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증가에 대한 연구

1,1'-Dioctadecyl-3,3,3',3'  
tetramethylindotricarbocyanine  
iodide-loaded poly(lactide-co-  
glycolide) nanoparticles increase  
local retention after paraspinal  
muscle injection

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# Abstract

## 1,1'-Dioctadecyl-3,3,3',3' tetramethylindotricarbocyanine iodide- loaded poly(lactide-co-glycolide) nanoparticles increase local retention after paraspinal muscle injection

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Poly(lactide-co-glycolide) (PLGA) nanoparticles are promising materials for the development of new drug releasing systems. In this study, PLGA nanoparticles were doped with the fluorescent material 1,1'-dioctadecyl-3,3,3',3' tetramethylindotricarbocyanine iodide (DiR), and the retention of the nanoparticles was evaluated in mice. Mice (n = 20) were injected with 0.1 mL DiR-loaded PLGA nanoparticles (200 nm) into the right paraspinal muscle, and the same volume of pure DiR solution was injected into the left paraspinal muscle. Fluorescence images were obtained using an IVIS Imaging System. Fluorescent images were taken 1 day after the injection, and seven more images were taken at 1-week intervals. After 7 weeks, 12 mice showed a right-sided dominant signal, representing the DiR-loaded PLGA nanoparticles; five mice showed a left-side dominant signal, representing the free DiR solution; and three mice showed no signal at all beginning 1 day after the injection. These last three mice were excluded from further analysis owing to apparent injection failure. Notably, DiR-loaded PLGA nanoparticles showed significantly higher fluorescence intensity over the 7-week period. In conclusion, the results of the current study suggested that therapeutic agents that bind to PLGA nanoparticles may exhibit prolonged retention times.

**Keywords:** PLGA nanoparticle, paraspinal muscle, intramuscular injection, local retention time, nude mouse, fluorescence, IVIS imaging system

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## Introduction

As the population ages, chronic back pain is becoming one of the most frequently reported symptoms in the industrialized world. The estimated prevalence of nonspecific chronic low back pain in adults is 15%; however, this increases with age, reaching as high as 44% in individuals who are 70 years of age [1, 2]. Back pain can be caused by fracture, malignancy, inflammation, infection, deformity, or systemic diseases [1, 2]. However, chronic pain, defined as persistent pain that last 6 months after injury, is associated with chronic pathologic processes, and continually causes intermittent pain for months or years, is a complex problem that may not be amenable to routine pain control methods, and healing may never occur [3].

Because facet joints refer pain to adjacent structures, these types of joints are often a common cause of chronic back pain [4, 5]. Pain innervations are also present in other local soft tissue structures adjacent to the joint; thus, joint inflammation affects other local tissues [4-6]. Facet joints can be blocked by intra-articular injections or by anesthetizing the medial branches of the dorsal rami, which innervate the target joint [6, 7].

If the initial clinical and imaging findings are nonspecific or are insufficient for diagnosis, spinal pain intervention allows a functional assessment of the anatomic structures that are suspected to be sources of pain [8, 9]. Moreover, spinal pain intervention is needed as an adjunct to conservative management for patients with inoperable conditions, for postoperative pain and recovery, if oral or systemic drug therapy is discontinued because of adverse effects, or based on the presence of

adjacent segment deterioration after spinal fusion [2].

Corticosteroids are commonly used in pain intervention and have both short-term and long-term effects. However, recurrent corticosteroid injection could reduce the degree of pain relief. Moreover, corticosteroids have severe systemic side effects, including infection, suppression of the pituitary-adrenal axis, hyperadrenocorticism, Cushing's syndrome, osteoporosis, avascular necrosis of the bone, steroid myopathy, epidural lipomatosis, weight gain, fluid retention, and hyperglycemia [10]. Therefore, guidelines suggest limiting the frequency and interval of this type of pain intervention. The American Society of Interventional Pain Physicians (ASIPP) recommends that facet joint injections be given at least 2–3 months apart assuming that more than 50% relief is obtained within 8 weeks.

