

**Rational and precise design of polymeric nanoparticles
for tumor imaging and internal radiation therapy**

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List of Abbreviations

^{18}F -SFB	succinimidyl 4- ^{18}F fluorobenzoate
^1H NMR	proton nuclear magnetic resonance spectroscopy
^{131}I -SIB	<i>N</i> -succinimidyl 3- ^{131}I iodobenzoate
ABC phenomenon	accelerated blood clearance phenomenon
AFM	atomic force microscope
ALT	alanine transaminase
APTES	3-aminopropyltriethoxysilane
AST	aspartate transaminase
CAC	critical association concentration
CBSA	cationic bovine serum albumin
CBSA-NP	CBSA conjugated PEG-PLA nanoparticle
CD	circular dichroism
COSY	correlation spectroscopy
DDS	drug delivery system
DLS	dynamic light scattering
DMAP	<i>N,N</i> -dimethyl-4-aminopyridine
DMEM	Dulbecco's modified Eagle's medium
DSC	differential scanning calorimetry
ELISA	enzyme-linked immunosorbent assay
EOB	end of bombardment
EPR	enhanced permeability and retention effect
FDG	2-deoxy-2- ^{18}F -fluoro-D-glucose

FO	follicles
GPC	gel permeation chromatography
HbV	hemoglobin vesicles
HCC	hepatocellular carcinoma curative
ICG	indocyanine green
IP	intraperitoneal
IV	intravenous
MM2	molecular mechanics 2
MPS	mononuclear phagocyte system
MZ	marzinal zone
NCA	<i>N</i> -carboxyanhydride
NIRF	near-infrared fluorescence
NS	not significant
OD	optical density
PCL	PEG-coated naked cationic liposome
PDI	polydispersity index
PDLA	poly(D-lactic acid)
PDLLA	poly(D,L-lactic acid)
PEGylated liposome	poly(ethyleneglycol)-modified liposome
PEIT	percutaneous ethanol injection therapy
PET	positron emission tomography
PG	poly(glycerol)
PLLA	poly(L-lactic acid)
poly(Sar) _m - <i>block</i> -PLLA _n	poly(sarcosine) _m - <i>block</i> -poly(L-lactic acid) _n

PS	poly(sarcosine)
PSCL	PEG-coated siRNA-lipoplex
PVP	poly(<i>N</i> -vinyl-2-pyrrolidone)
RFA	radiofrequency ablation
ROI	region of interest
SEC	size exclusion chromatography
SLS	static light scattering
TD	T-cell dependent
TEM	transmission electron microscopy
TI	T-cell independent

List of the Measurement Instruments

^1H - or ^{19}F -NMR spectra	DPX400 (Bruker, Billerica, MA, USA)
AFM	Agilent 5500 AFM (Agilent Technologies Inc., CO, USA)
CD	JASCO J600 spectropolarimeter
DLS	Nano-ZS (Malvern, UK) Photal DLS-8000KS (Otsuka Electronics Corp., Osaka, Japan)
dn/dc, multi-angle light scattering	DRM-3000 and DLS-6800 (Otsuka Electronics Co., Ltd., Osaka, Japan)
fluorescence intensity	RF-5300 Fluorescence Spectrophotometer (Shimadzu Corp. Japan).
Fluorine-18	ultracompact cyclotron (CYPRIS model 325R; Sumitomo Heavy Industry Ltd., Tokyo, Japan) cyclotron (HM-18, Sumitomo Heavy Industry Ltd., Tokyo, Japan)
NIRF images	Clairvivo OPT (Shimadzu Corp. Japan) IVIS-Spectrum (Caliper – PerkinElmer, USA)
PET images	Clairvivo PET (Shimadzu Corp., Kyoto, Japan)
Radioactivity	CRC-30 radioisotope calibrator (Capintec Inc., Ramsey, NJ, USA) ARC-2000 gamma-counter (Aloka, Tokyo, Japan)

SLS	Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK)
TEM	JEM-2000EX II (JEOL Ltd., Japan)
UV assay	Multiscan Spectrum Microplate Photometer (Thermo Scientific, USA) Microplate Reader on iMark (Bio-Rad Laboratories Inc., CA, USA)
UV spectra	BioPhotometer plus 6132 (Eppendorf, Hamburg, Germany) DU530 Life Science UV/Vis Spectrometer (Beckman Coulter Inc, Fullerton, CA, USA)
γ -Counter	COBRA II (Packard Instrument, USA)

General Introduction

General Introduction

Nanoparticles for drug delivery system

Nanoparticles such as polymeric micelles and liposomes are highly potential as a vehicle for diagnostic and therapeutic agents in the field of drug delivery system (DDS) [1-5]. The nanoparticles loading the medicinal reagents are expected to be applicable for evaluation of pathological conditions and clinical treatment in a non-invasive manner. Researches on DDS carrier have been explored intensively in the last decade, and new knowledge and various technologies using polymeric micelles and liposomes for diagnosis and therapy have become reported [6-8]. These nanoparticles can be used as not only diagnostic but also therapeutic agents depending on the loading chemical agents on them [6, 9].

Application of nanoparticles for chemotherapy has been actively investigated to attain suitable biodistribution and blood circulation behavior of drugs through their encapsulation into nanoparticles [10, 11]. Doxil[®] is a typical example for this type of nanocarrier, which is poly(ethyleneglycol)-modified (PEGylated) liposome loading doxorubicin in the inner aqueous phase [1].

Enhanced permeability and retention (EPR) effect

The accumulation of nanoparticles in solid tumors is explained by the EPR effect [11-13], which allows nanoparticles in the size range from 10–100 nm to leak out from blood vessels having submicron-sized defects at tumor regions where the lymphatic drain system is generally immature as well (Figure 1). Indeed, polymeric micelles loading anti-tumor drugs have been widely studied for clinical applications [14, 15].

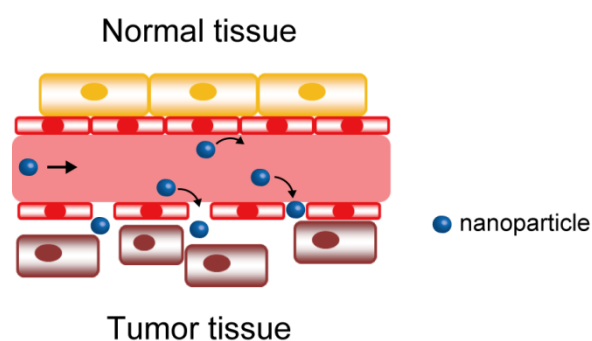


Figure 1. Schematic illustration of the EPR effect

Lactosome

The author focuses her attention especially on polymeric micelles composed of amphiphilic polydepsipeptides, poly(sarcosine)-*block*-poly(L-lactic acid), named as “Lactosome” [16]. The average block sizes of hydrophilic poly(sarcosine) and hydrophobic poly(L-lactic acid) (PLLA) were 60–90-mer and 30-mer, respectively (Figure 2). Lactosome with a diameter of ca. 35 nm has been shown to accumulate specifically in solid tumors through the EPR effect [17].

Poly(sarcosine) is a water soluble peptoid, which is in general resistant to endogenous proteinases, and is easily excreted via kidney. Further, the component sarcosine, *N*-methylglycine, is a kind of natural amino acids found in muscles and other body tissues, and is metabolized endogenously by sarcosine dehydrogenase [18]. PLLA is a well-known biocompatible and biodegradable polymer [19, 20]. Therefore, Lactosome is considered to be biocompatible materials.

Besides, Lactosome is hardly recognized by the mononuclear phagocyte system (MPS) leading to a long life time in blood stream. Indeed, human liver carcinoma cells orthotopically implanted on liver were successfully imaged by Lactosome carrying a near-infrared fluorescence (NIRF)-dye, indocyanine green (ICG), because of

low-background signal in liver due to the escape ability of Lactosome from the healthy region of the liver [17]. On the basis of these favorable points, Lactosome is expected to be a potential candidate for DDS nanocarrier.

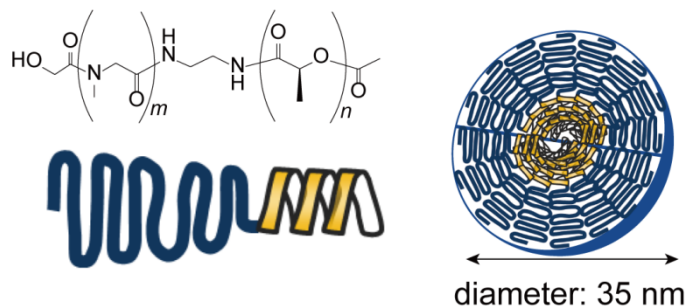


Figure 2. Schematic illustration of Lactosome

Accelerated blood clearance (ABC) phenomenon

Nanoparticles as DDS carrier are required to have a long-circulating property. However, the biodistribution of Lactosome at the second dose was changed to show a rapid clearance from the blood stream by entrapment in liver. The similar pharmacokinetic alteration was also observed with PEGylated liposome (Figure 3), which is called as the ABC phenomenon [21-24].

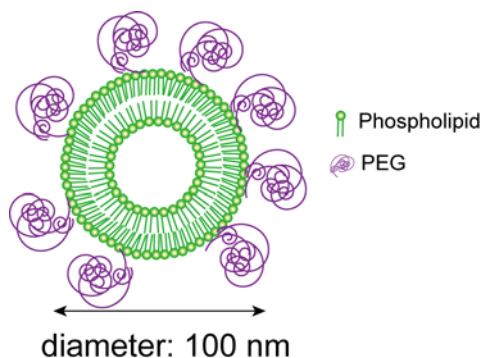


Figure 3. Schematic illustration of the PEGylated liposome

First report on the ABC phenomenon appeared in 2000. Dams and coworkers showed that weekly injections of ^{99m}Tc -PEG liposomes dramatically influenced the circulatory half-life in rats and a rhesus monkey within one month after first administration, and they indicated that this phenomenon was caused by a soluble serum factor [21]. Furthermore, Laverman and coworkers pointed out two phases in the enhanced clearance: the induction phase; the period following the first injection during which the biological system is “primed”, and the effectuation phase; the period following the second injection during which the nanoparticles are rapidly cleared from the blood stream [25]. This report showed that non-PEGylated liposomes were also cleared rapidly after the first injection, and liposomes therefore with a short circulation time also induced the phenomenon. Moreover, they examined the enhanced clearance effect of the commercially available formulation of doxorubicin in PEGylated liposomes (Caelyx[®] or Doxil[®]). Caelyx[®] was not able to induce the enhanced clearance of Caelyx[®] at the second dose at 1 week after the first dose. However, the first injection of the empty PEGylated liposome did induce rapid clearance of subsequently administered Caelyx[®]. The authors indicated that the presence of doxorubicin inside the PEGylated liposome inhibited the induction of the enhanced clearance effect.

Ishida and coworkers observed the same phenomenon in rats and mice in 2002 and named its phenomenon the ABC phenomenon [22, 26-28]. They reported that the extent of PEGylation and the lipid dose affected the ABC phenomenon, but the surface charge was irrelevant [22]. The extent of the ABC phenomenon induced by mPEG₂₀₀₀-liposomes decreased with increasing first dose, meaning that tolerance appeared with high doses [29].

In 2005, Semple and coworkers reported that PEGylated liposome encapsulating antisense oligodeoxynucleotide or plasmid DNA elicited a strong immune response to induce the rapid blood clearance of subsequent doses in mice [30]. In addition, IgM binding to PEGylated liposomes was observed. Judge and coworkers also reported that IgG as well as IgM were produced against PEGylated liposome [31]. Srode and coworkers showed that anti-PEG IgG was generated in response to the first injection of empty PEGylated liposome in rabbits [32].

Ishida and coworkers found out that significant IgM and C3 bound to PEGylated liposomes in 2006 [33]. They proposed a mechanism for the ABC phenomenon based on previous studies (Figure 4). Further, they proved that the spleen played a critical role in the induction phase of the ABC phenomenon with using splenectomy mice models [34]. They suggested that the encapsulated doxorubicin and a high lipid dose abrogated the immune response against PEGylated liposomes probably because B cells might be killed by doxorubicin and become anergic due to the excess amount of PEG [35].

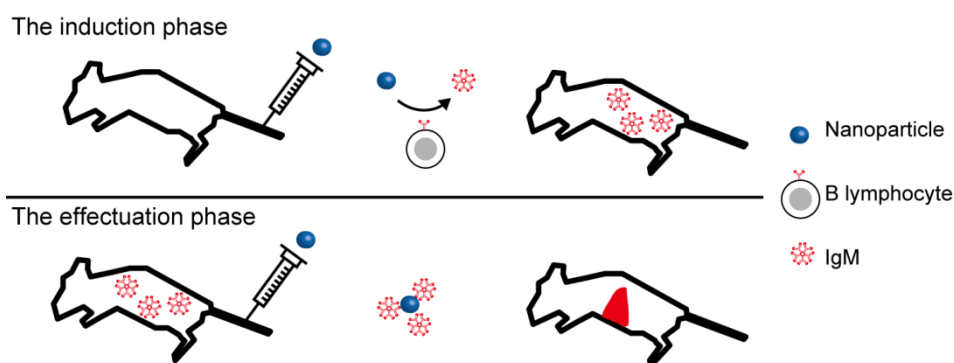


Figure 4. Schematic illustration of the ABC phenomenon

In 2007, Romberg and coworkers reported that the second dose of poly(hydroxyethyl-L-asparagine)-liposomes at 1 week after the first injection was less rapidly cleared from the blood circulation than the second dose of PEGylated liposomes [36]. Wang and coworkers showed that nonspecific anti-PEG IgM was elicited by injection of pure liposomes, which induced the enhanced blood clearance of the subsequent dose of PEGylated liposomes [37]. They suggested that the immunogenicity of the empty liposome would become a serious concern although the epitopes of the liposome remained to be determined. Further, Ishida and coworkers demonstrated that the anti-PEG IgM production was detected in T-cell deficient nude BALB/c mice following intravenous injection of the “empty” PEGylated liposome, suggesting that the “empty” PEGylated liposome should initiate the immune response against PEG in a T cell-independent manner [23]. Lu and coworkers also found the ABC phenomenon with cationic bovine serum albumin conjugated PEG-PLA nanoparticle (CBSA-NP) upon post-injection after single dose or over a periodic successive high doses of CBSA-NP [38]. The ABC phenomenon induced by the periodic successive high doses of CBSA-NP might not be attributed to the specific anti-PEG IgM, because such doses of CBSA-NP may introduce a high level of PEG density causing immune response anergic by B-1 B cells. They supposed the ABC phenomenon following the successive high doses of CBSA-NP might be attributed to humoral immune response to CBSA, as it was a T-cell dependent antigen (TD-antigen).

In 2008, Ishida and Kiwada reviewed that the physicochemical properties of the injected liposomes at the first dose, time interval between injections, lipid dose, and drug-encapsulation were the factors relevant with the ABC phenomenon [39].

Furthermore, Ishida and coworkers claimed that complement activation caused by anti-PEG IgM elicited by the first dose was a major cause of the initiation of the accelerated blood clearance of the subsequent dose of the PEGylated liposome in the ABC phenomenon [40]. In the same year, Koide and coworkers have suggested a new factor of the nanoparticle size affecting the ABC phenomenon [41]. Koide showed that the ABC phenomenon was not observed with 9.7–31.5 nm polymeric micelles. Moreover, Cui and coworkers found that the repeated injections of the PEGylated liposomal antitumor drugs (doxorubicin and mitoxantrone) did not induce the ABC phenomenon, and the rapid distribution phase due to the accumulation into blood-rich organs disappeared [42]. The disappearance of the rapid distribution phase and, in connection with that, the decrease of the nanoparticle's accumulation in spleen at the early phase might be caused by the mononuclear phagocyte system (MPS) being killed by anti-tumor drug.

Tagami and coworkers suggested that the accumulation in the spleen and the apoptotic effect of the PEG-coated substances on splenic B cells could affect the potency of anti-PEG IgM production because the PEG-coated siRNA-lipoplex (PSCL) induced apoptosis on IgM-expressing splenic cells more strongly than PEG-coated naked cationic liposome (PCL) in 2009 [43]. Ishihara and coworkers presented an evidence that small PEG-PLA (particular size 69 nm) has a lower propensity than large PEG-PLA (particular size 119 nm) about the induction of the ABC phenomenon [44]. Taguchi and coworkers suggested that hemoglobin vesicles (HbV) that the PEGylated liposome encapsulating hemoglobin (HbV) didn't induce the ABC phenomenon in mice due to high dose injection (1400 mg Hb/kg) even though low dose of HbV (0.1 mg

Hb/kg) induced the phenomenon [45].

Tagami and coworkers showed that the presence of CpG motifs, which are known to have a potent immune-stimulatory, could enhance the anti-PEG IgM production by the PEG coated pDNA-lipoplexes in 2010 [46]. Kiwada group emphasized that the PEGylated liposome was T-cell independent antigen (TI-2 antigen), and spleen was proven to be a primary organ about the secretion of anti-PEG IgM [24, 47]. Ishihara and coworkers demonstrated that the use of poly(*N*-vinyl-2-pyrrolidone) (PVP), instead of PEG, as a coating material for colloidal carriers could evade the ABC phenomenon [48]. And then, Ma and coworkers hypothesized that the hydrophobic core of the micelle or lipid bilayer of the PEGylated liposome should play an important role to trigger the ABC phenomenon because the phenomenon was not induced by a Gadolinium-containing PEG-poly(L-lysine)-based polymer micelle due to the hydrophilic core region [49]. Xu and coworkers showed that cleavable PEG-cholesterol derivatives could evade from the ABC phenomenon because of PEG cleavage [50].

Kaminskas and coworkers also suggested that smaller PEGylated micelles (particular size: 20 nm) could reduce the induction ability of the ABC phenomenon in 2011 [51]. The authors suggested the PEG-micelles might stimulate the limited production of anti-PEG IgM. About the PEGylated wrapsome incorporating siRNA (Figure 5), Tagami and coworkers indicated that the anti-PEG IgM production was dependent on the siRNA sequence which was encapsulated in the PEGylated lipid nanocarrier [52]. The selective modification of siRNA might attenuate its adjuvant effect on anti-PEG IgM. Furthermore, they showed that the PEGylated wrapsome

incorporating β -galactosidase, which has an immune stimulatory activity and produces many inflammatory cytokines, induced strongly the anti-PEG IgM production. About HbV, Taguchi and coworkers showed that the ABC phenomenon was induced by the repeated injections of hemoglobin vesicles (1400 mg Hb/kg) under conditions of massive hemorrhage using a rat model of hemorrhagic shock although HbV didn't induce the ABC phenomenon in normal mice model due to high dose injection [53].

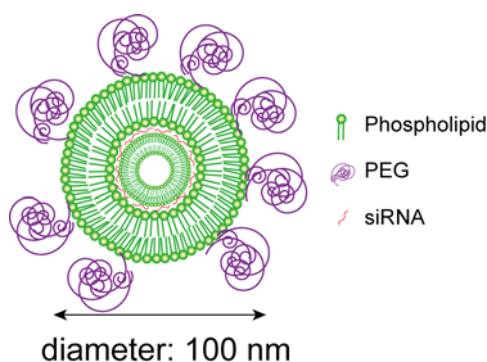


Figure 5. Schematic illustration of the wrapsome

Abu Lila and coworkers showed that the repeated administrations of the PEGylated liposomal oxaliplatin did not induce a significant anti-PEG IgM production due to a potent apoptotic response in 2012 [54]. Koide and coworkers examined again the relation between the nanoparticle size and the ABC phenomenon. The results indicated that the ABC phenomenon was less significant with a smaller polymeric micelle with 33.6 nm diameter than that with 76.2 nm diameter [55]. Suzuki and coworkers provided an evidence that Doxil[®] at a therapeutic dose (20 mg doxorubicin/m²) did not cause the ABC phenomenon, but Doxil[®] at lower doses (<2mg doxorubicin/m²) induced the anti-PEG IgM production and thereby a rapid clearance of the second and/or third dose of Doxil[®] in beagle dogs [56]. Two groups in China demonstrated that the ABC

phenomenon was induced when the PEGylated liposomal topotecan formulations were repeatedly administrated in Wistar rats, beagle dogs, and mice, even though the PEGylated liposomal doxorubicin did not induce the ABC phenomenon [57, 58]. Topotecan is the topoisomerase I inhibitor, which can hold cells at S phase in the cell cycle. Therefore, the drug may be ineffective to affect B cell. On the other hand, Zhang and coworkers showed that the PEGylated recombinant canine uricase induced the ABC phenomenon under the particulate size of PEGylated cross-linked conjugates being smaller than 40-60 nm [59]. Careful removal of the uricase aggregates and the PEG diol contaminant, and modifying with small PEG reagents successfully abolished the ABC phenomenon. Zhao and coworkers reported that the repeated injections of the PEGylated solid nanoparticles composed of glycerol monostearate and PEG induced the ABC phenomenon in mice and beagles [60]. In addition, they showed the first subcutaneous injection of the PEGylated solid nanoparticles into the footpad of rats also induced the following intravenously administered PEGylated particles to be cleared very rapidly from the blood circulation [61].

Abu Lila and coworkers demonstrated that the use of polyglycerol (PG), instead of PEG, prevented induction of the ABC phenomenon in 2013 [62]. Previous reports have emphasized that, upon first injection, a fraction of PEG-coated liposomes reaching the spleen should bind specifically to the immunoglobulins, recognizing the PEG chain, on the surface of the reactive B cells in the splenic marginal zone (MZ), and trigger the production of anti-PEG IgM [34]. Therefore, they speculated that the hydroxymethyl side group in the repeating $-(O-CH_2-CH(CH_2OH))_n-$ subunits of PG sterically hinders the interaction and/or effective binding to surface immunoglobulins on the reactive

splenic B cells. Furthermore, Nagano and coworkers showed that the PEGylated liposomes encapsulating S-1, which is one of the orally administered fluoropyrimidine derivatives, and oxaliplatin attenuated the ABC phenomenon due to the simultaneous dose of anti-tumor drug and phospholipids [63]. Shimizu and coworkers also reported that splenic MZ-B cells were crucial for the PEGylated liposome-mediated ABC phenomenon [64]. In addition, the authors demonstrated this stimulated MZ-B cells rapidly migrated from the MZ into the follicle (FO) region while carrying the trapped PEGylated liposomes. Moreover, Abu Lila and coworkers have shown that splenic cells incubated with the PEGylated liposomes *in vitro* as well as those obtained from mice pre-treated with the PEGylated liposomes *in vivo* succeeded in producing anti-PEG IgM [65]. In addition, they revealed that IgM⁺/CD19⁻/CD45R⁻-B cells, which are presumed to be the MZ-B cells, were responsible for the production of anti-PEG IgM. On the other hand, Shiraishi and coworkers proposed that highly water-soluble PEG-related compounds did not affect IgM production, since they have observed no induction of the ABC phenomenon upon the first dose of a high molecular weight of pure PEG [49, 66]. In this connection, it is notable that the interaction between antigens and antibodies is based on the noncovalent bonds of van der Waals force, hydrophobic force, hydrogen bond, and Coulomb force, etc. [67, 68] (Figure 6). Li and coworkers also found that the ABC phenomenon could be induced by PEGylated liposomal topotecan for dogs [69]. Wang and coworkers examined the relation between antibody induction and a variety of PEGs with a terminal of either methoxyl (OCH₃), hydroxyl (OH), amino (NH₂), carboxyl (COOH), or thiol (SH) [70]. Their study suggested that thiols might stimulate the proliferation and differentiation of B cells to induce the fastest clearance at the second intravenous administration as a result of the synthesis of the cell membrane and

cytosolic proteins or reacting with FO dendritic cells. Saadati and coworkers have shown that pre-injection of encapsulated etoposide at different doses still induced the ABC phenomenon [71].

