospheres containing FITC and the released FITC.

Furthermore, it was found that little FITC distributed in the spleen, pancreas and brain (data not shown). In this study, the distribution of PLGA nanospheres in the blood or each tissue

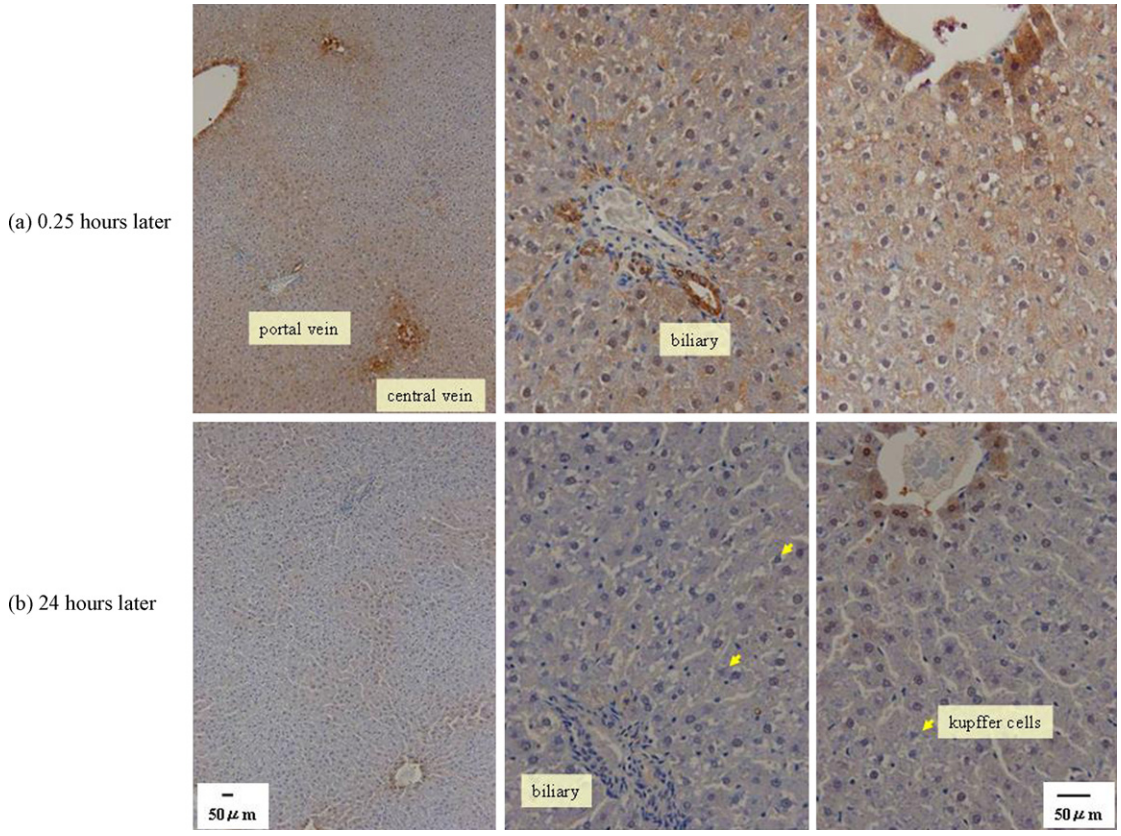


Fig. 6. Microscopic features of liver.

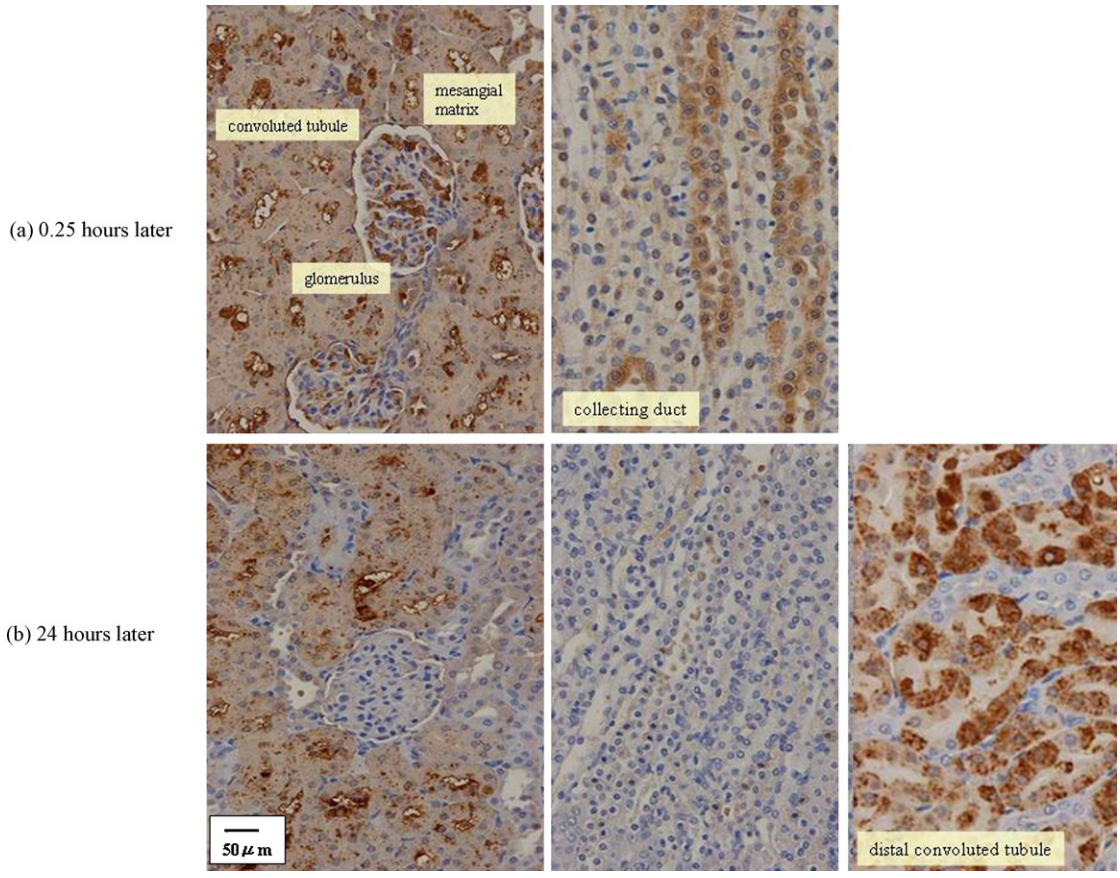


Fig. 7. Microscopic features of kidney.

could not be determined, because we could not differentiate the FITC released by the PLGA nanospheres from the FITC encapsulated in the nanospheres. It is worthwhile to carry out further examinations in the future to study the dynamic state of PLGA nanospheres in vivo.

4. Conclusion

In this study, we pathologically and histologically examined several tissues of rats after intratracheal instillation of PLGA nanospheres for the purpose of applying drug encapsulated PLGA nanospheres for pulmonary administration.

The results within the scope of this study are as follows.

(1) PLGA nanospheres remained in type 1 alveolar epithelium cell, alveolar space and blood basement membrane at least for 1.5 h after the drug administration.
(2) PLGA nanospheres were absorbed immediately through type 1 alveolar epithelium cell, because FITC was found in liver and kidney 0.25 h after the drug administration.
(3) PLGA nanospheres hardly distributed in spleen, pancreas and brain.
(4) PLGA nanospheres were difficult to uptake by macrophage. The possibility of causing excessive immune response and inducing tissue damage at the specific deposition site by PLGA nanospheres was very low, because the nanospheres were not considered as foreign substances biologically.

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