Accordingly, there is an increased need to develop a new drug delivery system for improved local steroid retention. Kim et al. [11] used a rat model of spinal cord injury to compare the efficacy of controlled, nanoparticle-enabled local delivery of methylprednisolone to the injured spinal cord with systemic delivery and a single local injection of methylprednisolone without nanoparticles. Based on histological and behavioral data, they report that local, sustained delivery of methylprednisolone via nanoparticles is significantly more effective than systemic delivery.

The goal in our laboratory is to develop a new drug delivery system using poly(lactide-co-glycolide) (PLGA) nanoparticle-loaded triamcinolone for applications in the clinical setting. However, it is first necessary to confirm that the in vivo retention time of nanoparticle-loaded material is longer than that of the

material alone. Therefore, the purpose of this study was to evaluate the in vivo retention time of materials loaded in nanoparticles as compared with that of the material alone by in vivo imaging in nude mice.

## **Materials and Methods**

### *Preparation of nanoparticles*

Positively charged PLGA nanoparticles doped with a fluorescent material, 1,1'-dioctadecyl-3,3,3',3' tetramethylindotricarbocyanine iodide (DiR), were synthesized by the College of Pharmacy, Chung-Ang University, South Korea. In order to elucidate the effects of particle size, nanoparticles were made in three different sizes: 200 nm, 10  $\mu\text{m}$ , and 100  $\mu\text{m}$ . The particle size, polydispersity index (PDI), and zeta potential were measured with a Zetasizer Nano-ZS instrument (Marlvern Instrument, Worcestershire, UK). The particle size distributions of 10 MP were measured using a Mastersizer 2000 instrument (Marlvern Instrument). The particle size distributions of 100 MP were measured by sieving the particles through a series of meshes ranging from 80 to 180  $\mu\text{m}$ . Nanoparticles containing DiR as the fluorescent probe were further characterized in terms of their particle size, zeta potential, and amount of DiR loading in aqueous medium (Table 1).

### *Animals*

A total of 23 hairless mice were obtained from Orient Bio (Kyunggi-do,

South Korea). Mice were housed under conditions that included a controlled light cycle and controlled temperature ( $23 \pm 1^\circ\text{C}$ ). Tap water and standard laboratory chow were available ad libitum. All animal experiments were performed in accordance with the Principles of Laboratory Animal Care (NIH publication number 85-23, revised 1996) and were approved by the Committee for Animal Experiments of Bundang Seoul National University Hospital (Kyunggi-do, South Korea).

### *Intramuscular injections*

For each animal, 0.1 mL DiR-loaded PLGA nanoparticles was injected into the right paraspinal muscles, and the same volume of pure DiR solution (50  $\mu\text{g}/\text{mL}$ ) was injected into the left paraspinal muscles. DiR solution was prepared by solubilizing the hydrophobic probe in 1% (w/v) sodium lauryl sulfate solution. Insulin syringes (31 G) were used for the injection, and all bilateral injections on the paraspinal back muscles were carried out by a single researcher.

### *Experimental design*

A pilot study was performed in three nude mice, with one size of nanoparticles used for each mouse, in order to determine whether there were differences in the in vivo characteristics of the nanoparticles according to particle size. The main experiment was performed with 20 nude mice, and the smallest nanoparticles (200 nm) were used.



### *In vivo optical imaging system (IVIS)*

Fluorescence images were obtained using an IVIS Imaging System (Lumina II, CALIPER, USA). Fluorescent images were taken 1 day after the injection (day 1), and seven more images were taken at 1-week intervals (weeks 1–7).

Fluorescence intensities were estimated using ImageJ, a public-domain Java image processing program, which can calculate area and pixel value statistics for user-defined selections. One region of interest (ROI), corresponding to the area of the highest fluorescence signal intensity, was chosen manually with ImageJ.

### *Statistical analysis*

Statistical analysis was performed using PASW Statistics 19 software (SPSS, Inc., Chicago, IL, USA). Statistical differences were examined using Student's *t* tests. Results with *p* values of less than 0.05 were considered significant.