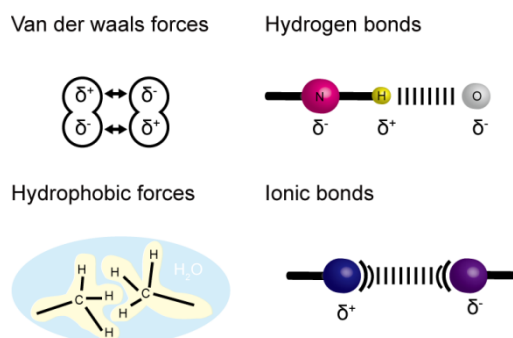


Figure 6. Noncovalent bonds for interaction between antibody and antigen

Abu Lila and coworkers have shown that the PG-coated pDNA-lipoplex attenuated the production of anti-polymer IgM in 2014 [72].

Evasion from the ABC phenomenon

Summarizing the above for evasion from the ABC phenomenon, there are several nanoparticles and methods for it; (1) the PEGylated polymeric micelles decreasing the particle size down to less than 30 nm, (2) coating of nanoparticles with PVP, (3) modification of liposome surface with a PG-derived lipid, (4) polymeric micelle with a PEG shell and a hydrophilic inner core (PEG-P(Lys-DOTA-Gd)), (5) anti-tumor drug or siRNA in PEGylated liposome, and (6) alteration of the administration regimen.

(1) The PEGylated polymeric micelles decreasing the particle size down to less than 30 nm

The ABC phenomenon was not observed with 9.7 nm and 31.5 nm PEGylated

polymeric micelles [41, 55]. This observation might be interpretable in terms of the adjuvant effect of nanoparticles. The smaller nanoparticles become, the less the adjuvant effect is elicited.

(2) Coating of nanoparticles with PVP

The use of PVP, instead of PEG, as a coating material for colloidal carriers could evade the phenomenon [48]. The PVP-coated nanoparticles had a shorter half-life in the blood circulation, and the chance to activate the immune response would be small. However, any clear explanation is not given.

(3) Modification of liposome surface with a PG-derived lipid

The use of PG, instead of PEG, prevented induction of the ABC phenomenon [62]. The authors claimed that $-(O-CH_2-CH(CH_2OH))_n-$ subunit of PG should sterically hinder the interaction and/or the effective binding to surface immunoglobulins on reactive splenic B-cells.

(4) Polymeric micelle with a PEG shell and a hydrophilic inner core

The Gadolinium-containing PEG-poly(L-lysine)-based polymer micelle could evade from the ABC phenomenon [49, 66]. The authors therefore hypothesized that the hydrophobic core of the polymeric micelles or the lipid bilayer of PEGylated liposome might be involved in the activation of the immune system.

(5) Anti-tumor drug or siRNA in PEGylated liposome

The ABC phenomenon wasn't observed with the PEGylated liposomal antitumor

drugs (doxorubicin, mitoxantrone, and oxaliplatin) [25, 42, 54] and the PEG-coated siRNA-lipoplex [43]. These nanoparticles could induce the apoptotic effect on B cells (Table 1).

Table 1. The relation of the contents in nanoparticles and the ABC phenomenon

ABC phenomenon	Contents
YES	plasmid DNA etoposide antisense oligodeoxynucleotide topotecan siRNA(dependent on sequence) uricase hemoglobin
NO	doxorubicin mitoxantrone siRNA(dependent on sequence) oxaliplatin

Red characters: anti-tumor drug

(6) Alteration of the administration regimen

T-cell independent antigens like PEGylated liposome could activate B cells and induced IgM antibodies production at the early stage of immunization [73, 74]. On the other hand, at high doses, they lacked the activation ability on B cells, and immune tolerance against PEGylated liposome was developed. The ABC phenomenon was therefore suppressed by high-dose PEGylated liposomes [29, 35, 37-39, 45]. On the other hand, the ABC phenomenon induced by the PEGylated liposome was ceased within one month [21].

Aim

Nanoparticles are highly potential in the field of DDS as a nanocarrier. Lactosome also has a high possibility for a DDS nanocarrier due to the selective accumulation in various solid tumors. However, Lactosome induced the ABC phenomenon similarly to

the PEGylated liposome. In the case of Lactosome, the surface of the polymeric micelle is covered densely with poly(sarcosine) chains. Both poly(sarcosine) and PEG are electrically neutral water-soluble polymers, but the former is composed of endogenously occurring sarcosine and the latter is a synthetic common polymer. When we look into the immune response in detail, Lactosome induced IgG₃ production as well as IgM, but the PEGylated liposome triggered only IgM production. There should be some differences between Lactosome and the PEGylated liposome about the immune response.

At first, the author therefore examined the ABC phenomenon induced by Lactosome. In order to clarify the property of Lactosome on the activation of the immune response, the author has also studied other nanoparticles composed of amphiphilic helical peptides with a hydrophobic helical block of (Leu-Aib)₆ (Aib represents α -aminoisobutyric acid) and poly(sarcosine) [75]. These nanoparticles are also covered densely with poly(sarcosine) chains but with a hydrophobic core of α -helices. Further, nanosheets could be prepared by the amphiphilic peptide, and the morphology effect of nanosheet or spheric polymeric micelle on the immune response was also examined. Since the hydrophobic region of nanoparticles may be involved in the immune response [49, 66], the surface density of poly(sarcosine) chains was increased to a maximum value by using A₃B-type (A; poly(sarcosine), B; poly(L-Lactic acid)) or poly(sarcosine)-*block*-(L-Leu-Aib)₆ amphiphiles to shield the hydrophobic region as much as possible.

On the other hand, the author examined the size effect of nanoparticles on the immune response. The nanoparticle size is also critical for the EPR effect [6, 76]. The dimension control of polymeric assemblies has been so far achieved primarily by

changing the molecular packing parameter of the hydrophilic and hydrophobic balance of amphiphilic block polymers [77-79]. Here, the author found that the polymeric micelle sizes composed of the A₃B-type polydepsipeptide could be precisely controlled by incorporation of the AB-type or poly(L-lactic acid) into the hydrophobic core.

The life-time of nanoparticles in blood circulation can be discussed from three points; 1) a high signal/background contrast in short time can be obtained with a rapid clearance, 2) a high accumulation in tumors by the EPR effect is attainable with a long circulation, and 3) less activation of the immune system is stochastically possible with a rapid clearance [80-82]. The author challenged this issue by making nanoparticles with different life times by using stereocomplex formation in the hydrophobic core. The right-handed and the left-handed helices of poly(lactic acid) are well known to form stereocomplex leading to tight molecular packing in the self-assemblies. The signal agent loaded on Lactosome, which is composed of poly(L-lactic acid), could be remained for a long duration when it was attached to poly(D-lactic acid). But the holding time could be shorten by using poly(D,L-lactic acid) for the signal agent. This nanoparticle design is applied for tumor imaging by PET. PET is one of the popular molecular imaging techniques for evaluations of tumors [83-85]. 2-Deoxy-2-¹⁸F-fluoro-D- glucose (FDG), which allows an evaluation of glucose metabolism, is one of the most commonly used radiopharmaceuticals in oncology. However, FDG-PET imaging is rather poor at detection of brain tumor, liver cancer, gastric cancer, prostate cancer, renal carcinoma and bladder cancer due to the active glucose metabolism at those organs or urination of FDG [86, 87]. To avoid these problems, many novel post FDG radiopharmaceuticals including nanomedicines such as liposomal nanoparticle drugs are currently developed for the diagnosis and detection of

tumors [88-91].

Finally, Lactosome was examined on the therapeutic agent. Lactosome was loaded with ^{131}I for radioactive iodine therapy. This inner radiation therapy was also combined with the percutaneous ethanol injection therapy (PEIT). Local tumor therapies like radiofrequency ablation (RFA) and PEIT have been developed for patients who couldn't have invasive surgery for their age and tumor region [92, 93]. PEIT uses ethanol injection into tumor regions to induce coagulative necrosis due to dehydration and protein denaturation [94, 95]. PEIT has inherent advantages in the low cost and the safe therapy, but, in some cases, risk of relapse is high when the safety margin is not taken wide enough [95, 96]. In order to improve the therapeutic efficiency, therefore, PEIT is frequently performed in combination with anti-tumor agents such as epirubicin, cisplatin, and 5-fluorouracil [97, 98]. Instead of chemical drugs, Lactosome is used and evaluated anti-tumor effect.

The outline of the present thesis is described as follows from Chapter 1 to 8.

Chapter 1 describes the effect of multiple administrations of Lactosome on the blood circulation behavior to reveal the relationship of the administration regime of Lactosome with the ABC phenomenon. The duration of the ABC phenomenon induced by Lactosome is studied. Various types of mice are examined to identify Lactosome as a TI antigen. Further, with competitive inhibition assay, the epitope is decided.

In Chapter 2, evasions of Lactosome from the ABC phenomenon before and after immunization are examined with high doses of Lactosome. It is based on that the

PEGylated liposomes induced immune tolerance when it was abundantly administered to mice [35]. In general, immune tolerance reflects that apoptosis or anergy of B cells are induced by administration of high dose.

In Chapter 3, peptide-nanosheet composed of poly(sarcosine)-*block*-(L-Leu-Aib)₆ (Figure 7) is analyzed on tumor imaging, the ABC phenomenon, and the IgM production, and is compared with those of Lactosome. In the previous study, molecular assemblies have been prepared from a series of amphiphilic helical peptides which have a hydrophobic helical block of (Leu-Aib)_n [75]. In these molecular assemblies, the regular packing of helices leads to form interdigitated monolayers which can generate various types of morphologies of nanotubes, vesicles, and round-bottom flask shapes. The spherical polymeric micelles have a disadvantage of the presence of loosely packed space at the most outer surface due to the curvature. On the other hand, the sheet structure is free from this concern because of the flat morphology.

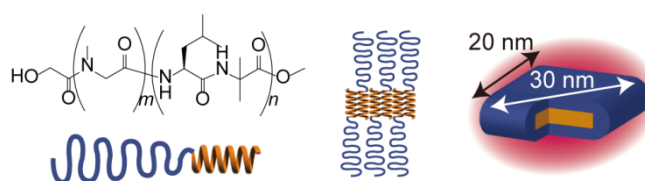


Figure 7. Schematic illustration of peptide-nanosheet

In Chapter 4, the A₃B-type Lactosome is prepared and examined on the IgM production. The effect of high local density, blood circulating and size are elucidated on the ABC phenomenon. In previous study, the nanoparticle of small size around 10 – 30 nm was not also observed the ABC phenomenon [41, 55]. The micelle size of A₃B-type

is 22 nm (Figure 8). The sizes composed of the AB-type or A₃B-type polydepsipeptide are made larger with incorporation of poly(L-lactic acid) into the hydrophobic core. With these micelles, the effect of size against ABC phenomenon is elucidated. Further, the effect of blood long-circulating is also analyzed because the PVP-coated nanoparticles which don't induce the ABC phenomenon had a shorter half-life than PEG-coated nanoparticles and don't induce the ABC phenomenon [48].

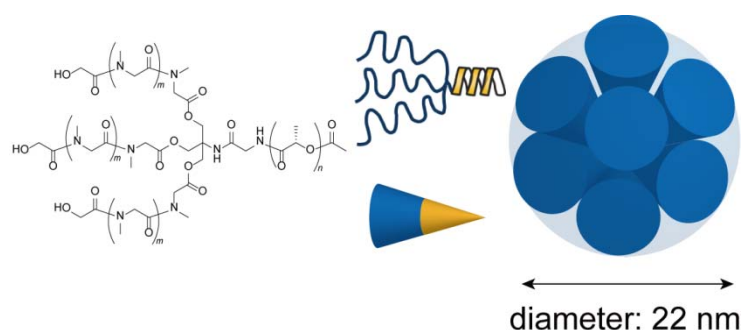


Figure 8. Schematic illustration of the A₃B-type Lactosome

In Chapter 5, nanoparticles composed of branched-type amphiphilic polydepsipeptides are studied the size control. In general, the constituent amphiphilic polymers are considered to take random structure in the assemblies, resulting in relatively a wide size distribution of the molecular assembly. Due to size control, branched A₂B- and A₃B-type amphiphilic polydepsipeptides having PLLA 30-mer as a hydrophobic block are prepared. Interestingly, the polymeric micelle sizes composed of the A₃B-type polydepsipeptide are made larger with AB-type polydepsipeptide (Figure 9). The size control is analyzed TEM and DLS.

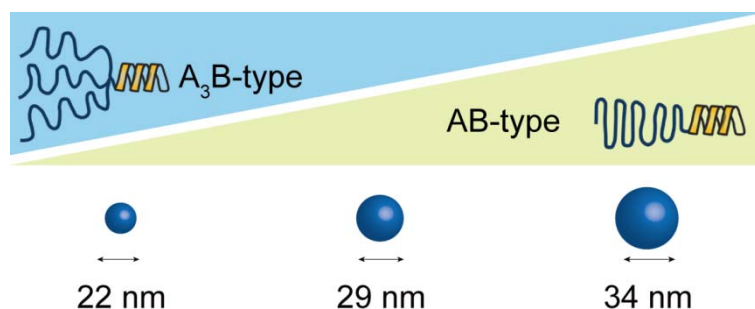


Figure 9. A size control of the A₃B-type lactosome with AB-type polydepsipeptide

In Chapter 6, the effect of stereochemistry is analyzed on *in vivo* blood clearance time of Lactosome for tumor imaging. It is known that stability of polymeric micelles *in vivo* is influenced by compounds entrapped in the hydrophobic core of the micelle [100-102]. NIRF image is generally taken by using Lactosome containing NIRF-labeled poly(L-lactic acid) (PLLA) of 1.5 mol%. PLLA takes a left-handed helical structure and is known to form stereocomplex with right-handed helical poly(D-lactic acid) (PDLA) providing materials with high mechanical strength [103-105] (Figure 10). Physical stability of Lactosome in the blood stream is therefore considered to increase with the addition of PDLA through stereocomplex formation. On the contrary, Lactosome may be destabilized by the incorporation of poly(D,L-lactic acid) (PDLLA) into the hydrophobic core through disturbing the helix molecular packing. Stereochemistry in the hydrophobic core of self-assemblies is therefore an important factor for determining properties of self-assemblies.

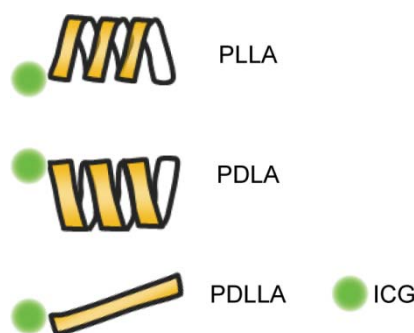


Figure 10. Schematic illustration of polylactic acid's stereocomplex

In Chapter 7, ^{18}F -PLLA is synthesized because the possibility of Lactosome is analyzed for PET imaging. The advantages of using polymers for the nanocarriers are the physical stability compared with liposome made of lipids or other metal [106], and their facile size adjustment in the range from a few ten nm to a hundred nm. In addition, instead of using PEG, which is pointed out to cause allergic reactions [107], poly(sarcosine) is used. If Lactosomes labeled with ^{18}F instead of NIRF agents are developed, they have the potential to be useful radiopharmaceuticals for tumor diagnosis by PET. ^{18}F -PLLA₃₀ is prepared by coupling reaction of NH_2 -PLLA₃₀ with ^{18}F -SFB and is incorporated into Lactosome (^{18}F -labeled Lactosome) (Figure 11). With the nanoparticle, *in vivo* biodistribution is performed.

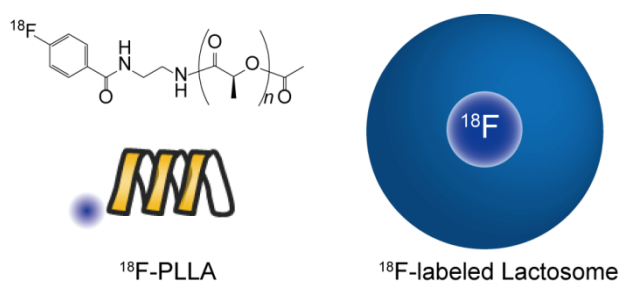


Figure 11. Schematic illustration of the ^{18}F -labeled Lactosome

In Chapter 8, the possibility of Lactosome is analyzed for tumor treatment with combination use of PEIT. Nanoparticles accumulate in tumor and inflammation regions by the EPR effect [12, 13]. This is because nanoparticles can leak out to tumor and inflammation regions from the leaky blood vessels and stay there for a long period due to an undeveloped lymphatic system. The accumulation of nanoparticles in solid tumors should be promoted by simultaneous treatment with RFA or PEIT. The author, therefore, hypothesizes that RFA or PEIT treatment induce petit inflammation in the tumor region and the marginal margin, resulting in further accumulation of the polymeric micelle on the basis of the promoted EPR effect. The beta decay radioisotope of ^{131}I [108, 109] is carried on Lactosome (Figure 12). It is expected to accumulate more effectively in tumor, leading to suppression of tumor proliferation with combination use of PEIT.

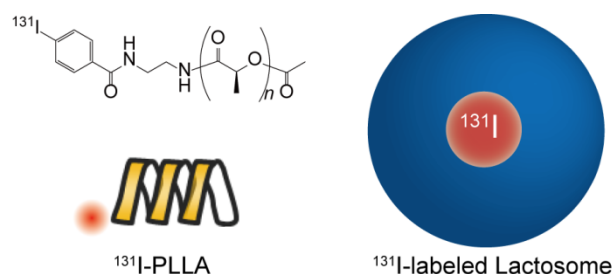


Figure 12. Schematic illustration of the ^{131}I -labeled Lactosome

The studies clearly demonstrate that *in vivo* pharmacokinetics of nanoparticles can be related with the molecular structure and the molecular assembling geometry of the constituent amphiphiles of the nanoparticles. Further, a strategy for the nanoparticle to evade from the ABC phenomenon, that is a serious concern for application of nanoparticles to medicinal field, is proposed. The details are described in the following chapters.

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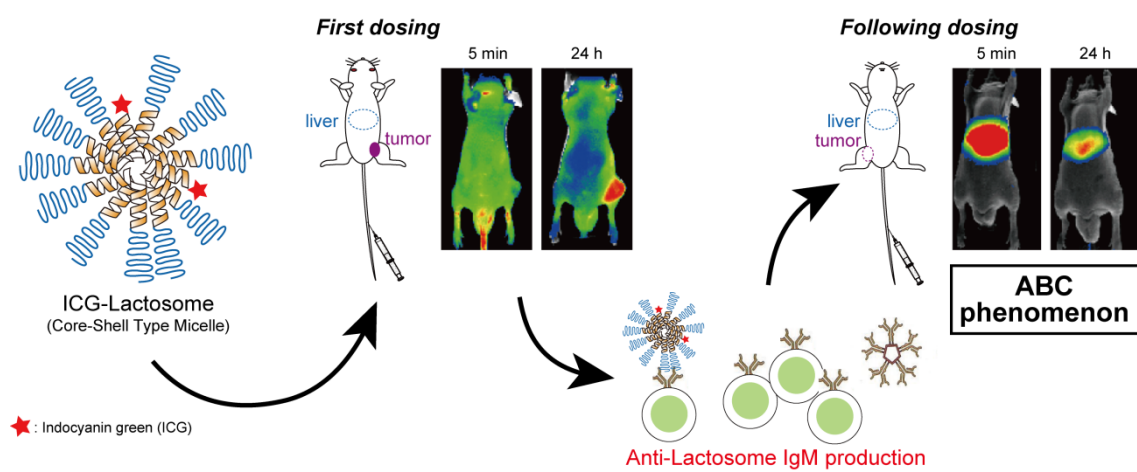
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Chapter 1

Pharmacokinetic Change of Nanoparticulate Formulation “Lactosome” on Multiple Administrations



Introduction

Molecular imaging is a technique for evaluation of pathological conditions easily in a non-invasive manner [1-5]. Researches on molecular imaging are increasing in the last decade, and new knowledge and various technologies using molecular imaging for diagnosis at the early stage of diseases have been reported [6-9].

Molecular probes, which are designed to accumulate in targeted pathological regions, are generally administered intravenously to construct images. The author has been examining nanoparticles labeled with near-infrared fluorescence chromophores as molecular probes for solid tumor imaging [10-12]. The selective accumulation of nanoparticles in solid tumors is explained by the enhanced permeability and retention (EPR) effect [13-15], which allows nanoparticles in the size range from 10 nm to 100 nm to leak out from blood vessels having submicron-sized defects at tumor regions where the lymphatic drain system is generally immature as well.

The author especially focuses on polymeric micelles composed of amphiphilic polydepsipeptides, poly(sarcosine)-*block*-poly(L-lactic acid), named as “Lactosome” [16]. Lactosome is designed to show prolonged *in vivo* blood clearance time based on the following two points; i) the diameter of the polymeric micelles is adjusted to be 35 nm with a narrow size distribution, ii) the surface of micelles is covered densely by hydrophilic poly(sarcosine) chains. These two characteristics make Lactosome sustain in the blood stream for a long period due to escape from the excretions through kidney and liver. As a result, Lactosome with labeling of a near-infrared fluorescent probe is successful for imaging of various solid tumors, especially hepatic cancer cells orthotopically implanted in liver [10]. Further, Lactosome is composed of the biodegradable and biocompatible polymer. These points are advantages against other

nanoparticles, which are mainly constituted from synthetic polymers such as poly(ethylene glycol) (PEG) [17, 18]. Therefore, Lactosome is expected to be a potential candidate for DDS nanocarrier, and can be competed with PEGylated nanoparticles, which is actively investigated in these days.

As a nanocarrier for anti-cancer drug, PEGylated liposome, which is covered with hydrophilic PEG chains, is now used clinically (Doxil[®]). PEGylated liposome is hardly recognized by the biological defense system and shows a long blood circulation property [4, 19, 20]. However, it has been pointed out, in some cases, that PEGylated liposome at the 2nd intravenous dosage changed the *in vivo* disposition from the 1st dosage through a rapid clearance from the blood stream. This pharmacokinetic change is called the “accelerated blood clearance (ABC)” phenomenon, which is explained that anti-PEG IgM is produced by the 1st dosage of PEGylated liposome through recognition of the immune system where spleen acts a major role [21-23]. In the present study, therefore, the effect of multiple administrations of Lactosome on its blood circulation behavior is investigated.

Material and methods

Materials

Synthesis of amphiphilic block polydepsipeptide, and Lactosome preparation was performed as previously reported [10, 16].

Instruments

The hydrodynamic diameters of Lactosome were analyzed by dynamic light scattering (DLS) on a Nano-ZS (Malvern, UK). Near infrared fluorescence (NIRF)

images were taken by Clairvivo OPT (Shimadzu Corp. Japan) and IVIS-Spectrum (Caliper, USA). Filter sets of *ex.* 785 nm and *em.* 845 nm for Clairvivo OPT and *ex.* 745 nm and *em.* 840 nm for IVISTM-Spectrum were used. The pseudo images were constructed from the photon counts. ELISA for anti-Lactosome antibody was performed using Multiscan Spectrum Microplate Photometer (Thermo Scientific, USA).

Mouse

A/J, BALB/c, BALB/c nu/nu, CBA/N, CDF-1, ddY, HR-1, and KSN mouse were purchased from Japan SLC, Inc. (Japan). ICR, ICR-nu, NOD-SCID, and SCID mouse were from Oriental Bioservice, Inc. (Japan). RAG2(KO) was kindly gifted from Haward Hughes Medical Institute (HHMI).

Preparation of tumor-bearing and inflammation mice

SUIT-2/pEF/luc cells (5×10^5 cells), which possess the luciferase gene cassette by using the method as previously reported [10, 16], were suspended in phosphate-buffered saline (PBS, 20 μ L), and subcutaneously inoculated into the right femoral region of 7-week-old male nude mice (BALB/c nu/nu; Japan SLC Inc., Hamamatsu, Japan). The mice were used for *in vivo* experiments after 2 weeks from the tumor transplantation. The inflammation model mice were prepared by injection of turpentine oil (50 μ L/body) to the right femoral region at 3 days before the imaging.

***In vivo* near-infrared fluorescence (NIRF) imaging**

To the mice, ICG-Lactosome was intravenously injected (50 μ g/mouse, Lactosome diameter: 35 nm). The concentration of the injected ICG was set to be 100 pmol/mouse.

At 5 min and/or 24 h after the administration, NIRF images were taken. During the imaging process, the mice were held on the imaging stage under anesthetized condition with 2.5% of isoflurane gas in air flow (1.5 L/min). The multiple dosing effects of Lactosome on the disposition were analyzed by four kinds of protocols as shown in Figures. 1e, 2b, 3d and 4b. Figure 1e shows the second dosage of ICG-labeled Lactosome which was injected 7 days after the first dosage of Lactosome. The effects of two and three times of Lactosome dosages on the anti-Lactosome IgM production were checked by the protocol shown in Figure 2b. To examine the effect of the interval period between the two dosages on the disposition, the interval period was varied from one day to five days from the first dosage of Lactosome (Figure 3d). Further, the lasting and the interval dosage effects on the disposition were evaluated according to the protocol shown in Figure 4b.

Collected plasma

Mouse blood was collected from inferior vena cava under anesthesia condition, and pooled by Microtainer[®] (BD, USA). The blood plasma was obtained by centrifugation (10 min, 3000 rpm) and stored at -40 °C.

ELISA experiment

Lactosome (0.5 µg) in 50 µL distilled water was added to 96-well plates and air dried completely for 1 day. Then, 150 µL of blocking buffer (2% BSA/PBS) was added and incubated for 2 h, and the wells were washed four times with washing buffer (PBS-T). On the Lactosome competition assay, Lactosome components such as sarcosine monomer, sarcosine dimer, poly(sarcosine), sarcosine-linker-(lactic acid),

L-lactic acid monomer, and L-lactic acid dimer were further added in the plasma. After the incubation, the wells were washed four times using the washing buffer. Peroxidase conjugated goat-anti-mouse IgM or goat-anti-mouse IgG₃ in 0.1% BSA/PBS (50 µL, Southern Biotech, USA) was added as the secondary antibody. The solution was incubated for 2 h, and then the wells were washed again four times. *o*-Phenylene diamine (0.5 mg/mL, Sigma, St. Louis, MO), which was dissolved in 0.0003% H₂O₂-0.1 M citrate phosphate buffer (pH 5.0), was added to the microplate. 10 minutes after the *o*-phenylene diamine addition, optical density (OD) was determined by UV absorbance at 490/620 nm. All mean values are expressed as the mean ± S.D. Differences in data were evaluated by Student's t-test.

Judgment criteria on ABC phenomenon appearance

ICG labeled Lactosome was intravenously injected to mice, and NIRF images were taken at 24 h after the administration. Region of interest (ROI) analyses were carried out at the two regions of the liver and the healthy left femoral regions. The fluorescence from the latter region was taken as the background signal. By using photon counts from the regions, the liver/background fluorescence intensity ratios were determined after the 1st dosage of Lactosome as $(L/B)_0$. At 7 days after the 1st ICG-Lactosome administration, the same amount of ICG-Lactosome was re-administrated to obtain the liver/background intensity ratios of (L/B) . Occurrence of the ABC phenomenon was judged by the increase of (L/B) from $(L/B)_0$. When $(L/B)/(L/B)_0$ was over 1.3, the ABC phenomenon is considered to be induced.

Ethics

All of her *in vivo* animal experiments were approved by the Animal Research Committee of Kyoto University. Animals were treated humanely.

Results

Pharmacokinetic change of Lactosome

In vivo disposition of Lactosome upon multiple dosages was studied (n =3). NIRF imaging was performed at 5 min and 24 h after the ICG-labeled Lactosome injection (Figure 1). Without Lactosome pre-administration, NIRF signals were spread over to the whole body via the blood stream during the beginning 5 min (Figure 1a). The tumor was clearly detected at 24 h after the dosage, because ICG-labeled Lactosome gradually accumulated in the transplanted tumor region by the EPR effect as reported in the previous paper (Figure 1b) [10]. On the other hand, *in vivo* disposition of ICG-labeled Lactosome of the pre-administrated group was changed drastically, and ICG-labeled Lactosome was rapidly trapped by the liver within the initial 5 min (Figure 1c). The ICG-labeled Lactosome accumulated in the liver was metabolized to show a low signal intensity at 24 h. As a result, the transplanted tumor could not be imaged at the 2nd dosage (Figure 1d). Pharmacokinetic alternation of Lactosome is induced by its multiple administration in a similar manner with PEGylated liposome, although surface property of the nanoparticles is covered with different polymers with poly(sarcosine) of Lactosome and poly(ethylene glycol) (PEG) of PEGylated liposome.

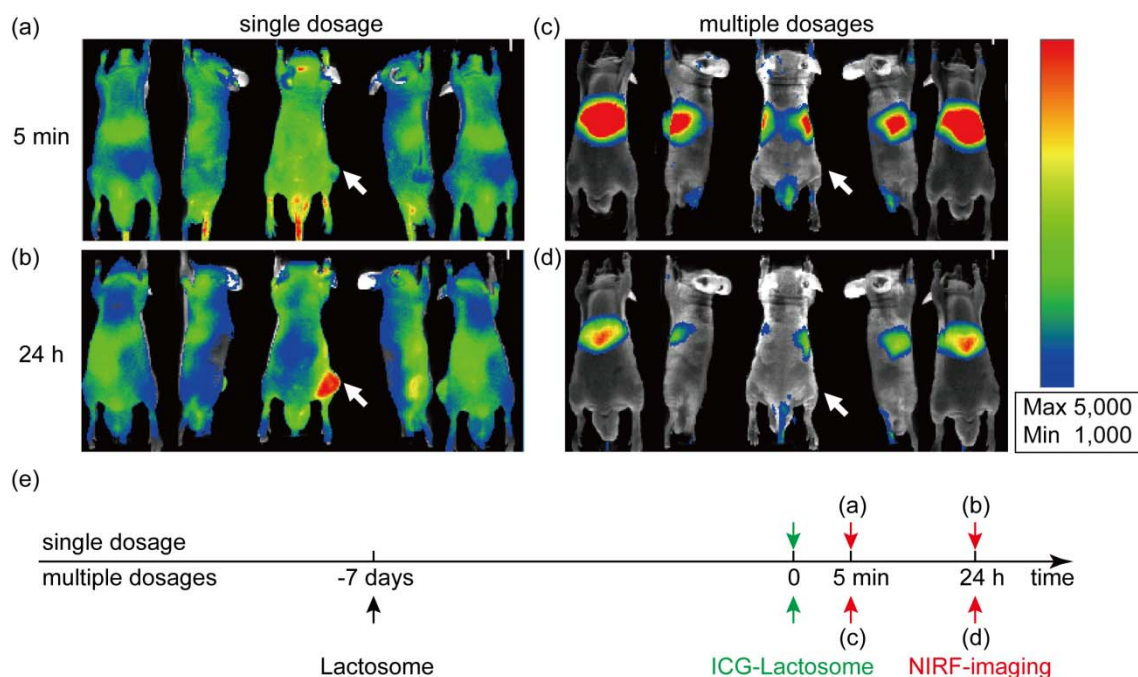


Figure 1. Pharmacokinetic change of Lactosome on the multiple administrations. (a–d) NIRF images of tumor-transplanted nude mice at 5 min or 24 h after ICG-labeled Lactosome administration ($n = 3$). (e) Time schedule for the NIRF imaging. Black and green arrows indicate the injection time points of Lactosome and ICG-labeled Lactosome to the tumor-transplanted nude mice, respectively. NIRF imaging was performed at red arrows. Time point of ICG-labeled Lactosome injection is indicated by green arrows to be set at 0 h.

It is reported that ABC phenomenon of PEGylated liposome is caused by anti-PEG IgM production, which prompted her to investigate on anti-Lactosome IgM production upon Lactosome dosage. The amount of anti-Lactosome IgM in the blood serum was evaluated by ELISA (Figure 2, $n = 3$). The anti-Lactosome IgM level became significantly higher at 1 week after Lactosome dosage than that at 5 min before (preimmune). Anti-Lactosome IgM was kept at high concentration for 1 week after two periodically injections. These results indicate that production of anti-Lactosome IgM is the reason for the pharmacokinetic change of Lactosome upon multiple dosages.

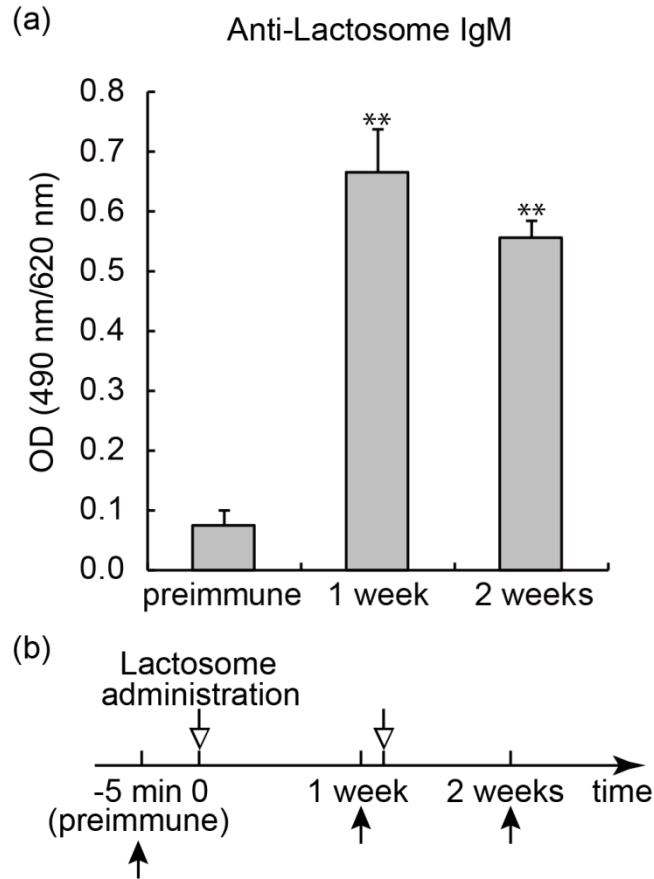


Figure 2. (a) Produced amount of anti-Lactosome IgM determined by ELISA ($n = 3$, $**p < 0.01$). (b) Time schedule for the experiment. Mouse blood was collected at three time points, which are indicated by black arrows. Time points Lactosome administrated are indicated by white arrows, and time for the first Lactosome dosage was set at 0 h.

Evaluation of anti-Lactosome IgM and IgG₃

Antibodies secreted into the blood stream are mainly IgM as the first response of B-cells and the following IgG. Among IgG subtypes, IgG₃ plays an important role in the cascade of the complement system through opsonization of antigens [24-27]. Figure 3 shows the time course analyses of IgM and IgG₃ productions in the blood stream ($n = 4$). The anti-Lactosome IgM production started 3 days after the dosage, and reached to a plateau level at the 4th day. Production of anti-Lactosome IgG₃ followed that of IgM.

NIRF images of mice were taken with injection of ICG-labeled Lactosome at the

same time of the blood collection for evaluation of antibody productions (Figure 3c, n = 1). Within 2 days after the 1st dosage of Lactosome, ICG-labeled Lactosome was distributed in the whole body despite the multiple dosages. However, *in vivo* disposition of ICG-labeled Lactosome was changed on the 3rd day after the 1st dosage to accumulate entirely in the liver. The biodistribution of ICG-labeled Lactosome at the 2nd dosage is therefore well correlated with the production of anti-Lactosome IgM. Utilizing photon counts of the imaging data, fluorescence intensity ratio at liver and background (L/B) was calculated, and (L/B)/(L/B)₀ value listed in Table 1 was used for the judgment of the Lactosome ABC phenomenon occurrence. In conjunction with the production of anti-Lactosome IgM, (L/B)/(L/B)₀ value was increased to 1.3 after 3 days from the dosage, and reached to 2.0 on day 4. Based on the results, certified (L/B)/(L/B)₀ value for the Lactosome ABC phenomenon appearance is defined to be above 1.3 in this paper.

Table 1. Observation of the ABC phenomenon based on ROI analysis at the 2nd dosage of Lactosome with different intervals from the 1st dosage.

Interval between two dosages /day	(L/B) ^{*1}	(L/B)/(L/B) ₀ ^{*2}	ABC phenomenon
1	1.1	1.0	×
2	1.5	1.2	×
3	1.6	1.3	×
4	2.4	2.0	○
5	2.5	2.1	○

^{*1} (L/B) represents the fluorescence intensity ratio of the liver region against the background of the healthy left femoral region.

^{*2} (L/B)₀ value is the (L/B) value at the 1st dosage and set to be 1.2, which was determined from NIRF images of mice with single administration of ICG-labeled Lactosome.

Next, the persistent period appearing the ABC phenomenon in mice was examined. ICG-Lactosome was administered to inflammation model mice every 1–6 months for 6 months, and NIRF imaging was performed. ICG-labeled Lactosome was trapped by the liver when pre-administration intervals of Lactosome were not exceeded for more than three months. In case that the pre-administration interval became six months, ICG-labeled Lactosome was again accumulated at the inflammation region (Figure 4a). $(L/B)/(L/B)_0$ values calculated from Figure 4a are listed in Table 2. Under the condition that ABC phenomenon was observed, $(L/B)/(L/B)_0$ values was over 2.0. With 6 months interval of Lactosome administration, the $(L/B)/(L/B)_0$ values was significantly decreased to 1.2.

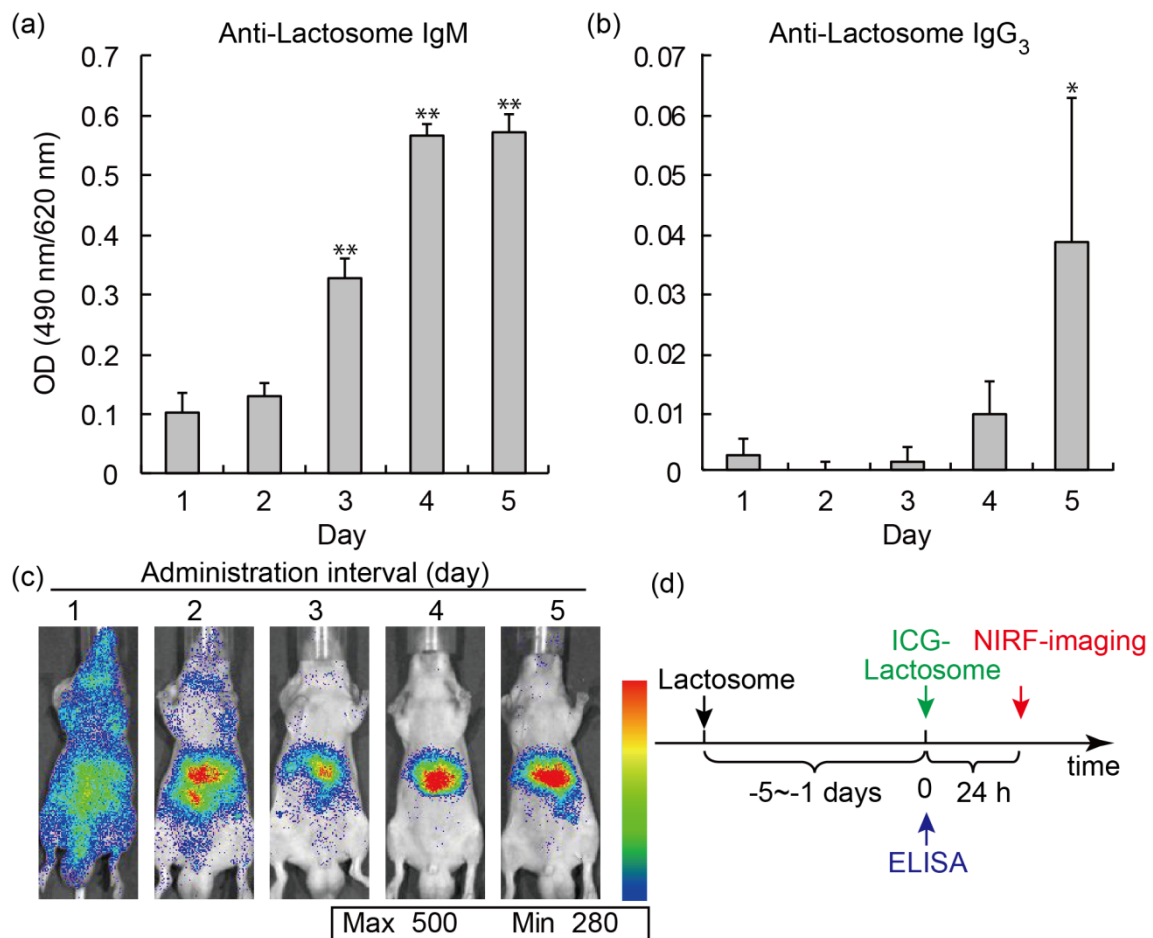


Figure 3. (a,b) Time course analyses of productions of anti-Lactosome antibodies (IgM and IgG₃) after Lactosome administration (n = 4, *p < 0.05, **p < 0.01). (c) NIRF images of mice with administration of ICG-labeled Lactosome. (d) Time schedule for the experiments. Time periods from the 1st Lactosome administration to the following ICG-labeled Lactosome administration were varied from 1 to 5 days. NIRF images were taken at 24 h after ICG-labeled Lactosome administration.

Produced amount of anti-Lactosome IgM and IgG₃, which was determined by ELISA, is displayed in Figures 4c and 4d. Amount of the produced anti-Lactosome IgM and IgG₃ was kept at high level within three months of administration intervals, but decreased within six months. Results obtained from ELISA were well-correlated with that from NIRF imaging, and supported that six months are needed for attenuation of the ABC phenomenon.

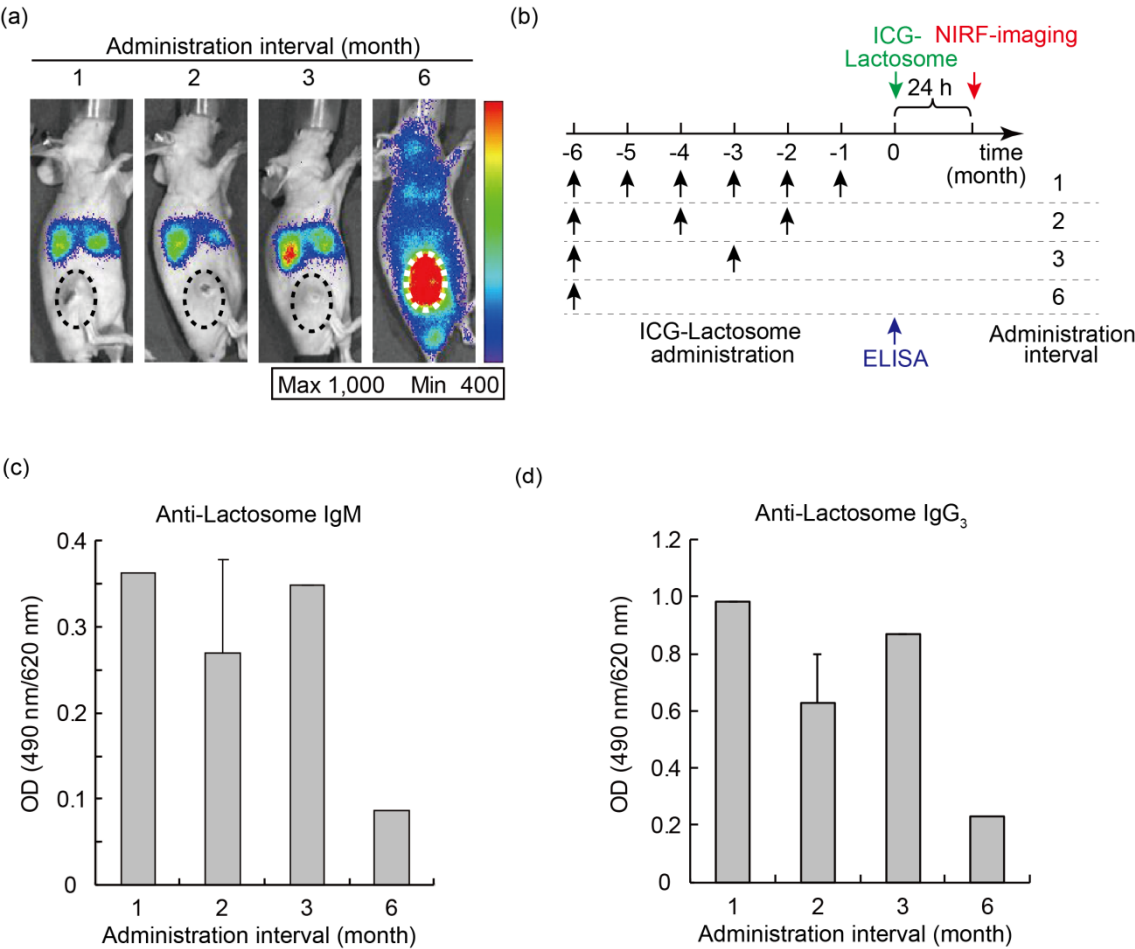


Figure 4. Effect of Lactosome administration interval on anti-Lactosome antibody duration (a) NIRF images after Lactosome administration for 6 months with various administration intervals (1 month: n = 2, 2 months: n = 2, 3 months: n = 2, 6 months: n = 3). Dotted circles indicate inflammation regions. (b) Time schedule for the experiment. (c,d) Amount of anti-Lactosome IgM and IgG₃ in blood after 6 months from the Lactosome first administration with various intervals (1 month: n = 1, 2 months: n = 3, 3 months: n = 1, 6 months: n = 2).

Table 2. Effect of Lactosome administration interval on NIRF imaging

Entry	Lactosome administration interval / month	(L/B) ₀	(L/B)	(L/B)/(L/B) ₀	ABC phenomenon
1	1	1.1	2.3	2.2	○
2	2	1.2	2.5	2.1	○
3	3	1.3	2.8	2.3	○
4	6	1.2 ± 0.1	1.4 ± 0.3	1.2 ± 0.3	×

Model mice having different immune systems

The immune response to Lactosome was further analyzed by using several types of mice having different immune systems. The types of model mice are listed in Table 3, where mice from entries 1 to 6 have natural immune system, entries 7–9 and entry 10 are knockout model mice of T-lymphocyte cells and B-lymphocyte, respectively, and entries 11–13 are double knockout model mice of T- and B-lymphocyte cells. On second ICG-Lactosome administration, NIRF images revealed that mice having B-lymphocyte cells (entries 1, 2, 4-9) trapped Lactosome in liver, and (L/B)/(L/B)₀ value was over 1.3, indicating occurrence of the ABC phenomenon. In the case of CDF1 mice (entry 3), the (L/B) value could not be obtained due to the cinnamon color hair mouse, but the accumulation of ICG-Lactosome at the 2nd dosage was observed at 5 min after the injection (Figure 5). On the other hand, B-lymphocyte cells knockout mice (entries

10-13) did not show the ABC phenomenon. In the cases of CBA/N and RAG2(KO) mice (entries 10 and 12), the agouti and black colors, respectively, were an obstacle for fluorescence detection, but ICG-Lactosome imaging of the inflammation region in these mice was possible upon multiple dosages, indicating no ABC phenomenon.

Table 3. Observation of the ABC phenomenon with using various mice having different immune systems.

Entry	Mouse type	(L/B) ₀	(L/B)	(L/B)/(L/B) ₀	ABC phenomenon
1	A/J	1.4 ± 0.1	2.4 ± 0.4	1.7 ± 0.4	○
2	BALB/c	1.2 ± 0.1	2.2 ± 0.6	1.9 ± 0.4	○
3	CDF1 ^{*1}	-	-	-	○
4	ddY	1.1 ± 0.1	1.7 ± 0.3	1.5 ± 0.2	○
5	HR-1	1.2 ± 0.1	2.9 ± 0.4	2.3 ± 0.1	○
6	ICR	1.1 ± 0.1	1.8 ± 0.2	1.6 ± 0.2	○
7	BALB/c-nu	1.2 ± 0.1	2.1 ± 0.3	1.8 ± 0.1	○
8	ICR-nu	1.1 ± 0.0	2.4 ± 0.3	2.2 ± 0.2	○
9	KSN	1.1 ± 0.1	3.1 ± 0.3	2.7 ± 0.4	○
10	CBA/N ^{*2}	-	-	-	×
11	NOD-SCID	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	×
12	RAG2(KO) ^{*3}	-	-	-	×
13	SCID	1.3 ± 0.1	1.3 ± 0.0	1.0 ± 0.0	×

(L/B) and (L/B)₀ represent the same meanings as those explained at Table 1.