## **Results**

### *Differences in the timing of fluorescence signals in the presence of nanoparticles*

Initially, three mice were injected with nanoparticles of three different sizes (200 nm, 10  $\mu$ m, or 100  $\mu$ m) loaded with the fluorescent material. The smallest particle used in this study, 200 nm, gave a very rapid increase in fluorescence signal,

reaching a maximum within 1 week (Figure 1). The fluorescence signal remained consistent throughout the entire test period of 7 weeks. In contrast, the two larger particles (10 and 100  $\mu\text{m}$ ) showed a much slower increase in the fluorescence signal, reaching maxima at about week 6, near the end of the test period of 7 weeks, and the maximum values were lower than that of the 200 nm particles.

### *In vivo retention times of the nanoparticles*

The retention profiles of DiR-loaded PLGA nanoparticles in the back muscles of nude mice were investigated by IVIS fluorescence imaging after intramuscular injection. In 14 of the 17 mice, free DiR solution showed stronger signal intensity than DiR-loaded PLGA nanoparticles on day 1 after injection (Table 2-1). After follow up of the 20 nude mice for 7 weeks, 12 mice showed a right-sided dominant signal (Table 2-2), representing the DiR-loaded PLGA nanoparticles; five mice showed a left-side dominant signal (Table 2-2), representing the free DiR solution; and three mice showed no signal at all beginning 1 day after the injection. These last three mice were excluded from further analysis owing to apparent injection failure. Notably, DiR-loaded PLGA nanoparticles showed significantly higher fluorescence intensity over the 7-week period (Table 3).

Figures 2-1, 2-2, and 2-3 show IVIS images of mice #6, #3, and #7 with the fluorescent signal of DiR-loaded PLGA nanoparticles and free DiR solution after intramuscular injection. During the 7-week period, the mean signal intensities

of the free DiR solution and DiR-loaded PLGA nanoparticles diverged gradually, as shown in Table 3. On day 1 and week 1, free DiR solution showed significantly higher signal intensities than the DiR-loaded PLGA nanoparticles ( $p < 0.001$ ). However, for the next 4 weeks, there were no significant differences. Finally, by week 7, there was a significantly increased amount of fluorescent probe incorporated in the PLGA nanoparticles in the paraspinal muscle ( $p = 0.035$ ).

## Discussion

Since the emergence of nanotechnologies, various fields have begun to utilize nanoparticles, including computer science, textile, cosmetic, and medical industries. Biodegradable nanoparticles are frequently used to improve bioavailability, retention times, and controlled delivery [12]. Nanomedicines have been evaluated in clinical trials of different phases for potential applications in the treatment of cancer, acquire immunodeficiency syndrome (AIDS), diabetes, tuberculosis, and malaria [13]. In this study, we found that PLGA nanoparticle-loaded materials exhibited increased retention times in vivo, supporting the potential applications of PLGA nanoparticle-loaded materials in spinal intervention.

Nanoparticles are generally composed of positively charged polymers made through an emulsion and evaporation procedure. The nanoparticles provide a cationic surface shell structure with a neutral polymer core in which drug or fluorescence conjugates bind through ester bonds. Previous studies have demonstrated prolonged intra-articular retention times through formation of cationic

nanoparticles with a diffuse ionically crosslinked network of nanoparticles and endogenous hyaluronate in the knee joint [14]. To the best of our knowledge, no studies have evaluated intramuscular injection of nanoparticles at the paraspinal muscles.

Nanoparticle size could be a key factor affecting the differences in biodistribution. Leclerc et al. reported that intramuscular injection of nanoparticles results in accumulation of nanoparticles in the circulatory system and testes and excretion of significant amounts of particles through the urine using mouse models [15]. However, they confirmed that no morphological alterations occurred in the testis as a result of exposure to nanoparticles [15]. In studies of the biodistribution of nanoparticles, researchers have found that smaller nanoparticles tend to reach the testes, whereas larger particles are unable to reach the testes [15-17]. De Jong et al. intravenously injected rats with gold nanoparticles having diameters of 10, 50, 100, or 250 nm and found that the distribution of gold nanoparticles was size dependent, with the smallest particles showing widespread distribution to the blood, heart, lungs, liver, spleen, kidney, thymus, brain, and reproductive organs [17]. Similarly, in our pilot study, we found that the smallest nanoparticles, having a size of 200 nm, showed the highest peak signal intensity within 1 week, which persisted throughout the test period. In contrast, 10- and 100- $\mu\text{m}$  particles showed delayed signal release, with peak signal intensity observed at around 6 weeks after injection. Thus, further optimization of particle size may be needed for different applications. Prior studies evaluating the intra-articular injection of nanoparticles also emphasized that particle

size as a key factor; particles smaller than 250 nm can freely escape from the joint cavity [18]. Similarly, another study reported that nanoparticles with a mean diameter of 265 nm are phagocytosed in the synovium by macrophages that infiltrate through the synovial tissues, whereas particles measuring 26.5  $\mu\text{m}$  in size are not phagocytosed. The phagocytosed nanoparticles are delivered to the deep underlying tissues; therefore, nanospheres are more suitable for delivery to inflamed synovial tissue than microspheres due to their ability to penetrate the synovium [19].

The peak signal intensity of the 100- $\mu\text{m}$  particles was lower than those of the 10- $\mu\text{m}$  and 200-nm particles. Because of the minor variations of the signal intensity within the first week, delayed IVIS scan of 100- $\mu\text{m}$  particles would be needed to provide a much higher signal intensity than the signal intensity observed at week 7. In this study, we used the 200-nm nanoparticles because we assumed that the rapid onset of the peak signal was more feasible when comparing with the signal intensity of the contralateral side.

Although prior studies [14, 18-20] focused on the intra-articular local retention time, our results also demonstrated that the nanoparticle-loaded material was retained for a prolonged period in vivo. This in vivo examination of DiR-loaded PLGA nanoparticles confirmed that the nanoparticles exhibited significantly higher fluorescence signal intensity over the 7-week period after injection.

The major limitation of this study is that we did not perform an in vitro study. Additionally, other therapeutic material, such as steroid-loaded nanoparticles,

could perform differently from our DiR-loaded nanoparticles; therefore further studies are required.

In summary, the results of the current study suggested that therapeutic agents that bind to PLGA nanoparticles may exhibit prolonged retention times. Further studies are needed to optimize particle size, evaluate drug-loading capacity, and perform in vivo retention assessments in animal models to more closely mimic the clinical setting.

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Table 1. Physicochemical characteristics of DiR-loaded PLGA nanoparticles

	200 NP	10 MP	100 MP
Particle size (nm)	258.9 ± 8.3 nm <sup>a</sup>	1.982 μm <sup>b</sup>	80–180 μm <sup>c</sup>
PDI	0.15 ± 0.01 <sup>a</sup>		
Zeta potential (mV)	-10.9 ± 0.1 <sup>a</sup>		
Loading amount (% , w/w)	0.474	1.344	3.046
	<sup>d</sup>		

Note: Values represent means ± SDs.

Abbreviations: DiR, 1,1'-dioctadecyl-3,3',3' tetramethylindotricarbocyanine iodide; PLGA, poly(lactide-co-glycolide); NP, nanoparticle; MP, microparticle; SD, standard deviation; PDI, polydispersity index.

<sup>a</sup> Particle size, polydispersity index (PDI), and zeta potential measured with Zetasizer Nano-ZS (Marlvern Instrument, Worcestershire, UK).

<sup>b</sup> The particle size distribution of 10 MP was measured using a Mastersizer 2000 (Marlvern Instrument)

<sup>c</sup> The particle size distribution of 100 MP was measured by sieving through a series of meshes ranging from 80 to 180 μm.

<sup>d</sup> Loading amount (% , w/w) = (weight of the encapsulated DiR) / (total weight of the PLGA particles) × 100

Table 2-1. Signal intensity on the day just after injection

Mouse no.	Free DiR solution	DiR-loaded PLGA nanoparticles
(n = 17)		
1	141.5	111.8
2	153.2	84.5
3	126.9	153.6
5	142.2	150.4
6	150.3	94.6
7	156.5	119.0
8	55.2	54.4
9	133.4	74.0
10	164.1	93.3
11	126.6	79.3
13	131.8	108.1
14	148.6	72.6
15	154.2	86.2
16	93.2	103.3
17	145.8	114.5
18	156.6	91.9
19	158.1	129.3

Note: mice 4, 12, and 20 were omitted owing to injection failure.