^{*1-3} These mice have colored hairs to interfere the fluorescence measurements. The accurate values therefore were not obtained. ^{*1} The occurrence of the ABC phenomenon with CDF1 mice was judged by the observation of the fluorescence accumulation in liver at five minutes after the 2nd dosage of ICG-Lactosome (Figure.5). ^{*2-3} CBA/N and RAG2(KO) mice showed the fluorescence accumulation in the inflammation region even at the 2nd dosage of ICG-Lactosome, indicating no ABC phenomenon.

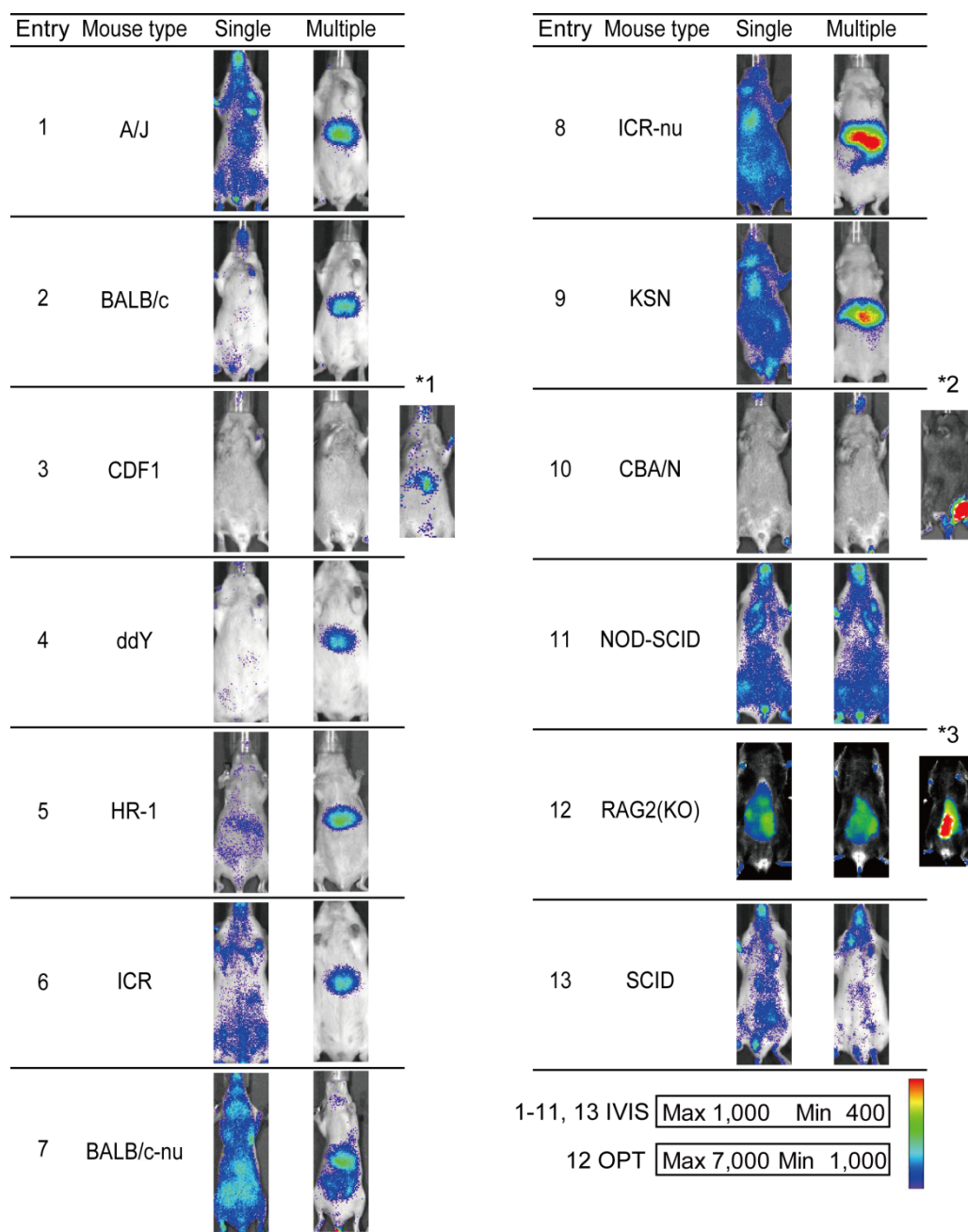


Figure 5. NIRF images of 13 types of model mice having various immune systems.

*¹ NIRF image taken after 5 min from ICG-Lactosome administration. Strong fluorescence can be detected from liver region. *² ICG-Lactosome was accumulated at inflammation region of CBA/N on multiple dosage. White dotted circle indicates inflammation region. *³ ICG-Lactosome was accumulated at inflammation region of RAG2(KO) on multiple dosage. Hair on RAG2(KO) mice back was removed before imaging. White dotted circle indicates inflammation region.

^{*1} CDF1 mice showed strong fluorescence at liver soon after the ICG-Lactosome administration (5 min). Further, ICG fluorescence could not be detected at the tumor region, when ICG-Lactosome was repeatedly injected to tumor transplanted CDF1 mouse. Therefore, it is concluded that CDF1 mice showed the ABC phenomenon against Lactosome. ^{*2, 3} When inflammation model mice of CBA/N and RAG2(KO) were used for NIRF imaging, ICG-Lactosome was accumulated at the inflammation region on the Lactosome multiple administrations.

Determination of epitope structure

Lactosome is a self-assembly of amphiphilic block polydepsipeptide, poly(L-lactic acid)-*block*-poly(sarcosine). Hydrophobic and hydrophilic blocks are connected by a 1,2-diaminoethane derivative linker. In order to search for the epitope moiety of Lactosome, sarcosine monomer, sarcosine dimer, poly(sarcosine), sarcosine-linker-(lactic acid), L-lactic acid monomer, and L-lactic acid dimer were synthesized and studied by the competitive inhibition assay with Lactosome (Figure 6). The addition of poly(sarcosine) of 500 µg to blood plasma containing anti-Lactosome antibodies clearly decreased the absorbance of the ELISA assay, indicating competitive binding of poly(sarcosine) to anti-Lactosome antibodies. The epitope moiety is therefore concluded to be poly(sarcosine), which is reasonable because poly(sarcosine) chains cover the surface of Lactosome and are easily accessible to the immune system.

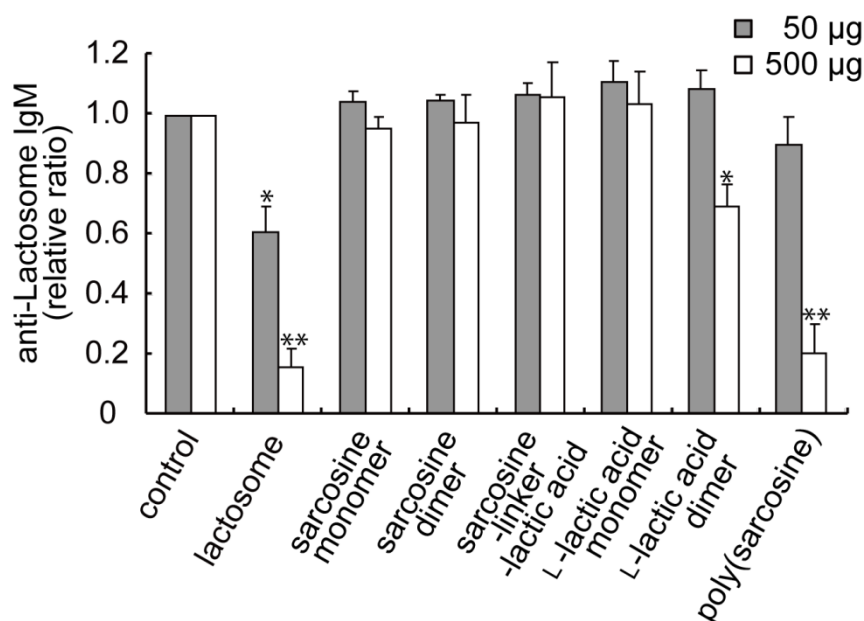


Figure 6. Competitive inhibition assay of various compounds by ELISA with using blood serum containing anti-Lactosome antibodies. Optical density (OD) without any compounds (control) was normalized to be a relative value of 1.0. Black and white bars show OD values with addition of the compound of 50 and 500 µg, respectively (n = 3, *p < 0.05, **p < 0.01).

Discussions

On multiple dosages of Lactosome, pharmacokinetic change was observed. The ABC phenomenon of Lactosome is explained by the immune response related with B-lymphocyte cells, which resembles the ABC phenomenon of PEGylated liposome [21-23]. Anti-Lactosome antibodies, IgM and IgG₃, were produced within 3–4 days after the 1st dosage of Lactosome. Lactosome at the following dosage was opsonized by these antibodies, and trapped by the reticuloendothelial system. The memory effect of B-lymphocyte cells lasted nearly for six months. Even though Lactosome and PEGylated liposome induced commonly the ABC phenomenon, there are two different points. One is the detection of IgG₃ production by Lactosome. In almost all cases, IgG production by PEGylated liposome is not detected [28] or remained at an extremely low

level [29]. The other is the persistence period of the memory effect, nearly six months of Lactosome and one month of PEGylated liposome [21, 22].

Both of Lactosome and PEGylated liposome induced the T-cell independent (TI) B-cell immune response [23]. Representative chemical properties of TI antigens are 1) high molecular weight, 2) slow metabolism and 3) multiple repeating antigenic determinants, and Lactosome meets these points [27]. TI antigens are further grouped into two classes (TI-1 or TI-2). TI-1 antigen is a potent B-cell (mature and immature) mitogen, and TI-2 only activates mature B cells. Since the ABC phenomenon was not observed with CBA/N mouse, which has only immature B cells, Lactosome is thus classified to be a TI-2 antigen [27]. Importantly, there are obvious physical differences between Lactosome and PEGylated liposome. Lactosome diameter of 35 nm is smaller than PEGylated liposome of 100 nm. Further, the surface of the Lactosome is covered densely by poly(sarcosine) chains like a dense polymer brush layer. On the other hand, PEG chains at the surface of PEGylated liposome take a mushroom configuration due to the limited mixing of PEG-lipid of less than 30% in the total lipids. In addition to these points, the different chemical property of poly(sarcosine) from PEG may be the reasons for the difference in the TI immune response triggered by Lactosome and PEGylated liposome.

However, these nanoparticles commonly show the ABC phenomenon. The ABC phenomenon has been shown with three different types of nanoparticles; PEGylated liposome, polymer micelle composed of PEG-*block*-polyAsp(OBzl) [30], and Lactosome. These nanoparticles showed a prolonged life time in the blood stream, resulting in the long exposure time of the nanoparticles to the immune system. Further, nanoparticles generally possess the adjuvant effect to trigger the immune system.

Taken together, nanoparticles in general might have a high ability to trigger the immune system with exhibiting molecules at the surface of nanoparticles as a potential epitope for the immune system.

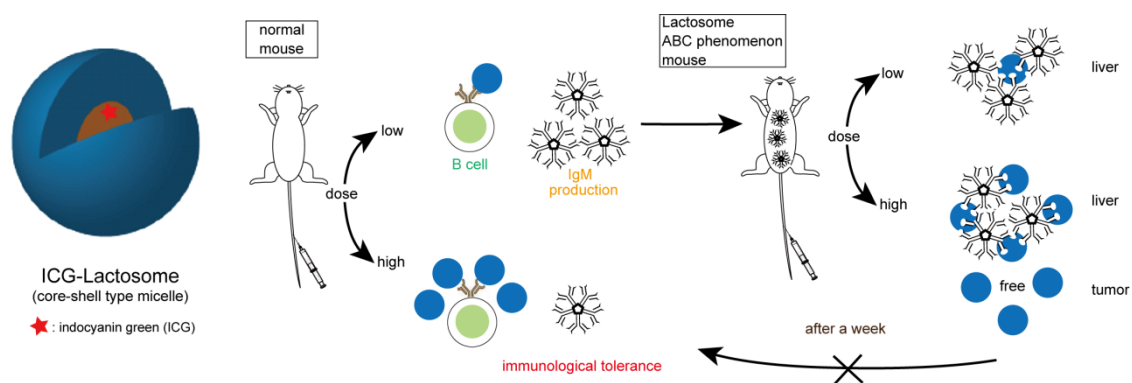
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Chapter 2

Evasion from Accelerated Blood Clearance of Nanocarrier Named as “Lactosome” Induced by Excessive Administration of Lactosome



Introduction

Application of nanoparticles for chemotherapy has been actively investigated on possible control of biodistribution and blood circulation behavior of drugs through their encapsulation into nanoparticles [1, 2]. For example, the applications of nanoparticles for the tumor targeted drug delivery of anticancer drugs is actively investigated utilizing phenomenon that nanoparticles are selectively accumulated into solid tumor regions by the enhanced permeability and retention (EPR) effect [3, 4].

Lactosome is a polymeric micelle with ca. 35 nm diameter, which is composed of amphiphilic block polydepsipeptide, poly(sarcosine)-*block*-poly(L-lactic acid) [5]. The average block sizes of hydrophilic poly(sarcosine) and hydrophobic poly(L-lactic acid) were 60–90-mer and 30-mer, respectively. Lactosome shows a long blood circulation behavior and Lactosome labeled with indocyanine green (ICG-Lactosome) successfully imaged tumor orthotopically implanted on liver, which is due to suppression of non-tumor associated capture of Lactosome by liver. The high contrast imaging of tumors in liver becomes possible due to the contribution of hydrophilic poly(sarcosine) chains covering the surface of Lactosome densely in a polymer brush state. However, the biodistribution of Lactosome at the second dose was changed to show a rapid clearance from the blood stream by entrapment in liver [6]. This drastic pharmacokinetic alteration is caused by productions of anti-Lactosome IgM and anti-Lactosome IgG₃ after first administration of Lactosome, because Lactosome induced the T-cell independent (TI) B cell immune response. The similar pharmacokinetic alteration was also observed with PEGylated liposome, which is called as the accelerated blood clearance (ABC) phenomenon [7-10]. The ABC phenomenon was also reported on various kinds of polymeric micelles [11, 12].

TI antigens like PEGylated liposome can activate B cells and induce IgM antibodies production at the early stage of immunization [13, 14]. On the other hand, at high doses, they lack the activation ability on B cells, and immune tolerance against PEGylated liposome was developed [15]. The ABC phenomenon is therefore suppressed by high-dose PEGylated liposome [15-19]. In this study, Lactosome with high dose is examined on the immune tolerance and the Lactosome ABC phenomenon.

Materials and methods

Materials

All reagents and solvents were purchased commercially and used without further purifications. Poly(sarcosine)₆₄-*block*-poly(L-lactic acid)₃₀ (poly(Sar)₆₄-*block*-PLLA₃₀) (MW=6872) and indocyanin green (ICG) labeled PLLA 30-mer (ICG-PLLA₃₀) were synthesized as previously reported [5, 20, 21]. For the preparation of Lactosome, poly(Sar)₆₄-*block*-PLLA₃₀ (1, 5, 10, 15, 25, 35 mg) in test tube was dissolved into chloroform, and then evaporated to form polymer film on the test tube. Water was added to the tube so as concentration of the amphiphilic polymer to be 1 mg/mL (1–10 mg) or 5 mg/mL (15–35 mg). The dispersion was heated at 85 °C for 20 min. After freeze-drying of the solution, saline (1 mL) was added, and the solution was filtered with using syringe filter (pore size: 0.20 µm) just before administration. For the preparation of ICG-Lactosome, ICG-PLLA₃₀ (0.5–1 nmol) was additionally mixed into chloroform solution on polymer film formation step. Molar number of ICG-PLLA₃₀ was determined using UV absorbance of ICG-Sulfo-OSu at 795 nm dissolved in DMSO. Subsequent protocol is the same with above.

Instruments

Hydrodynamic diameter of Lactosome was measured by dynamic light scattering (DLS) on a Nano-ZS (Malvern, United Kingdom). Near infrared fluorescence (NIRF) images were taken by Clairvivo OPT (Shimadzu Corp. Japan, *ex.* 785 nm/*em.* 845 nm). The pseudo images were constructed from the photon counts. ELISA assay for anti-Lactosome antibody was performed using Multiskan Spectrum Microplate Photometer (Thermo Scientific, USA).

Single-dose toxicity study

BALB/c mice (Body weight: 20–22 g) were purchased from Japan SLC, Inc. (Japan). Lactosome of 2000 mg/kg/400 μ L, which is a 400-fold amount of the dose for imaging, was intravenously or intraperitoneally injected to 7-week-old male and female mice. The dosage amount was determined based on OECD 423, which is a standard method for acute oral toxicity test [22]. This is because intraperitoneal (IP) and intravenous (IV) injections are considered to be more toxic than oral administration. The mice behavior and body weight were traced for 11 days.

Blood-half life time of ICG-Lactosome

Mice were divided into 4 groups ($n = 4$). ICG-Lactosome (1 mg/mL/100 μ L, amount of ICG 5 nmol/kg) was intravenously injected to 9-week-old male mice. Mice blood were collected in Microstrainer[®] tube (BD Corp. USA) from inferior vena cava exunder anesthesia condition at 2, 8, 24, and 48 h after Lactosome administration. The serum was obtained by centrifugation (10 min, 3000 rpm). Fluorescence intensity of ICG-Lactosome in the serum was measured by fluorescence spectrophotometer

RF-5300 (Shimadzu Corp. Japan, ex. 730 nm/em. 813 nm). The data indicates time courses of ICG-Lactosome concentration in serum and was fitted using IGOR Pro 4.09A.

Preparation of tumor-bearing mice

BALB/c nu/nu mice (Body weight: 20–22 g) were purchased from Japan SLC, Inc. (Japan). SUI-2/pEF/luc cells (5×10^5 cells) were dissolved in phosphate-buffered saline (PBS, 20 μ L), and subcutaneously inoculated into right femoral region of 7-week-old male nude mice [5]. The mice were used for *in vivo* experiments after 2 weeks from the tumor transplantation.

***In vivo* near-infrared fluorescence (NIRF) imaging**

On experiment, which evaluate effect of first Lactosome dosage amount to the Lactosome ABC phenomenon, tumor bearing mice were divided into 7 groups ($n = 3$). To the mice of each group, Lactosome of 35 nm in diameter was intravenously injected (5, 25, 50 mg/kg/100 μ L, 150, 250, 350 mg/kg/200 μ L). To the control mice (Lactosome 0 mg/kg), saline (100 μ L) was injected. After 7 days from the first Lactosome dosage, ICG-Lactosome (diameter: 35 nm) was intravenously injected (100 μ L, 5 mg/kg) to the mice. Injected ICG amount was set to be 5 nmol/kg. NIRF images were taken at 5 min and 24 h after the administration. During the imaging process, the mice were held on the imaging stage under anesthetized condition with 2.5% of isoflurane gas in air flow (1.5 L/min).

On experiment, which evaluate effect of second Lactosome dosage amount to the Lactosome ABC phenomenon, tumor bearing were divided into 6 groups ($n = 3$). To the

all mice except control group ones, Lactosome of 35 nm in diameter was intravenously injected (100 μ L, 5 mg/kg). To the mice of control group (Lactosome 0 mg/kg), saline (100 μ L), was injected. At 7 days after the first Lactosome administration, ICG-Lactosome (diameter: 35 nm) was intravenously injected (5, 25, 50 mg/kg/100 μ L, 150, 350 mg/kg/200 μ L) to the mice. Injected ICG amount was set to be 5 nmol/kg on all groups. NIRF images were taken in the same method with above.

Preparation of serum

On experiment, which evaluate effect of first Lactosome dosage amount to the Lactosome ABC phenomenon, mice blood was collected after 7 days from the first Lactosome (diameter: 35 nm, injection volume: 100 μ L (5, 25, 50 mg/kg), 200 μ L (100, 150, 200, 250 mg/kg)) dosage. Collected blood from inferior vena cava under anesthesia condition was transferred into Microstrainer[®] tube (BD Corp. USA). Blood serum was separated by centrifugation (10 min, 3000 rpm) and saved at -40°C .

On experiment, which evaluate effect of second Lactosome dosage amount to the Lactosome ABC phenomenon, mice were occurred ABC phenomenon to be injected Lactosome (5 mg/kg). To the mice, Lactosome (diameter: 35 nm, injection volume: 100 μ L (5, 25, 50 mg/kg), 200 μ L (150, 350 mg/kg)) was injected at 7 days after first injection. The mice blood were collected at 7 days after second administration, and treated in the same way with above.

Enzyme-linked immunosorbent assay (ELISA) experiment

Lactosome (0.5 μ g) in 50 μ L distilled water was added to 96-well plates and air dried completely for 1 day. Then, 150 μ L of blocking buffer (2% BSA/PBS) was added and

incubated for 2 h. The wells were washed four times with washing buffer (PBS-T: 0.05% Tween 20 in PBS). Mice serum were added to the wells and incubated for 2 h. After the incubation, the wells were washed four times using PBS-T. Peroxidase conjugated goat-anti-mouse IgM in 0.1% BSA/PBS (50 μ L, Southern Biotech, USA) was added as the secondary antibody. After incubation of the solution for 2 h, and then the wells were washed again four times with PBS-T. *o*-Phenylenediamine (0.5 mg/mL, Sigma, St. Louis, MO), which was dissolved in 0.0003% H₂O₂-0.1 M citrate phosphate buffer (pH 5.0), was added to the microplate. 10 minutes after the *o*-Phenylenediamine addition, optical density (OD) was determined from UV absorbance at 490/reference at 620 nm.

Ethics

All of her *in vivo* animal experiments were approved by the Animal Research Committee of Kyoto University. Animals were treated humanely.

Results

Preparation of Lactosome and ICG-Lactosome

Lactosome, which is composed of poly(Sar)₆₄-*block*-PLLA₃₀ amphiphilic polydepsipeptide, was prepared by the film rehydration method. ICG-Lactosome was prepared by the same method with Lactosome but with an appropriate amount of ICG-labeled poly(L-lactic acid) 30mer (ICG-PLLA₃₀) in addition to the amphiphilic polydepsipeptide on polymer film preparation (Figure 1). Lactosome showed no toxicity up to 2000 mg/kg (Figure 2) and blood-half time of ICG-Lactosome was calculated to be 17.8 h in mice (Figure 3)

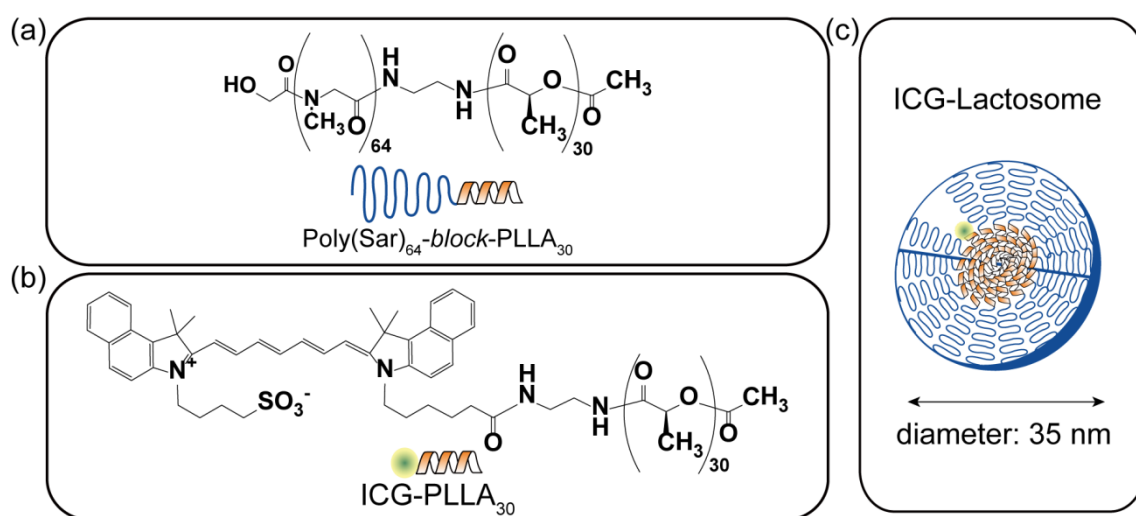


Figure 1. Chemical structure of (a) poly(Sar)₆₄-block-PLLA₃₀ (b) ICG-PLLA₃₀. (c) Illustration of polymeric micelle of ICG-Lactosome.

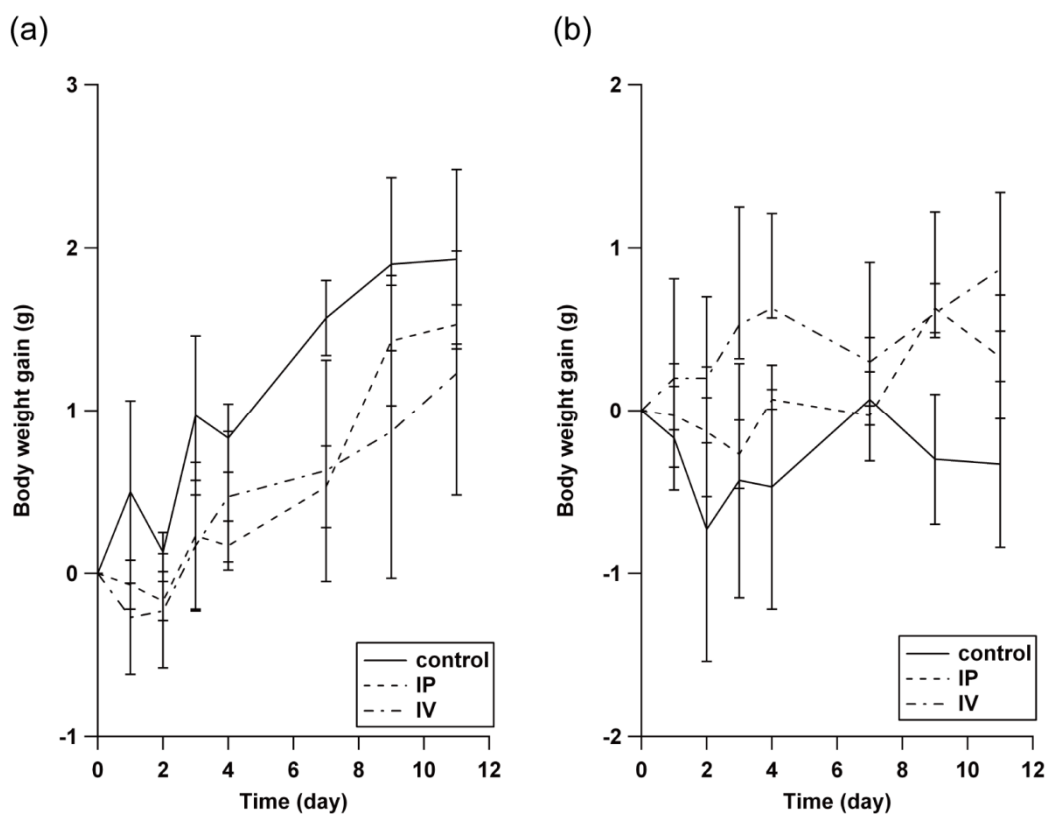


Figure 2. Time courses of body weight gain of mice. Lactosome of 2000 mg/kg was injected IP and IV to male (a) and female (b) mice (n = 3).

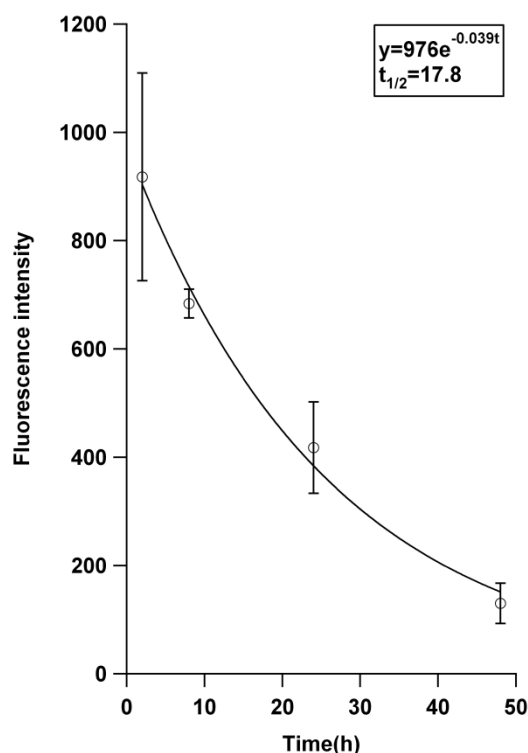


Figure 3. Time courses of the residual ICG-Lactosome concentration in serum. Blood-half life time of ICG-Lactosome was calculated by IGOR Pro 4.09A.

Effect of the first Lactosome dose on the ABC phenomenon

Tumor bearing mice were divided into seven groups and Lactosome was pre-administrated at different doses of 5, 25, 50, 150, 250, and 350 mg/kg. As a control group, only saline was administrated. At 7 days after the first Lactosome administration, ICG-Lactosome (5 mg/kg) was injected, and NIRF images were taken at 15 min and 24 h after the second injection (Figures 4a and 4b). The Lactosome ABC phenomenon, which is indicated by accumulation of NIRF signal at liver instead of spreading over the whole body, was observed with the groups of the first Lactosome doses in the range of 5–50 mg/kg. With increasing the amount of Lactosome pre-dose over 150 mg/kg, the NIRF signals were found to spread over the whole body via blood stream, followed by accumulation in the transplanted tumor regions in the NIRF images at 24 h.

The region of interest (ROI) analyses at liver were carried out with taking the fluorescence intensity at the healthy left femoral region as background using NIRF images, which were taken 15 min after ICG-Lactosome administration (Figures 4a, 4d, and Figure 5). The signal intensity ratios at liver against background (L/B) are summarized in Table 1 with those at the single Lactosome dose (corresponding to the case of 1st dose of 0 mg/kg), $(L/B)_0$. Occurrence of the Lactosome ABC phenomenon can be evaluated by the numerical values of $(L/B)/(L/B)_0$ taking more than the critical value of 2.0 according to Table 1.

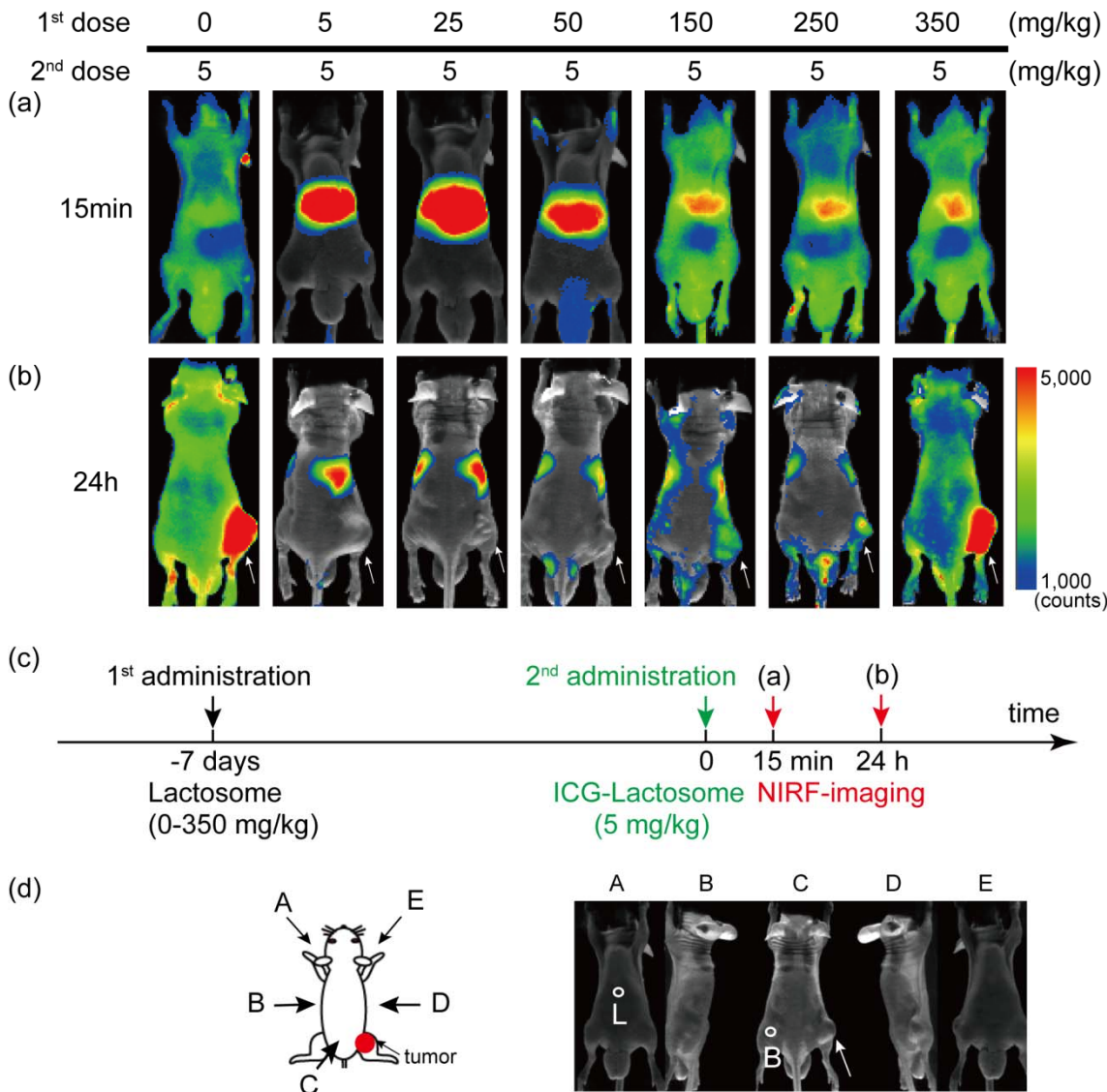


Figure 4. The effect of the first Lactosome dose on the Lactosome ABC phenomenon. NIRF images of mice at (a) 15 min (view from A in Fig. 4d) and (b) 24 h (view from C in Fig. 4d) after ICG-Lactosome of 2nd dose. Lactosome (5, 25, 50 mg/kg/100 μ L, 150, 250, 350 mg/kg/200 μ L) were injected to the mice 7 days before the ICG-Lactosome administration. SUIT-2/pEF/luc cells were transplanted at the right femoral region of mice, which tumor sites are indicated by white arrows. The fluorescein signal ranges were set to be the same for all the images from max count 5000 to min count 1000. (c) Time schedule for the NIRF imaging. Black and green arrows indicate the injection time points of Lactosome and ICG-Lactosome, respectively. NIRF imaging was performed at red arrows. (d) NIRF images were taken with using Clairvivo OPT (Shimadzu. Corp), which can take five images from different directions (A–E) with one time shot. The white circles indicate positions of ROI (L: liver, B: Background).

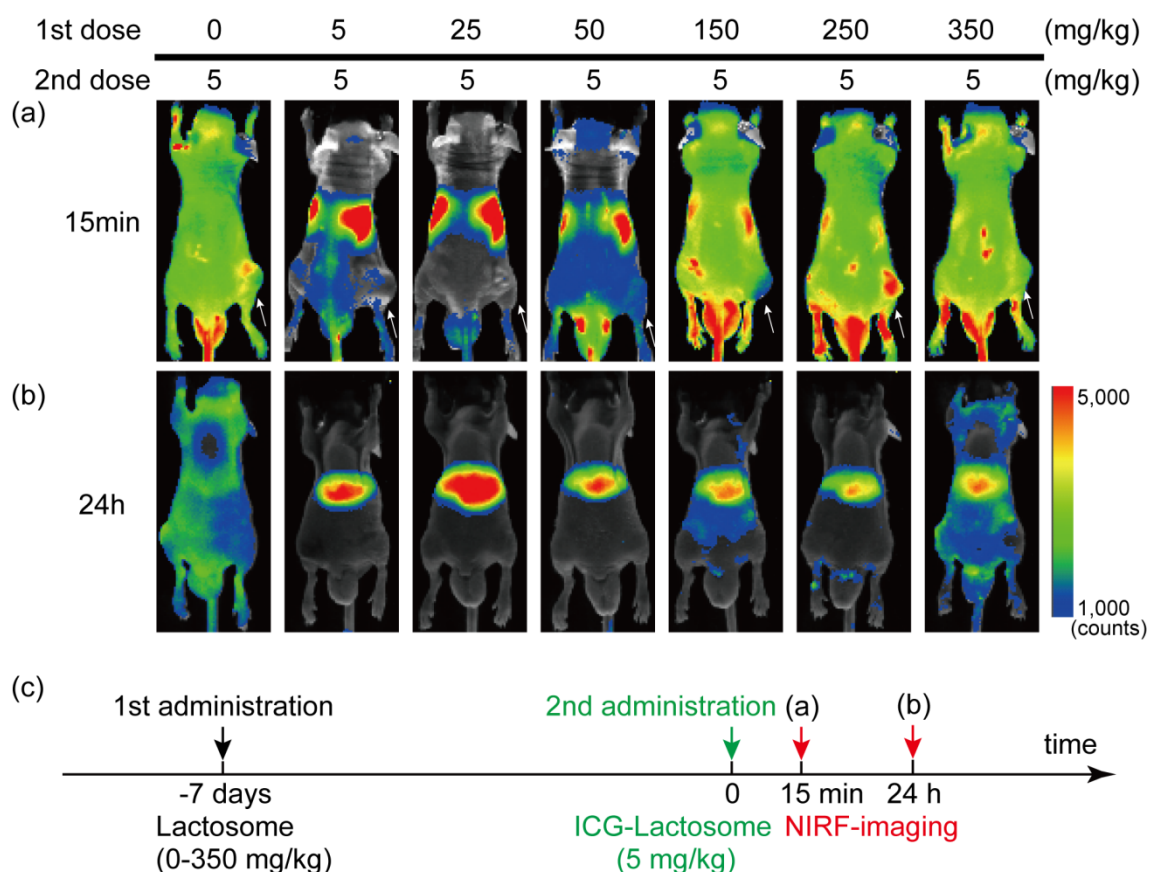


Figure 5. Effect of the Lactosome amount on the first dosage to the Lactosome ABC phenomenon. NIRF images of mice at (a) 15 min (left underpart: position C in Fig. 4d) and (b) 24 h (upperpart: position A) after ICG-Lactosome administration as 2nd dose. Lactosome (5, 25, 50 mg/kg/100 μ L, 150, 250, 350 mg/kg/200 μ L) were injected to the mice 7days before ICG-Lactosome administration. Tumor of SUIT-2/pEF/luc cells is

transplanted on right femoral region of mice, which is indicated by white arrows. The figure fluorescein signal range was represented to the max count 5000 from the min count 1000. (c) Time schedule for the NIRF imaging. Black and green arrows indicate the injection time points of Lactosome and ICG-Lactosome, respectively. NIRF imaging was performed at red arrows.

Table 1. The effect of first Lactosome dose on Lactosome biodistribution at second administration

Lactosome administration (mg/kg)	(L/B)			(L/B)/(L/B) ₀ ^{*1}			ABC phenomenon ^{*2}
0	1.03	±	0.08	-			-
5	11.1	±	5.5	10.8	±	5.4	Yes
25	8.67	±	6.42	8.40	±	6.22	Yes
50	6.57	±	5.09	6.36	±	4.93	Yes
150	1.80	±	0.53	1.74	±	0.52	No
250	1.15	±	0.17	1.12	±	0.17	No
350	1.47	±	0.22	1.43	±	0.22	No

^{*1} The (L/B)₀ value was determined to be 1.03 from NIRF images of mice at 15 min after single administration of ICG-Lactosome (Lactosome pre-dosage of 0 mg).

^{*2} The (L/B)/(L/B)₀ values taking more than 2.0 are indication of occurrence of the ABC phenomenon.

Since the Lactosome ABC phenomenon is well related with production of anti-Lactosome IgM induced by the first Lactosome administration irrespective of ICG labeling [6], the levels of anti-Lactosome IgM at 7 days after the dosing in the range of 5–250 mg/kg were evaluated by ELISA (Figure 6). The group of the Lactosome dose of 5 mg/kg showed the highest IgM production. As the Lactosome dose was increased, the amount of anti-Lactosome IgM was inversely decreased.

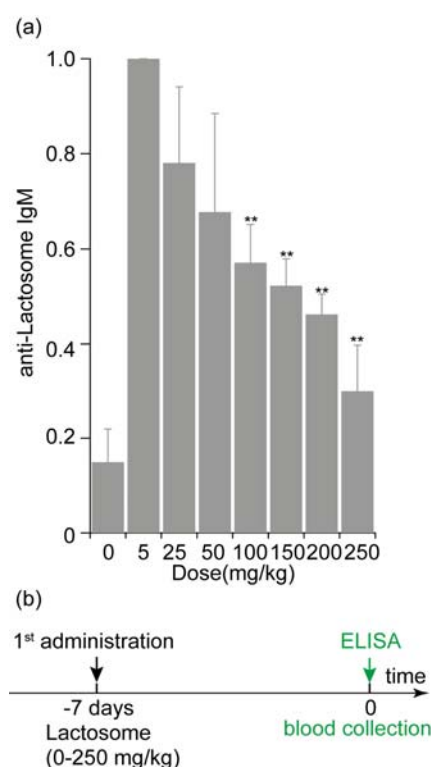


Figure 6. (a) The anti-Lactosome IgM level at 7 days after the Lactosome administration with varying the amounts (5, 25, 50 mg/kg/100 μ L, 100, 150, 200, 250 mg/kg/200 μ L). Each value was normalized with taking the IgM production with 5 mg/kg dose as a reference. (b) Time schedule for the experiment. Black arrow indicates the injection time point of Lactosome. The serums were collected at 7 days after the injection (the green arrow) and subjected to ELISA. ** represents significant difference from 5 mg/kg dose groups with $p < 0.01$.

The dose dependence of Lactosome pharmacokinetics at the second administration

With using mice immunized with the Lactosome pre-dose of 5 mg/kg (Figure 4), ICG-Lactosome (5–350 mg/kg with keeping ICG concentrations the same for all the groups) was administered to evaluate the dose dependence of the pharmacokinetics. NIRF images were taken at 15 min and 24 h after the administration (Figures. 7a, 7b, and Figure. 8). When the second Lactosome dose was as low as 5 or 25 mg/kg, the Lactosome ABC phenomena were observed. On the other hand, with the second Lactosome doses over 50 mg/kg, ICG-Lactosome spread over the whole body, and ICG

signal was detected from the transplanted tumor region at 24 h after the Lactosome second administration. Table 2 shows the results of the ROI analyses on NIRF imaging at 15 min after ICG-Lactosome administration (Figures 7a and Figure 8). When the second Lactosome doses were 5 and 25 mg/kg, $(L/B)/(L/B)_0$ became 10.8 and 6.32, respectively, indicating occurrence of the ABC phenomenon. Whereas, $(L/B)/(L/B)_0$ decreased below 2.0 in the cases that the second doses were over 50 mg/kg.

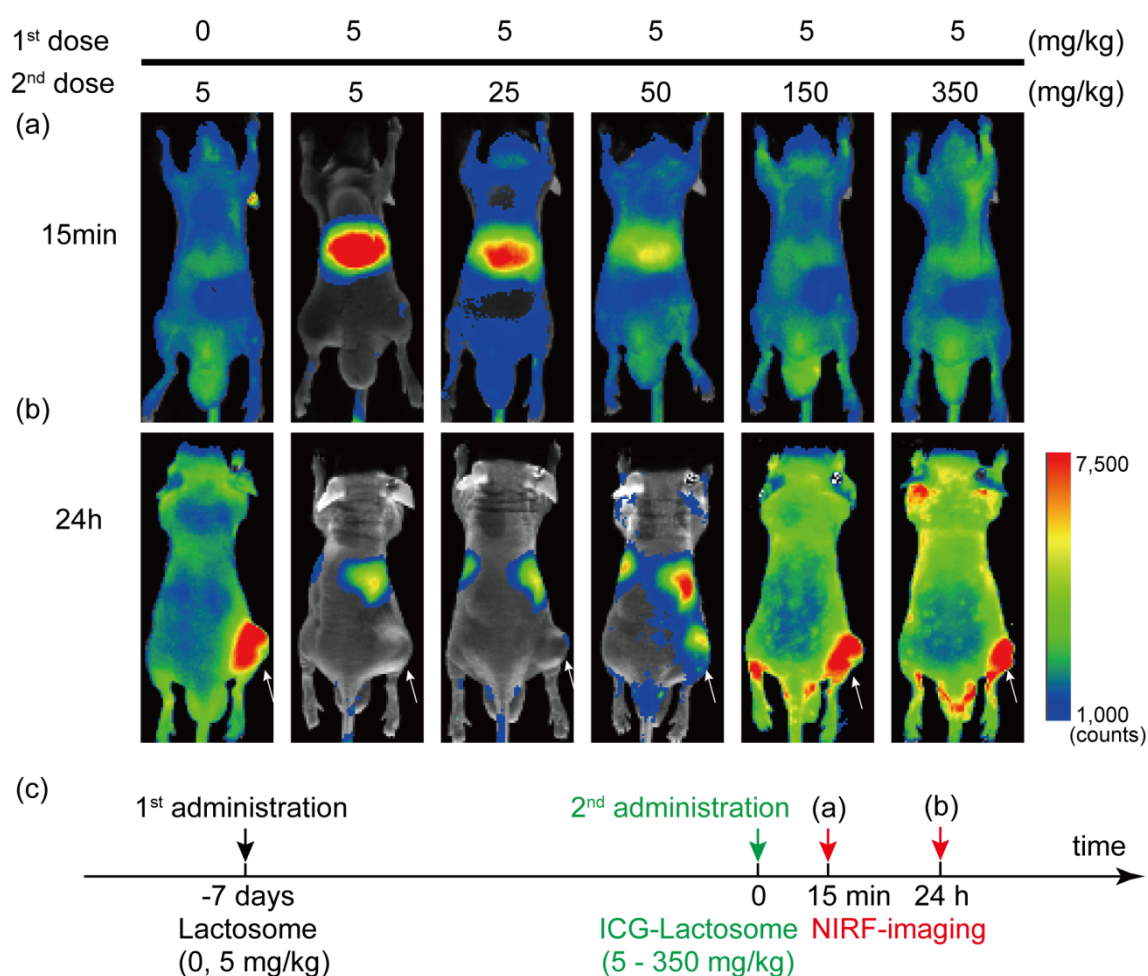


Figure 7. The second dose dependence of the Lactosome pharmacokinetics. The second Lactosome doses were varied from 5 to 350 mg/kg (5, 25, 50 mg/kg/100 μ L, 150, 350 mg/kg/200 μ L). The injected ICG amounts were kept the same to be 5 nmol/kg for all the groups. NIRF images of tumor-transplanted mice at 15 min (a) and 24h (b) after ICG-Lactosome administration. The white arrows indicate the tumor region. Fluorescence ranges were set to be the same for all the images from max count of 7,500

to min count of 1000. (c) Time schedule for the NIRF imaging. The black and green arrows indicate the injection time points of Lactosome and ICG-Lactosome to the tumor-transplanted mice, respectively. NIRF imaging was performed at the time points of red arrows.