Table 2-2. Signal intensity at the end of week 7

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Mouse no.	Free DiR solution	DiR-loaded PLGA nanoparticles
(n = 17)		
1	56.5	148.4
2	159.3	37.0
3	-	148.9
5	48.0	114.0
6	50.7	121.3
7	61.6	116.0
8	-	78.4
9	68.3	83.2
10	99	97.3
11	155.1	112.6
13	68.2	89.3
14	79.3	90.6
15	50.6	103.4
16	-	146.6
17	114.9	56.6
18	72.4	135.8
19	129.3	66.0

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Note: mice 4, 12, and 20 were omitted owing to injection failure.

The injected free DiR solution in mice 3, 8, and 16 showed no signal at the end of the day.

Table 3. Comparison of the mean signal intensity values between free DiR solution and DiR-loaded PLGA nanoparticles.

	Free DiR solution	DiR-loaded PLGA nanoparticles	<i>p</i> value
Day 1	137.5	96.4	< 0.001
Week 1	137.5	97.9	0.015
Week 2	125.6	125.2	0.979
Week 3	121.3	127.2	0.664
Week 4	110.4	133.9	0.081
Week 5	110.9	119.0	0.591
Week 6	94.5	122.9	0.052
Week 7	71.4	102.7	0.035

Figure 1. Changes in the fluorescence signal with time

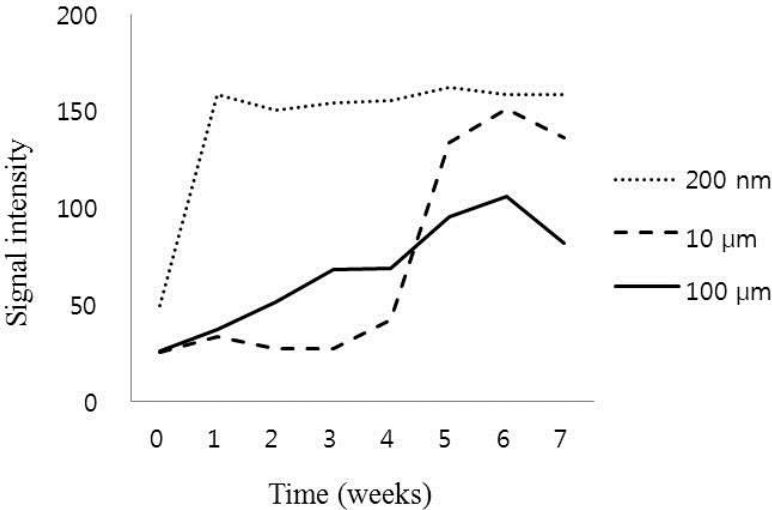


Figure 2-1. In vivo fluorescence images of the mouse 6

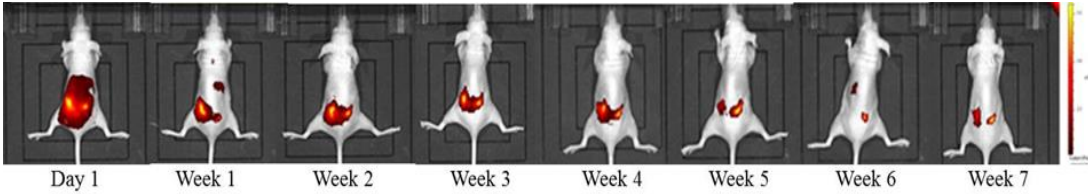


Figure 2-2. In vivo fluorescence images of the mouse 3.