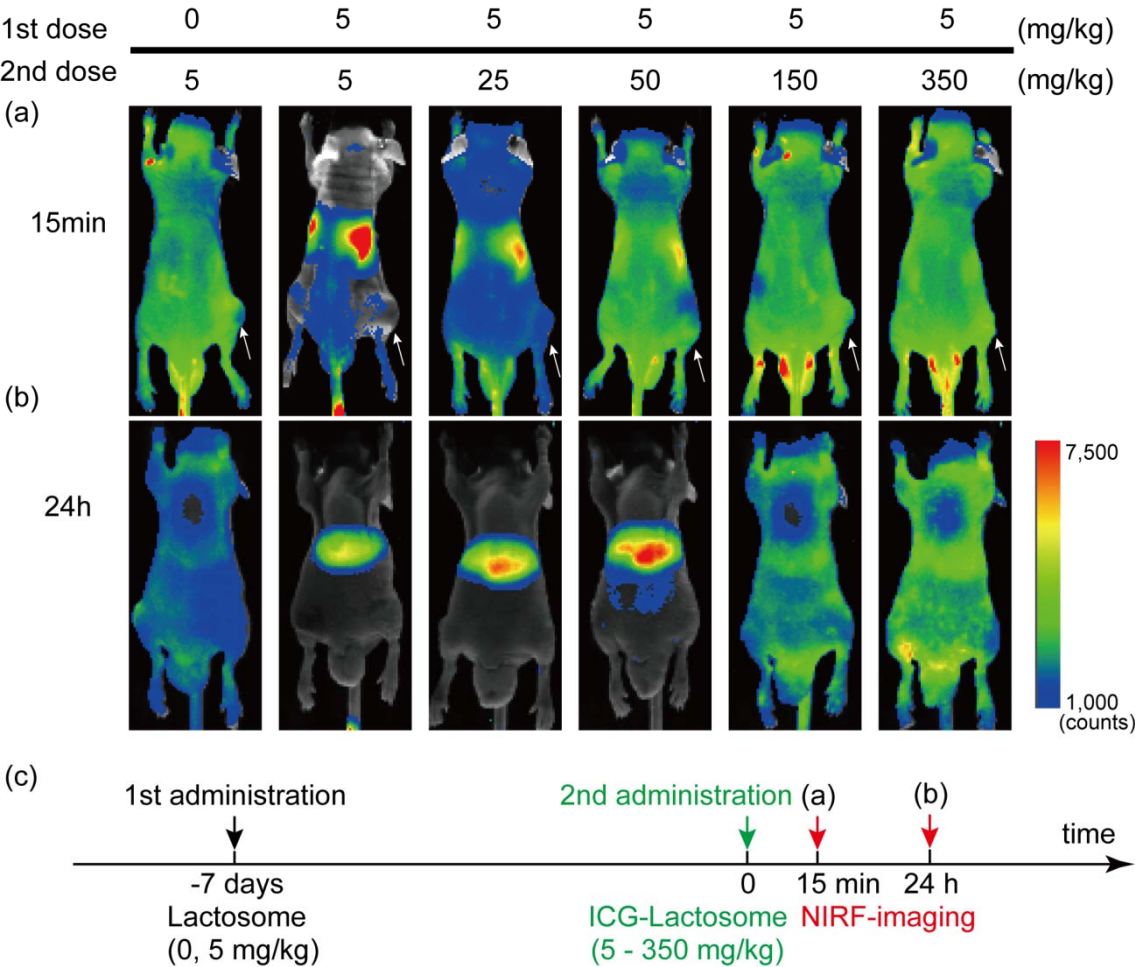


Figure 8. The second dose dependence of the Lactosome pharmacokinetics. The total Lactosome second doses were varied from 5 to 350 mg/kg (5, 25, 50 mg/kg/100 μ L, 150, 350 mg/kg/200 μ L). Injected ICG amount was set to be 5 μ mol/kg. NIRF images of tumor-transplanted mice at 15 min (a) and 24h (b) after ICG-Lactosome administration. The white arrows indicate the tumor region. Fluorescence range was represented from the max count of 7500 to the min count of 1000. (c) Time schedule for the NIRF imaging. The black and green arrows indicate the injection time points of Lactosome and ICG-Lactosome to the tumor-transplanted mice, respectively. NIRF imaging was performed at the time points of red arrows.

Table 2. The effect of second Lactosome dose on its biodistribution

Lactosome administration (mg/kg)	(L/B)			(L/B)/(L/B) ₀ ^{*1}			ABC phenomenon ^{*2}
0	1.03	±	0.08	-			-
5	11.1	±	5.5	10.8	±	5.4	Yes
25	6.52	±	1.85	6.32	±	1.80	Yes
50	1.65	±	0.67	1.60	±	0.64	No
150	0.91	±	0.01	0.88	±	0.01	No
350	0.91	±	0.06	0.88	±	0.06	No

^{*1} The (L/B)₀ value was determined to be 1.03 from NIRF images of mice at 15 min after single administration of ICG-Lactosome (Lactosome pre-dosage of 0 mg).

^{*2} The (L/B)/(L/B)₀ values taking more than 2.0 are indication for occurrence of the ABC phenomenon.

The second dose dependence of anti-Lactosome IgM productions was evaluated by ELISA. At 7 days after the Lactosome second administration with varying the doses from 5 to 350 mg/body, mice serums were collected to evaluate the anti-Lactosome IgM level. At any doses, anti-Lactosome IgM was found to maintain the high level (Figure 9).

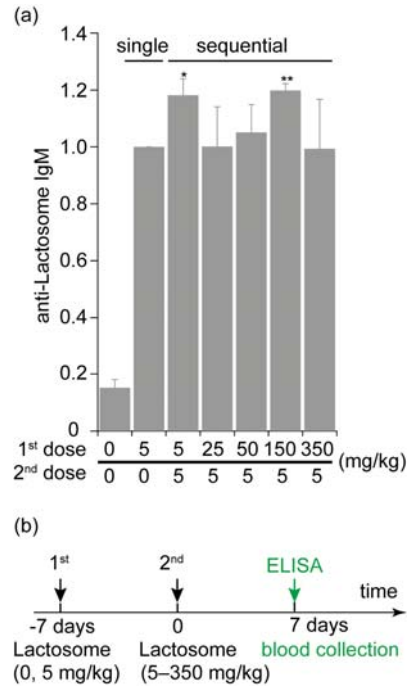


Figure 9. (a) The effect of second Lactosome dose on the produced anti-Lactosome IgM level. Each value was normalized by anti-Lactosome IgM production level when 5 mg/kg of Lactosome was singly administrated to the mice. (b) Time schedule for the experiments. The black arrows indicate the injection time points of Lactosome. The serums were collected at 7 days after the second Lactosome administration (5, 25, 50 mg/kg/100 μ L, 150, 250, 350 mg/kg/200 μ L), which is indicated by the green arrow. * and ** represent significant difference from the groups of single administration of 5 mg/kg with $p < 0.05$ and $p < 0.01$, respectively.

Discussion

The first Lactosome dose dependence of the ABC phenomenon shows a critical value of 50 mg/kg dose. When the first Lactosome dose was below 50 mg/kg, the ICG-Lactosome at the second administration was entrapped in liver (Figure 4) with high $(L/B)/(L/B)_0$ values (Table 1), indicating occurrence of the Lactosome ABC phenomenon as previously reported [6]. On the other hand, when the first Lactosome dose was over 150 mg/kg, the ICG-Lactosome at the second administration spread over the whole body followed by the accumulation in the tumor region by the EPR effect

(Figure 4) with low $(L/B)/(L/B)_0$ values below 2.0 (Table 1). These results suggest that tolerance to immunogenic Lactosome was developed with high dose similarly to the report that high-dose PEGylated liposome evaded from the ABC phenomenon [23]. However, the anti-Lactosome IgM levels in serums decreased just monotonously with increasing the first Lactosome doses without showing any critical value (Figure 6), meaning that the development of immune tolerance is insufficient with these doses even though the anti-Lactosome IgM level diminished with a large amount of Lactosome dose. In order to analyze the immune response more quantitatively, Lactosome doses at the second administration were varied.

When the ICG-Lactosome dose over 50 mg/kg was administered to the immunized mice, the NIRF signals spread over the whole body followed by accumulation in the tumor region (Figure 7). However, anti-Lactosome IgM in serum kept high level irrespective of the second doses in the range from 5 to 350 mg/kg (Figure 9). The high doses therefore did not develop the immune tolerance. The ABC phenomenon was still there, but anti-Lactosome IgM should be consumed with excessive doses of over 50 mg/kg at the second Lactosome administration, leading ICG-Lactosome free from binding of anti-Lactosome IgM.

The concentration of anti-Lactosome IgM in serum at 5 days after the first Lactosome administration was roughly determined to be 49 $\mu\text{g/mL}$ by the Mouse IgM ELISA quantitation set (Table 3). With using a total blood pool of a nude mouse and the molecular weight of IgM of ca. 2.0 mL and 970 kDa, respectively [24], the total amount of anti-Lactosome IgM in blood is calculated to be ca. 5 nmol/kg. Under the assumption that Lactosome is consisted of ca. 200 amphiphilic polydepsipeptide, Lactosome of 50 mg/kg corresponds to 35 nmol/kg. The Lactosome dose over 50 mg/kg is thus far

excessive to the amount of anti-Lactosome IgM. In other words, anti-Lactosome IgM does not work sufficiently against the high dose of Lactosome.

Table 3. Concentration of anti-Lactosome IgM in blood serum

Interval Between two dosages /day	Concentration ($\mu\text{g/mL}$)
0	-
1	-
2	-
3	-
4	21.44
5	49.16

Taken together, all the data presented here can be interpretable as follows. With increasing the first Lactosome dose, the anti-Lactosome IgM level decreases gradually due to partial immune tolerance. When the anti-Lactosome IgM level becomes lower than the amount required for opsonization of Lactosome of 5 mg/kg, the ABC phenomenon disappears at the second administration of the ICG-Lactosome of 5 mg/kg. On the other hand, even mice, which produce the high level of anti-Lactosome IgM with Lactosome pre-dose of 5 mg/kg, do not show the ABC phenomenon when the ICG-Lactosome is administered together with excessive amount of Lactosome, which should consume anti-Lactosome IgM produced in immunized mice.

It is considered that immune tolerance is induced by frequent administration of low dose antigen or administration of high dose antigen [25, 26]. As far as TI antigens are concerned, PEGylated liposome was shown to induce immune tolerance with administration of high dose PEGylated liposome [15-19]. Similarly, TI antigen of Lactosome is shown here to induce partial immune tolerance at higher doses of first administration. In this case, the immune tolerance is usually interpretable as B cell's

anergy or apoptosis [24]. In order to get more information on it, the regular dose of Lactosome was administered to the partially immunized mice with a high dose at first administration (Figure 10 and Table 4). The NIRF imaging using ICG-labeled Lactosome clearly shows the occurrence of the ABC phenomenon upon the second regular dose. It is therefore suggested that B cell's apoptosis is a more plausible explanation than the establishment of negative signaling to B cell against the immunogenic Lactosome.

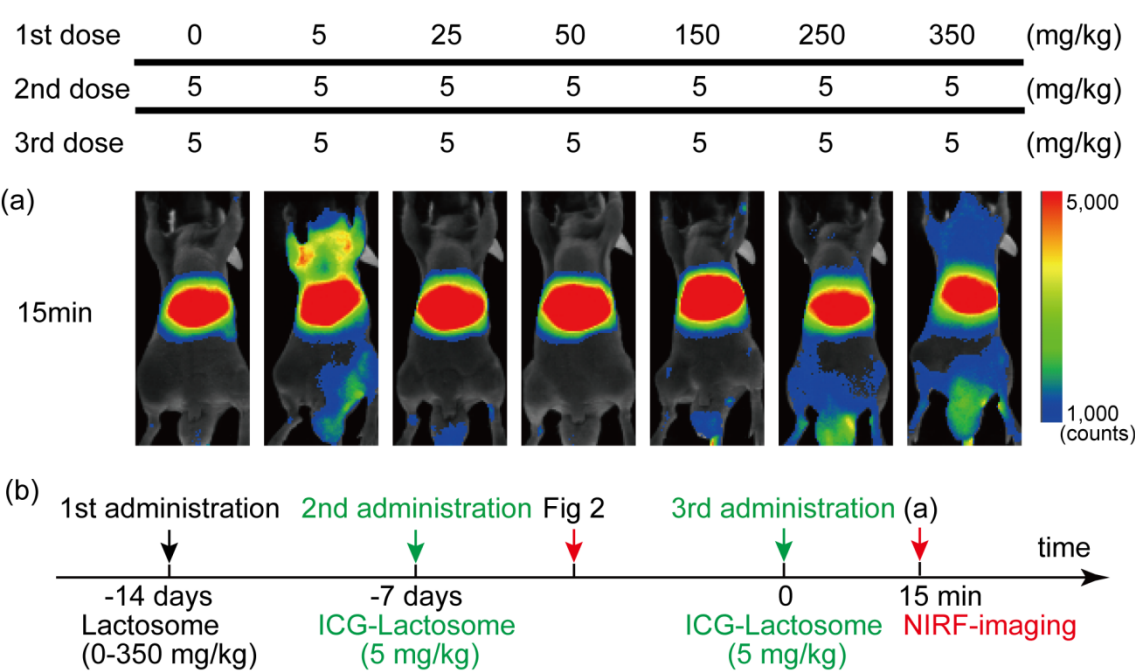


Figure 10. The pharmacokinetics of Lactosome at the third dosage after the first amount dosage of Lactosome and the second regular dosage. NIRF images of mice at (a) 15 min after ICG-Lactosome administration as 3rd dose at 7 days after 2nd administration as Fig. 2. Lactosome (5 mg/kg/100 μ L) were injected to the mice. The figure range was represented to the max count 5000 from the min count 1000. (b) Time schedule for the NIRF imaging. Black and green arrows indicate the injection time points of Lactosome and ICG-Lactosome respectively. NIRF imaging was performed at red arrows.

Table 4. The effect of first Lactosome dose on Lactosome biodistribution at third administration

Lactosome administration at first (mg/kg)	(L/B)			(L/B)/(L/B) ₀ ^{*1}			ABC phenomenon ^{*2}
0	17.7	±	4.1	18.7	±	4.3	Yes
5	7.57	±	3.60	7.97	±	3.79	Yes
25	12.0	±	1.2	12.6	±	1.3	Yes
50	11.6	±	7.8	12.2	±	8.2	Yes
150	12.7	±	5.7	13.4	±	6.0	Yes
250	8.87	±	1.92	9.34	±	2.02	Yes
350	24.9	±	26.6	26.2	±	28.0	Yes

^{*1} The (L/B)₀ value was determined to be 1.03 from NIRF images of mice at 15 min after single administration of ICG-Lactosome (Lactosome pre-dosage of 0 mg).

^{*2} The (L/B)/(L/B)₀ values taking more than 2.0 are indication of occurrence of the ABC phenomenon.

Once anti-Lactosome IgM level was raised by Lactosome administration, the production could not be suppressed by following Lactosome administration with high doses. Lactosome is found to be one of strong TI-2 antigens, which may be ascribed to the long clearance time from the blood stream and also to the repetitive and regular epitope presentation on the surface of nanoparticles. Lactosome, therefore, effectively activates B1 to generate IgM with the first administration of Lactosome, which may lead to keep the memory effect [27, 28]. The repeated exposure to Lactosome may induce the strong immune response of B cells, but which details remain to be studied.

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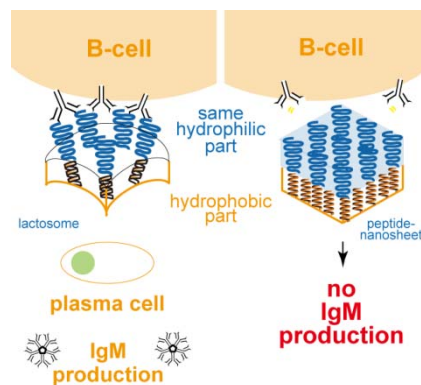
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Chapter 3

Suppressive Immune Response of Poly(sarcosine) Chains in Peptide-nanosheets in Contrast to Polymeric Micelles



Introduction

Liposome and polymeric nanoparticles have been frequently examined as carriers in the field of drug delivery system (DDS) [1, 2]. These nanocarriers can be used as diagnostic and/or therapeutic agents depending on the loading of chemical agents [3, 4]. Especially, nanocarriers in the size range of 10–100 nm may show the enhanced accumulation in solid tumors, that is so called the enhanced permeability and retention (EPR) effect, because of the high permeability of blood vessels and immature lymphatic drain systems [5, 6]. Doxil[®] is a typical example for this type of nanocarrier, which is polyethyleneglycol-modified (PEGylated) liposome loaded with doxorubicin in the inner aqueous phase [7].

Lactosome is a polymeric micelle composed of amphiphilic polydepsipeptide, poly(sarcosine)_m-*block*-poly(L-lactic acid)_n (poly(Sar)_m-*block*-PLLA_n; *m* and *n* are mean values) [8]. Lactosome with a diameter of ca. 35 nm has been shown to accumulate specifically in solid tumors through the EPR effect. Notably Lactosome is effective in evading from capture by the mononuclear phagocyte system (MPS) of liver and spleen. Accordingly, human liver cancer implanted orthotopically in mouse liver was imaged successfully with Lactosome labeled by the near infrared fluorescence probe indocyanine green (ICG), because the capture by healthy regions of liver was suppressed significantly [9]. Further, Lactosome is composed of a biodegradable and biocompatible polymer [10, 11]. Lactosome is therefore expected to be a potential anti-tumor drug formulation based on a block copolymer.

However, there was a problem with Lactosome which showed a drastic change of biodistribution between the first dose and the following doses. Lactosome could image various types of solid tumors at the first dose, but at the second dose after one week,

Lactosome was found to be captured immediately by the liver [12]. This phenomenon is called the accelerated blood clearance (ABC) phenomenon, which was reported with PEGylated liposomes [13-15] as well as with PEGylated micelles [16, 17]. The ABC phenomenon was already demonstrated to depend on the anti-PEG IgM production, stimulated by the first dose. Therefore, the nanoparticles of the second dose are marked by IgM and captured by Kupffer cells in liver, with the help of the complement system [18].

Lactosome was therefore recognized by the immune system even though Lactosome was not captured significantly by the MPS. The author hypothesized that nanoparticles would evade from the immune system when the nanoparticle surface is covered densely by hydrophilic polymer chains like a high-density polymer brush structure. The dense hydrophilic shell will not allow capture by B-cell receptors, thus preventing B-cell receptor recognition. In this regard, the spherical structures of micelles and small vesicles have a disadvantage because there always exist relatively looser spaces at the outer surface than that at the inner regions due to the large curvature. However, this concern may be solvable with using a sheet structure instead of the spherical structure. The author has studied molecular assemblies prepared from a series of amphiphilic helical peptides which have a hydrophobic helical block of (Leu-Aib)_n (Aib represents α -aminoisobutyric acid) [19]. In these molecular assemblies, the regular packing of helices leads to formation of interdigitated monolayers which can generate various types of morphologies of nanotubes, vesicles, and round-bottom flask shapes. Recently, the author found that the sheet structure of ca. 20 nm could be prepared from poly(Sar)_m-*block*-(L-Leu-Aib)_n.

As described before, the nanosheets should have a high density of hydrophilic

poly(sarcosine) chains on the surface. In the present study, nanosheets of poly(Sar)_m-*block*-(L-Leu-Aib)_n were analyzed on their tumor imaging ability, the ABC phenomenon, and the IgM production, and compared with those of Lactosome having the similar size but with a lower surface density of poly(sarcosine) chains, in order to clarify the relationship of the ABC phenomenon with the poly(sarcosine) chain density.

Materials and Methods

Instruments

Transmission electron micrographs (TEM) images were taken using JEM-2000EX II (JEOL Ltd., Japan). Atomic force microscope (AFM) images were taken with Agilent 5500 AFM (Agilent Technologies Inc., CO, USA). Hydrodynamic diameter and molecular weight of molecular assemblies were measured by dynamic light scattering (DLS) and static light scattering (SLS) on a Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK). dn/dc and multi-angle light scattering were measured by DRM-3000 and DLS-6800, respectively (Otsuka Electronics Co., Ltd., Osaka, Japan). UV assay was performed by Microplate Reader on iMark (Bio-Rad Laboratories Inc., CA, USA). NIRF images were taken by Clairvivo OPT (Shimadzu Corp., Kyoto, Japan, *ex.* 785 nm/*em.* 845 nm).

Preparation of Lactosome

Poly(Sar)_m-*block*-PLLA_n and indocyanin green (ICG) labeled PLLA_n were synthesized as previously reported [8, 9]. One amino group of 1,2-ethylenediamine was protected by benzyloxycarbonyl (Z) group and the other amino group was utilized as an initiator for preparation of the PLLA block. A mixture of the initiator, L-lactide, and 0.2

wt% tin(IV) octanoate as catalyst was heated up to 120 °C for 12 h. After the deprotection of the Z group, the amino terminal was used as initiator for polymerization of sarcosine *N*-carboxyanhydride (NCA). The N terminal of poly(sarcosine) was capped with glycolic acid.

The degree of polymerization of the poly(Sar) block (poly(Sar)_{*m*}-*block*-PLLA_{*n*}) was determined to be 64 from the relative areas of Sar *N*-CH₃ signal against the CH₃CO signal and the PLLACCH₃ signal in the ¹H NMR spectra. ¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.32-1.70 (m, 90H, PLLA CCH₃), 2.10 (s, 3H, CH₃CO), 2.70-3.25 (m, 196H, Sar *N*-CH₃), 3.75-4.48 (m, 128H, Sar NCH₂CO), 5.02-5.28 (m, 30H, PLLA CH).

GPC of poly(Sar)₆₄-*block*-PLLA₃₀ was performed with two Asahipak GF-1G 7B (7.5× 50 mm) and Asahipak GF-310 HQ columns (7.5× 300 mm) with chloroform (40 °C, 0.3 mL/min) as eluent.

The ¹H NMR and GPC data of Poly(Sar)_{*m*}-*block*-PLLA_{*n*} are shown in Figure 1a and Figure 1c. Poly(Sar)_{*m*}-*block*-PLLA_{*n*}, where *m* is about 64 and *n* is about 30 from the result of NMR (mean values). ICG-PLLA was obtained by labeling the amino group in PLLA block (30mer, a mean value) with ICG-Sulfo-OSu (ICG-PLLA₃₀). For the preparation of Lactosome, poly(Sar)₆₄-*block*-PLLA₃₀ (1 mg) in test tube was dissolved in chloroform, and evaporated to form a polymer thin film on the test tube wall. Water was added to the tube so that the concentration of the amphiphilic polymer became 1 mg/mL. The dispersion was heated at 85 °C for 20 min. After freeze-drying the solution, saline (1 mL) was added, and the solution was filtered by using a syringe filter (pore size: 0.20 μm) just before administration. For the preparation of ICG-Lactosome, the concentration of ICG-PLLA₃₀ was determined by using UV absorbance of ICG-Sulfo-OSu at 795 nm dissolved in DMSO. poly(Sar)₆₄-*block*-PLLA₃₀ (1 mg) and

ICG-PLLA₃₀ (2 nmol) in a test tube were dissolved in chloroform and the molecular assemblies were prepared similarly to Lactosome. The ICG amount was set to be 5 nmol/kg on a mouse.

Preparation of peptide-nanosheets

Poly(Sar)_m-*block*-(L-Leu-Aib)_n was synthesized as previously reported [19, 20]. Briefly, (L-Leu-Aib)_n was prepared by fragment condensation, whereas the poly-sarcosine extension at the N-termini of the helical segments was obtained by the Sar-NCA (*N*-carboxyanhydride) polymerization.

The degree of polymerization of the poly(Sar) block (poly(Sar)_m-*block*-(L-Leu-Aib)_n) was determined to be 60 from the relative areas of SarN-CH₃ signal against the OCH₃ signal and the LeuCH₂CH(CH₃)₂ signal in the ¹H NMR spectra. ¹H NMR (400 MHz, MeOH-d) δ (ppm) 8.2–7.3 (m, 12H, amide), 4.6–3.8 (br, 130H, HOCH₂CH₂CO, LeuC^αH, SarCH₂), 3.66 (s, 3H, OCH₃), 3.3–2.8 (m, 180H, Sar N-CH₃), 1.9–1.3 (m, 54H, LeuCH₂, LeuC^γH, AibCH₃), 1.1–0.8 (m, 36H, Leu(CH₃)₂).