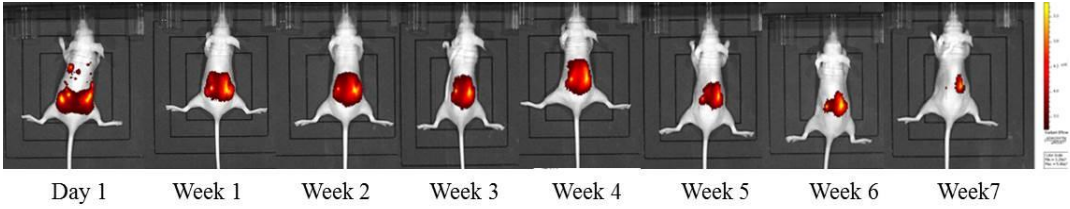
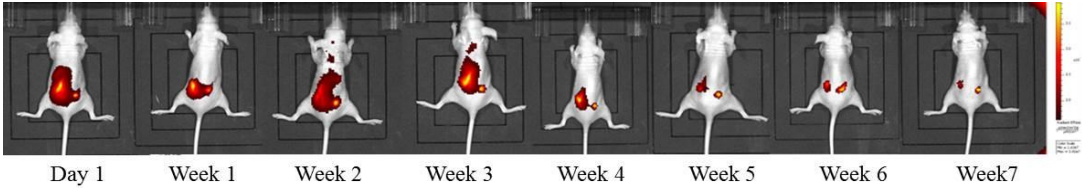




Figure 2-3. In vivo fluorescence images of the mouse 7



## 국문요약

PLGA 나노 파티클은 신체 내 국소 약물 방출 시간을 늘이기 위해서 개발되고 있는 촉망받는 물질 중 하나이다. 우리 연구에서는, PLGA 나노 파티클을 형광물질 (1,1'-dioctadecyl-3,3,3',3' tetramethylindotricarbocyanine iodide) 에 탑재하여 제조하여 근육 주사에 이용하였다. 총 23 마리의 누드 마우스가 실험에 사용되었으며, 3마리는 pilot 연구, 20 마리는 main 연구에 사용하였다. 체내 형광물질을 관찰하기 위해 IVIS Imaging System을 사용하였고, 주사 다음날, 그로부터 1주의 간격으로 총 7주 동안 이미지를 촬영하였다. Pilot 연구는 각각 200 nm, 10  $\mu$ m, 그리고 100  $\mu$ m의 세가지 다른 크기의 파티클이 탑재된 형광물질 0.1 cc 를 각각 쥐의 오른쪽 척추 주위 근육에 주사하여 크기에 따른 체내 방출 특성을 관찰하였다. 이 중 가장 크기가 작은 200 nm 파티클이 가장 빨리 방출되기 시작하였으며, 7주째에 가장 높은 형광시그널을 보였다. Main 연구에서는 200 nm 용액을 사용하였고, 20마리 누드마우스의 오른쪽 척추 주위 근육에는 200 nm PLGA nanoparticle이 탑재된 형광물질을, 왼쪽 척추 주위 근육에는 오로지 형광물질을 각각 0.1 cc씩 주사하였다. 7 주 후 촬영에서 12 마리는 오른쪽 척추 주위 근육에서 높은 시그널을 나타냈고, 5 마리는 왼쪽 척추 주위 근육에서 높은 시그널을 나타냈으며, 3 마리는 주사 다음날부터 지속적으로 아무 시그널을 보이지 않아서, 주사 실패로 간주하였다. 주목할 만한 점은, 주사 다음날 촬영한 이미지에서는 17 마리 중 오직 3마리만이 오른쪽 (나노파티클이 탑재된) 이 미세한 시그널을 보였지만, 7주를 추적 관찰하는 동안 점차적으로 12 마리에서 나노파티클이 탑재된 오른쪽이 미세한 시그널을 보였다는 것이다. 결론적으로, 우리 연구는 PLGA 나노 파티클에 탑재된 어떠한 물질을 근육주사 하였을 때, 체내 잔류 시간을 늘리는 데 이용될 수 있다는 것을 보여주었다.

**주요어:** 나노파티클, 척추 주위 근육, 근육주사, 체내 잔류 시간, 누드마우스, 형광 물질, 형광이미지분석기

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