GPC of Poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ was performed with two consecutive Gram columns with N,N-dimethylformamide (DMF) (40 °C, 1 mL/min) as eluent.

The synthetic product was characterized by ¹H NMR experiments and GPC (Figure 1b and Figure 1d). Poly(Sar)_m-*block*-(L-Leu-Aib)_n, where m is about 60 (a mean value) and n is 6 from the result of NMR. ICG-poly(Sar)_m-*block*-(D-Leu-Aib)_n was synthesized by the reaction of the ICG-Sulfo-OSu with the *N*-terminus of the poly(sarcosine) chain of the amphiphilic polypeptide (poly(Sar)₅-*block*-(D-Leu-Aib)₆) as previously reported [8, 9].

A stock solution of poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ (12 mg) in ethanol (240 μL) was

prepared. An aliquot (20 μ L) of the stock solution was injected to a buffer (10 mM Tris-HCl, pH of 7.4) (1 mL) under stirring at 4 $^{\circ}$ C. After stirring for 30 min, the dispersion was heated at 90 $^{\circ}$ C for 1 h. For the preparation of ICG-peptide-nanosheets, poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ (1 mg) and ICG-Poly(Sar)₅-*block*-(D-Leu-Aib)₆ (2 nmol) in ethanol solution were injected in buffer. ICG amount was set to be 5 nmol/kg on a mouse.

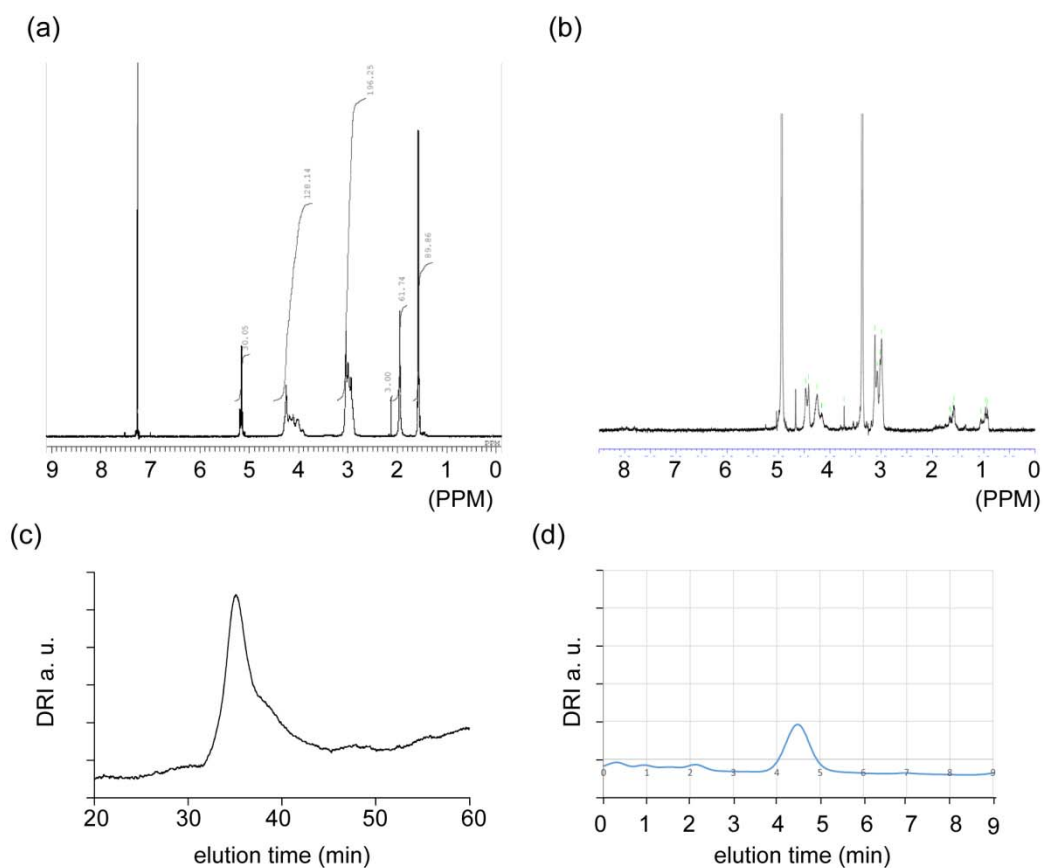


Figure 1. The characterizations of poly(Sar)₆₄-*block*-PLLA₃₀ and poly(Sar)₆₀-*block*-(L-Leu-Aib)₆. The NMR of poly(Sar)₆₄-*block*-PLLA₃₀ (a) and poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ (b). The GPC of poly(Sar)₆₄-*block*-PLLA₃₀ (c) and poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ (d) .

Transmission electron micrographs (TEM)

The dimension of the molecular assemblies was analyzed by TEM. A drop of dispersion was mounted on a carbon-coated Cu grid and stained negatively with 2% uranyl acetate, followed by suction of the excess fluid with a filter paper. TEM images were obtained at an accelerating voltage of 100 kV.

Atomic force microscope (AFM)

AFM images were measured in water with Agilent 5500 AFM in magnetic AC mode (MAC mode) using magnetic coated silicon nitride cantilevers (N9865A MAC Lever, Agilent Technologies Inc., CO, USA). The Si-wafers were modified by 3-aminopropyltriethoxysilane (APTES). The freshly prepared peptide-nanosheet solution was incubated on APTES-modified Si-wafer at room temperature for 1 h. Scanning rate was 0.5 Hz, the set point was in the range of 1.3 – 1.5 V and the free amplitude was in the range of 1.4 – 1.8 V.

Static light scattering (SLS)

Molecular weights of Lactosome and peptide-nanosheets were determined by SLS measurements using Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK) at 25 °C. Saline was used as a solvent and for a dilution of the stock solution (1.0 mg/mL). Sphere model ($R_g = 0.774 \times R_h$), was applied for a shape correction. The refractive index increments, dn/dc , of Lactosome and peptide-nanosheets were measured with using the differential refractometer, DRM-3000 (Otsuka Electronics Co., Ltd., Osaka, Japan). R_g/R_h was also evaluated by the multi-angle light scattering using DLS-6800.

Critical association concentration (CAC)

To estimate CAC values for Lactosome and peptide-nanosheet, the method was used based on the solubilization of water-insoluble fluorescent dye, pyrene, in micelles [21]. In case of Lactosome, 100 μ l of pyrene solution in chloroform (10 mg/ml) and 200 μ l of serial dilutions of Lactosome in test tubes were dried under vacuum. Then, 2 ml of saline was added to each tube with the dried pyrene. The dispersion was heated at 85 °C for 20 min and the solution was filtered with using a syringe filter (pore size: 0.20 μ m) to remove the non-solubilized pyrene. Final concentration of Lactosome solutions were $2 \times 10^{-4} - 2 \times 10^{-10}$ M. The fluorescence intensity of solubilized pyrene was measured at an excitation wavelength of 339 nm and emission wavelength of 390 nm using an RF-5300 Fluorescence Spectrophotometer (Shimadzu Corp. Japan). In case of peptide-nanosheet, 100 μ l of pyrene solution in chloroform (10 mg/ml) in test tubes were dried under vacuum. Then, 2 ml of serial dilutions ($10^{-5} - 10^{-8}$ M) of peptide-nanosheet in saline was added to each tube with the dried pyrene. The dispersion was ultrasonic at room temperature for 10 min. After that is above method.

IgM assay

For enzyme-linked immunosorbent assay (ELISA), Lactosome or peptide-nanosheets (5 mg/kg, 100 μ L) was injected to the BALB/c nu/nu mouse, which were purchased from Japan SLC, Inc. (Japan) (n=4 per group). Mouse blood was collected in 1.5 mL tube from inferior vena cava under anesthesia condition at 1 week after the administration. The serum was kept overnight at 4 °C. Next day the serum was obtained by centrifugation (10 min, 3000 rpm) and saved at -40 °C.

Lactosome in 50 μ L distilled water was added to 96-well plates (0.5 μ g/well) and air dried completely for 1 day. Then, 150 μ L of blocking buffer (2% BSA/PBS) was added and incubated for 2 h. The wells were washed four times with washing buffer (PBS-T: 0.05% Tween 20 in PBS). The sera with serial dilution were added to the wells and incubated for 2 h. After the incubation, the wells were washed four times using PBS-T. Peroxidase-conjugated goat-anti-mouse IgM in 0.1% BSA/PBS (50 μ L, Southern Biotech, USA) was added as the secondary antibody. After incubation of the solution for 2 h, and the wells were washed again four times with PBS-T. *o*-Phenylenediamine (0.5 mg/mL, Sigma, St. Louis, MO) dissolved in 0.0003% H₂O₂-0.1 M citrate phosphate buffer (pH 5.0) was added to the well. At 10 minutes after the *o*-phenylenediamine addition, optical density (OD) was determined at 490 nm with OD at 620 nm as reference.

Cell culture

Pancreatic carcinoma (SUIT-2/pEF/LUC) cell line was maintained at 37 °C with 5% FBS (Nacalai Tesque, Inc. Kyoto, Japan) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen Corp. USA) supplemented with GlutaMAX™-I Supplement (2 mmol/L, Gibco, Invitrogen Corp. USA), Plasmocin™ prophylactic (5 μ g/mL, Nacalai Tesque, Inc. Kyoto, Japan), penicillin (100 U/mL), and streptomycin (100 μ g/mL).

***In vivo* NIRF-imaging with ICG-nanocarriers**

SUIT-2/pEF/LUC cells (5×10^5 cells) were dissolved in phosphate-buffered saline (PBS, 20 μ L), and subcutaneously inoculated into right femoral region of 7-week-old

BALB/c nu/nu mice (n=3 per group). Lactosome or peptide-nanosheet (5 mg/kg, 100 μ L) was injected via the tail vein to the mice 1 week after the tumor transplantation. The second dose of ICG-Lactosome or ICG-peptide-nanosheet (5 mg/kg, 100 μ L) was injected to the mice 1 week after the first dose. The injected ICG amount was 5 nmol/kg. NIRF images were taken at 15 min, 1 h, 3 h, 6 h, 9 h, and 24 h after the second dose. During the imaging process, the mice were held on the imaging stage under anesthetized condition with 2.5% of isoflurane gas in air flow (1.5 L/min). The pseudo images were constructed from the photon counts.

Statistical analysis

All results are expressed as mean $\pm$ SD. Differences between groups in IgM production and NIRF-imaging studies were assessed by the t-test for independent samples. P-value < 0.05 and < 0.01 are considered statistically significant and designated by * and **, respectively.

Ethics

All of her *in vivo* animal experiments were approved by the Animal Research Committee of Kyoto University. Animals were treated humanely.

Results

Properties of Lactosome and peptide-nanosheet

Poly(Sar)₆₄-*block*-PLLA₃₀ (Figure 2a) formed a polymeric micelle with a diameter of ca. 35 nm as determined by DLS measurement in agreement with the TEM observation (Figure 2e) [8]. The SLS measurement revealed that one nanoparticle of Lactosome

contained ca. 294 molecules of poly(Sar)₆₄-*block*-PLLA₃₀ (Table 1). The surface density of poly(sarcosine) chains at the most outer surface of the nanoparticle was therefore calculated to be 0.07 chains /nm².

The authors has previously reported that poly(Sar)₂₅-*block*-(L-Leu-Aib)₆ formed curved sheets with a side length of ca. 250 nm [20] which, however, is too large compared with 35 nm of the polymeric micelle composed of poly(Sar)₆₄-*block*-PLLA₃₀. One way to reduce the sheet size should be to use a longer hydrophilic block. Indeed, poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ (Figure 2c) formed nanosheets (named peptide-nanosheet) as revealed by the TEM and AFM observations (Figure 2f, Figure 3, and Figure 4).

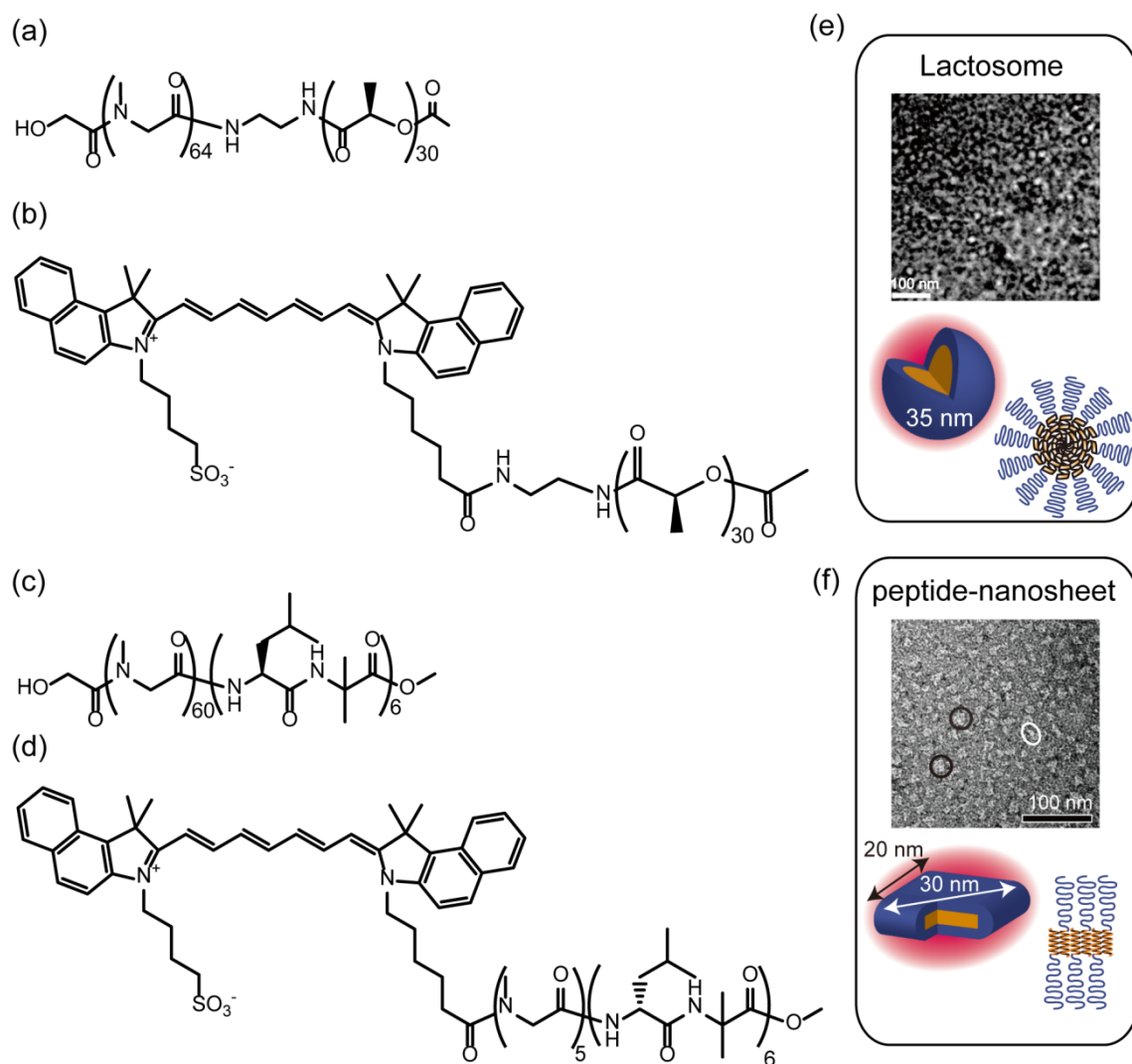


Figure 2. Structural formula and illustration of Lactosome and peptide-nanosheet. Structural formula of poly(Sar)₆₄-block-PLLA₃₀ (a), ICG- poly(L-lactic acid)₃₀ (b), poly(Sar)₆₀-block- (L-Leu-Aib)₆ (c), and ICG-(Sar)₅-block-(D-Leu-Aib)₆ (d). TEM image and the illustration of Lactosome (e) and peptide-nanosheet (f). Black circles and a white ellipse represent the images of peptide-nanosheet side length and thickness, respectively.

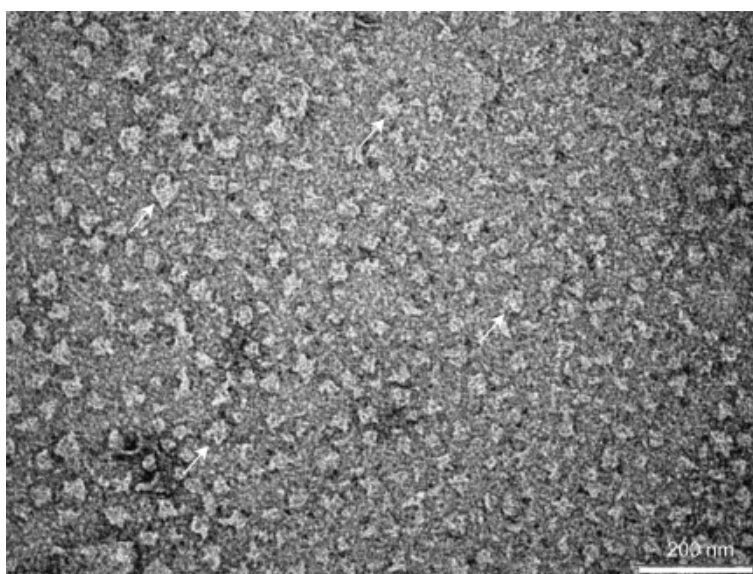


Figure 3. The TEM images of the peptide-nanosheet. The three white arrows indicate the typical nanosheet structure. The scale bar indicates 200 nm.

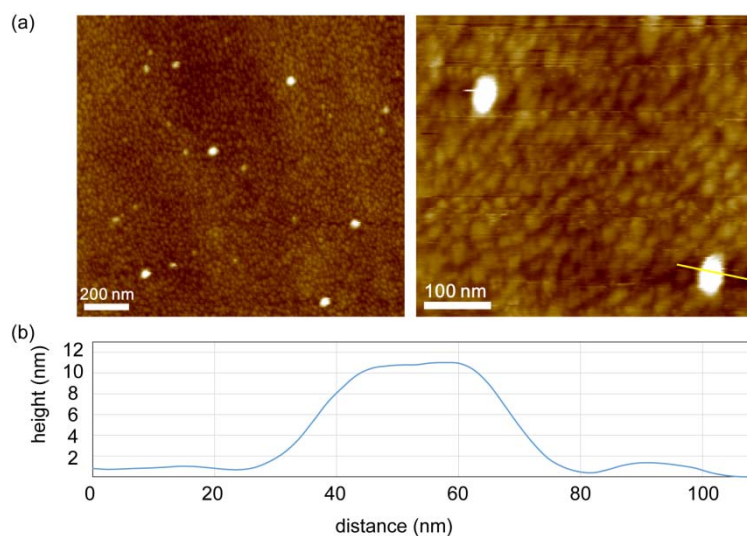


Figure 4. AFM image of peptide-nanosheet on APTES-modified Si-wafer (a). Height profile along the yellow line in (a) is indicated in (b).

The R_g/R_h value was determined by the multi-angle light scattering method to be 1.067 (Table 1), indicating that the shape should be a sheet [22]. The images in the TEM picture suggest the thickness of the nanosheets of about 6 nm which is reasonable for the interdigitated monolayer formation. When the cross-section of peptide-nanosheet is

approximated to be a square, the side length of ca. 20 nm was derived from the TEM images, which is consistent with the hydrodynamic diameter of 30 nm by the DLS measurement. Since one molecular assembly of peptide-nanosheet was found to contain ca. 263 molecules of poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ by the SLS measurement, the surface density of poly(sarcosine) chains of 0.33 chains/nm² (Table 1) is derived.

Table 1. Properties of Lactosome and peptide-nanosheet

	Lactosome	peptide-nanosheet
diameter/diagonal length (pdi)	36 nm (0.05) ^{a)}	30 nm (0.24) ^{a)}
molecular weight of one nanoparticle	2023 kDa ^{b)}	1456 kDa ^{b)}
molecular weight of amphiphilic block polymer	6872 Da	5541 Da
number of amphiphilic block polymers in one nanoparticle	294	263
multiangle light scattering (R_g/R_h) ^{c)}	0.749	1.067
surface area	4069 nm ²	800 nm ²
poly(sarcosine) density	0.07 chains/nm ²	0.33 chains/nm ²

^{a)} The hydrodynamic diameter obtained by DLS measurement.

^{b)} SLS measurement.

^{c)} Sphere ~0.75, Sheet 1.1

The surface density indicates that one poly(sarcosine) chain occupies the area of 3.2 nm² in the peptide-nanosheet. The peptide-nanosheet is considered as an interdigitated monolayer with extending poly(sarcosine) chains alternately to the both sides of the nanosheet. Accordingly, one amphiphilic peptide occupies the area of 1.6 nm², which is very close to the cross-section of the (Leu-Aib)₆ helical rod (1.4 nm²). This result totally

supports our interpretation that the nanosheet should consist of a tight helix association.

However, there are some uncertainties on the nanosheet morphology in regard to the AFM image, which may show an ellipsoid micelle (Figure 5). The long hydrophilic poly(sarcosine) chain may distort the sheet structure. The interpretation described above therefore remains within a qualitative speculation.

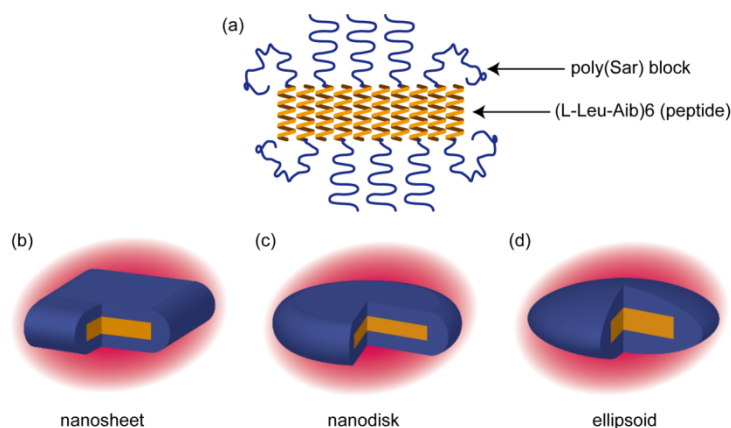


Figure 5. The illustrations of peptide-nanosheet. The molecular packing in the nanosheet (a). The orange block indicates (L-Leu-Aib)₆. The blue block indicates Poly(Sar). The morphologies of nanosheet (b), nanodisk (c), and ellipsoid (d).

The critical association concentrations (CAC) of Lactosome and peptide-nanosheets were found to be 1.58×10^{-7} M and 3.37×10^{-8} M, respectively, on the basis of the general evaluation method using fluorescence from pyrene (Figure 6). Since the dose to the mice corresponds to the concentration in the blood stream of $7 - 9 \times 10^{-6}$ M, the amphiphilic block polymers should preserve the molecular assembly formulation.

The structural formula of ICG-PLLA₃₀ and ICG-poly(Sar)₅-*block*-(D-Leu-Aib)₆ for NIRF-imaging are shown in Figures 2b and 2d. These molecules are designed to locate the ICG moiety at the relatively inner region of the molecular assemblies.

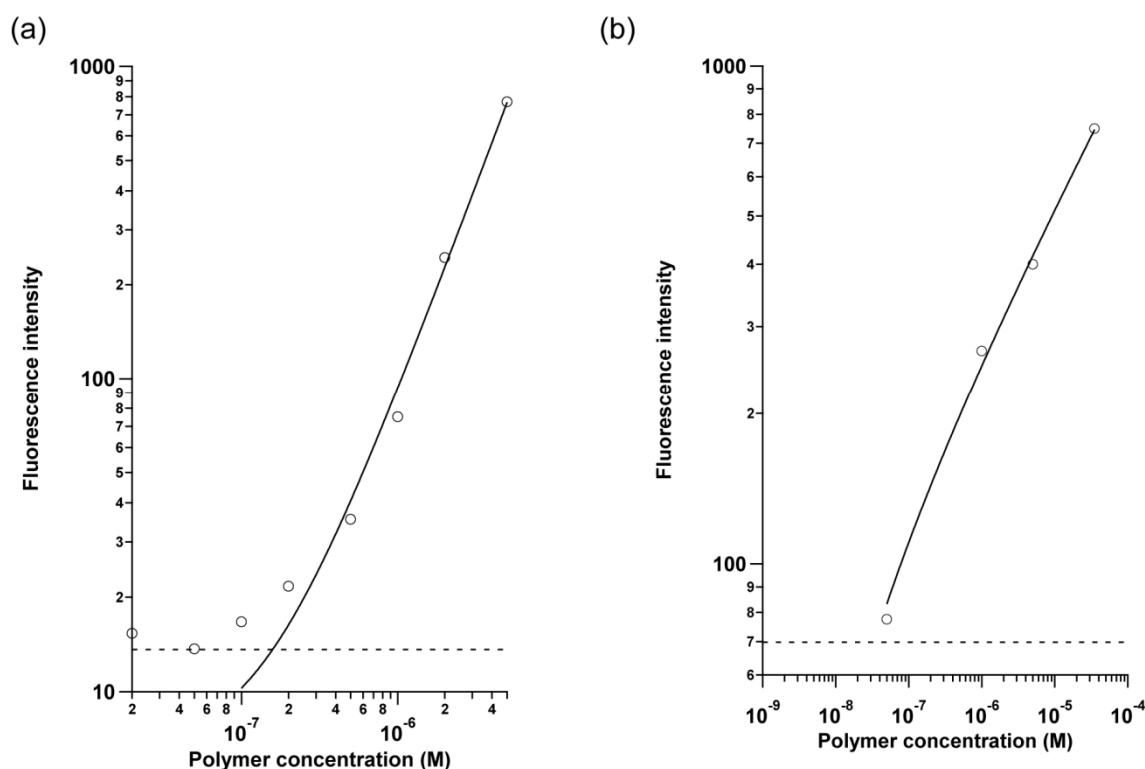


Figure 6. CAC of Lactosome(a) and peptide-nanosheets(b)

IgM Production

The production of anti-Lactosome IgM and anti-peptide-nanosheet IgM at 7 days after the first administration of Lactosome or peptide-nanosheet was evaluated by ELISA (Figure 7). Lactosome strongly induced the anti-Lactosome IgM production as shown by using the Lactosome plate in ELISA (Figure 7a). On the other hand, peptide-nanosheet did not induce an anti-peptide-nanosheet IgM production as shown by using the peptide-nanosheet plate in ELISA (Figure 7b). The IgM production by Lactosome administration was also detected with the peptide-nanosheet plate in ELISA (Figure 7b), suggesting that the antigen part of Lactosome should be the poly(sarcosine) chain as poly(sarcosine) is the only common part of poly(Sar)₆₄-*block*-PLLA₃₀ and poly(Sar)₆₀-*block*-(L-Leu-Aib)₆.

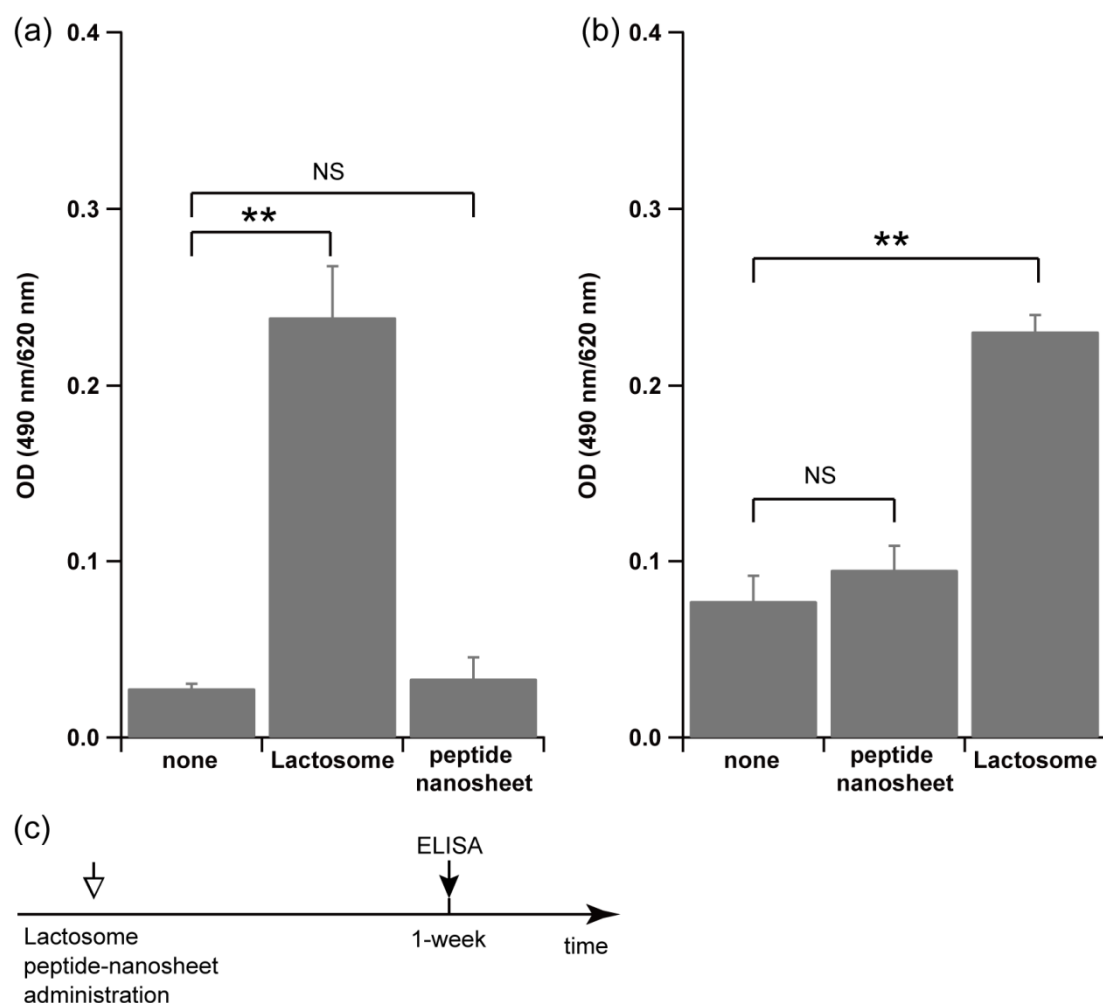


Figure 7. The value of anti-Lactosome IgM on the Lactosome plate (a) and the peptide-nanosheet plate (b). The serum was taken at 7 days after saline (none), Lactosome, and peptide-nanosheet administrations. Time schedule for the experiment (c). The white arrow indicates Lactosome and peptide-nanosheet administrations. The black arrow indicates blood collection (n=4). The p value: not significant (NS), * $p < 0.05$, ** $p < 0.01$.

ABC Phenomenon

In vivo distribution of Lactosome and peptide-nanosheet were analyzed by NIRF imaging by using their ICG labeled molecular assemblies (Figure 8). Table 2 summarizes the multiple-dose schedule of Lactosome, peptide-nanosheet, and their

combinations. Lactosome at the first dose accumulated in the tumor region as previously reported (Figure 8c) [9]. But Lactosome at the second dose was completely trapped in the liver due to the ABC phenomenon (Figure 8d). On the other hand, peptide-nanosheet at the first dose accumulated in the tumor region as well as liver (Figure 8f), indicating that the escape ability of peptide-nanosheet from the MPS was not so good as that of Lactosome. In sharp contrast to Lactosome, peptide-nanosheet at the second dose accumulated in the tumor region again (Figure 8g), indicating the absence of the ABC phenomenon in this case. This result is consistent with the ELISA showing no production of the anti-peptide-nanosheet IgM with peptide-nanosheet administration (Figure 7).

Table 2. The groups of ICG-Lactosome and ICG-peptide-nanosheet on multiple administrations

entry	1st dose		2nd dose	
	type	dose	type	dose
1	saline	-	ICG-Lactosome	5 mg/kg
2	Lactosome	5 mg/kg	ICG-Lactosome	5 mg/kg
3	peptide-nanosheet	5 mg/kg	ICG-Lactosome	5 mg/kg
4	saline	-	ICG-peptide-nanosheet	5 mg/kg
5	peptide-nanosheet	5 mg/kg	ICG-peptide-nanosheet	5 mg/kg
6	Lactosome	5 mg/kg	ICG-peptide-nanosheet	5 mg/kg

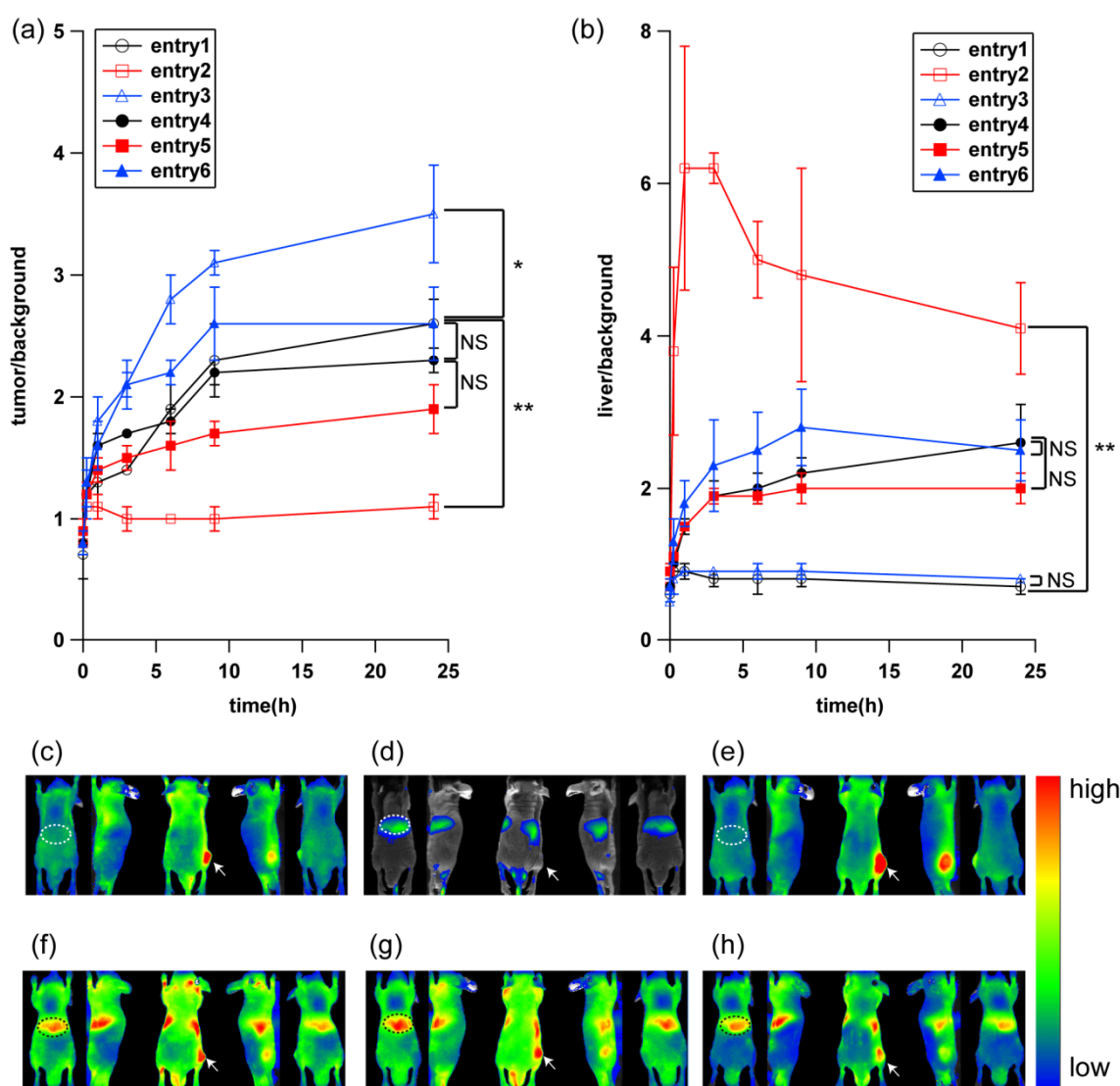


Figure 8. NIRF imaging of ICG-Lactosome and ICG-peptide-nanosheet with mice (n=3). NIRF images were taken at 15 min, 1 h, 3 h, 6 h, 9 h, and 24 h after the administration (Table 2). The ratio of ROI at tumor (a) and liver (b) against background. The p value: not significant (NS), * $p < 0.05$, ** $p < 0.01$ (Time point is 24 h). NIRF images were taken at 24h after administration (entry 1–6:c–h). The Fluorescence ranges were set to be from a maximum count of 7000 to a minimum count of 1000. The white arrows indicate the tumor sites. The dotted circles indicate liver.

The NIRF images at various times after the second dose were used for calculation of the accumulation amounts in tumor, liver, and background (the region of interest (ROI)) (Figure 8a, 8b). The ratios of tumor to background were lowered with the second

administration of Lactosome, but remained at a similar level with the second administration of peptide-nanosheet (Figure 8a). The reduction of the tumor/background ratio is explained by the drastic increase of liver capture of the second Lactosome administration, which was not observed after the second peptide-nanosheet administration (Figure 8b). *In vivo* distribution of ICG-peptide-nanosheet was the same irrespective whether the first dose was of saline, Lactosome, or peptide-nanosheet (Table 2 entry 4-6) and showed an accumulation in the tumor region (Figure 8f, 8g, and 8h), supporting the absence of ABC phenomenon with peptide-nanosheet. On the other hand, the accumulation in the tumor region of the first Lactosome administration (Figure 8c) was completely abolished with the second Lactosome administration (Figure 8b, 8d). These results are summarized in Table 3. The liver capture became significant only in the case of the second Lactosome administration after the first Lactosome administration. On the other hand, the liver capture of the second Lactosome administration at 7 days after the first peptide-nanosheet administration was significantly lower like that after the first Lactosome administration (Figure 8e, Table 3 entry 3). Peptide-nanosheet at the second administration after the first Lactosome administration accumulated in the tumor region as much as in liver (Figure 8h), but the liver capture of peptide-nanosheet was not changed even with the first Lactosome administration (Table 3 entry 6). All the data totally support the interpretation of the absence of the ABC phenomenon with peptide-nanosheet, and that the anti-Lactosome IgM could not recognize the peptide-nanosheet. Even though ICG-poly(Sar)₅-*block*-(D-Leu-Aib)₆ may affect the interaction between antibody and poly(Sar), poly(Sar)₅ is far shorter than the hydrophilic block of poly(Sar)₆₀-*block*-(L-Leu-Aib)₆ which should shield the ICG moiety to minimize the

ICG effect. In addition, the peptide-nanosheet with DY-776-(Leu-Aib)₆ did not induce the ABC phenomenon (Figure 9).

Table 3. Intensity ratio at liver against background of two carriers on multiple administrations

entry	(L/B)	(L/B)/(L/B) ₀ ^{a)}	ABC phenomenon
1	0.88 ± 0.12	-	-
2	6.17 ± 1.63**	7.00 ± 1.85	○
3	0.93 ± 0.01	1.05 ± 0.01	×
4	1.51 ± 0.14	-	-
5	1.48 ± 0.02	0.98 ± 0.01	×
6	1.80 ± 0.32	1.19 ± 0.21	×

^{a)} The (L/B)₀ value was determined to be 0.88 and 1.51 from NIRF images of mice 1 h after single administration of ICG-Lactosome and ICG-peptide-nanosheet, respectively.

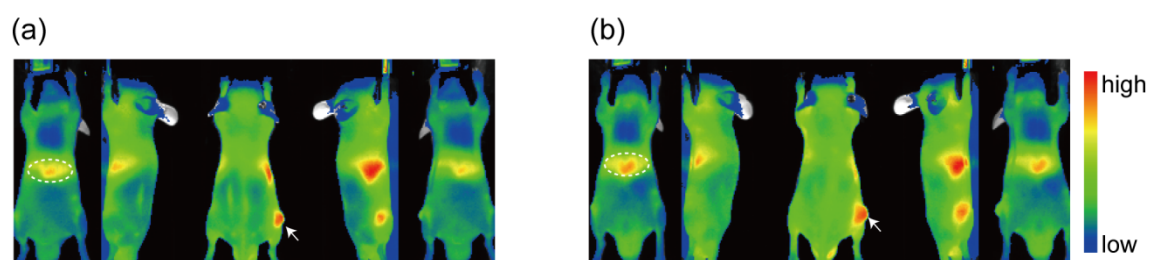


Figure 9. NIRF images of Dy776-peptide-nanosheet with mice (n=3). NIRF images were taken at 24h after administration. The images of First administration (a) and second administration at 7 days after first administration (b). The Fluorescence ranges were set to be from a maximum count of 10000 to a minimum count of 1000. The white arrows indicate the tumor sites. The dotted circles indicate liver.

Discussion

The nude mice of BALB/c nu/nu (T cell-deficient) were employed due to the *in vivo* NIRF imaging, and therefore the observations here are related with T-independent B-cell responses [12, 15]. T-independent lymphocytes are generally understood to respond to antigens having multiple repeating antigenic determinants [23]. In the case of the PEGylated liposome, the hydrophilic PEG chains covering the liposome surface have a repeated oxyethylene sequence, which may become the T-independent antigen to induce the ABC phenomenon (however, there is an ambiguity that a bare liposome could induce the ABC phenomenon [14]). When the surface densities of the PEG chains were increased up to 15 mol%, the ABC phenomenon was reported to be reduced [24, 25]. Ishida et al. pointed out that the surface density influenced the magnitude of the ABC phenomenon (the amount of IgM production), but it was difficult to suppress the ABC phenomenon just by increase of the surface density [25].

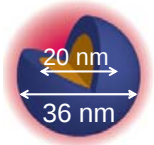
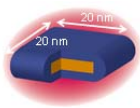
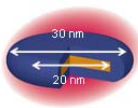
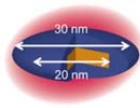
Peptide-nanosheet did not induce the ABC phenomenon. Indeed, the no IgM production was triggered. Furthermore, the anti-Lactosome IgM could not bind to peptide-nanosheet, which is deduced from the observation that the peptide-nanosheet of the second dose is accumulated in the tumor region after a first dose of Lactosome (Figure 8h). This observation is in clear contrast to the anti-Lactosome IgM binding to peptide-nanosheet on the ELISA plate (Figure 7b). This difference of the anti-Lactosome IgM binding to peptide-nanosheet can be explained by the different physical situation of poly(sarcosine) chains between the intact peptide-nanosheet and the ELISA plate. In the latter case, the nanosheet structure should be disassembled into a disordered structure due to the strong interaction with the ELISA plate, resulting in exposure of poly(sarcosine) chains to the anti-Lactosome IgM. On the other hand, poly(sarcosine) chains should be so crowded on the intact peptide-nanosheet that the

anti-Lactosome IgM could not get in the shell layer of peptide-nanosheet leading to no binding (Table 4).

The CAC differed between Lactosome and peptide-nanosheet, suggesting the possibility that the unimer (single polymer chain) concentration may be different to affect the immune response. However, in general, T-independent lymphocytes are considered to respond to antigens having multiple repeating antigenic determinants. It is therefore considered that the unimers should be far less effective as an antigen than the molecular assemblies.

Not only the anti-Lactosome IgM could not bind with poly(sarcosine) chains on peptide-nanosheet but also B-cells could not recognize poly(sarcosine) chains on peptide-nanosheet as a T-independent antigen. Accordingly, the Lactosome at the second dose accumulated in the tumor region after the first dose of peptide-nanosheet (Figure 8e).

Table 4. Properties of Lactosome and peptide-nanosheet

	Lactosome	peptide-nanosheet		
illustrations	 sphere	 nanosheet	 nanodisk	 ellipsoid
diameter/diagonal length (pdi)	36 nm (0.05) ^{a)}	30 nm (0.24) ^{a)}		
molecular weight of one nanoparticle	2023 kDa ^{b)}	1456 kDa ^{b), c)}		
molecular weight of amphiphilic block polymer	6872 Da	5541 Da		

number of amphiphilic block polymers in one nanoparticle	294	263		
multiangle light scattering (R_g/R_h) ^{d)}	0.749	1.067		
surface area outer	4069 nm ²	800 nm ^{2 e)}	1413 nm ^{2 e)}	1413 nm ^{2 e)}
poly(sarcosine) density outer	0.07 chains/nm ²	0.33 chains/nm ²	0.18 chains/nm ²	0.18 chains/nm ²
surface area corona	1256 nm ²	800 nm ^{2 e)}	800 nm ^{2 e)}	800 nm ^{2 e)}
poly(sarcosine) density corona	0.23 chains/nm ²	0.33 chains/nm ²	0.33 chains/nm ²	0.33 chains/nm ²

a) The hydrodynamic diameter obtained by DLS measurement.

b) SLS measurement.

c) The data were analyzed by the equipped software, which limited the available models to three types; the sphere ($R_g/R_h = 0.774$), the coil ($R_g/R_h = 0.816$), and the cylinder ($R_g/R_h = 1.732$) models. With using these models, MWs of the peptide-nanosheets were calculated to be 1,456 kDa, 1,464 kDa, and 1,631 kDa, respectively.

d) Sphere ~0.75, Sheet 1.1

e) The edge part is excepted.

This observation might be apparently contrast the results of the liposome having an increased PEG fraction [25]. The author speculates that even the liposome with the increased PEG fraction should be insufficient for shielding the hydrophobic core of PEGylated liposome because of the limited amount of the PEG-lipid. In contrast, the peptide-nanosheet could have a high density of hydrophilic poly(sarcosine) chains on the surface due to the regular molecular packing of helices in the interdigitated monolayers.

Several papers reported evasion from the ABC phenomenon [16, 26, 27]. Koide and others remarked that small-size particles (31.5 nm or less) can escape from the ABC

phenomenon. Shiraishi et al. suggested that the hydrophobic core of PEGylated micelles should be involved in the induction of the ABC phenomenon. According to their suggestion, the hydrophobic core of the peptide-nanosheet was completely shielded by the dense poly(sarcosine) chains to prevent the ABC phenomenon. However further studies are needed to elucidate the detailed mechanism of the ABC phenomenon.

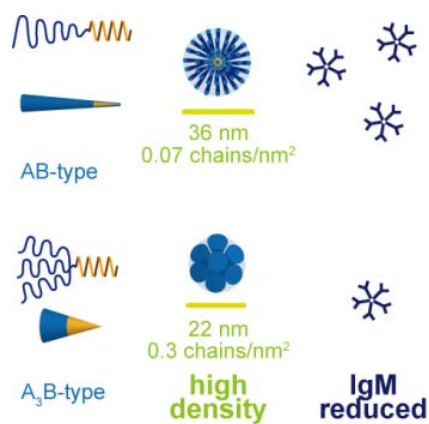
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Chapter 4

Factors Influencing *in Vivo* Disposition of Polymeric Micelles on Multiple Administrations



Introduction

Nanoparticles of polymeric micelles have highly potential for tumor imaging and therapy [1, 2]. These micelles accumulate in solid tumors through the enhanced permeability and retention (EPR) effect of high permeability across blood vessels and immature lymphatic drain systems out of the blood vessels [3, 4]. Indeed, polymeric micelles loading anti-tumor drugs have been widely studied for clinical applications [5]. Lactosome is a polymeric micelle, which is composed of amphiphilic polydepsipeptide with a hydrophilic poly(sarcosine) block and a hydrophobic poly(L-lactic acid) block (AB-type) [6]. The amphiphilic polydepsipeptide is biodegradable and biocompatible [7, 8]. Further, Lactosome is hardly recognized by the mononuclear phagocyte system (MPS) leading to a long lifetime in the bloodstream. On the basis of the long *in vivo* blood clearance time, the near-infrared fluorescent probe (NIRF)-labeled Lactosome was successful for imaging of various solid tumors. Especially, the orthotopic liver tumor was clearly imaged due to the low background of the healthy liver region showing highly selective accumulation of Lactosome in tumors [9]. Lactosome has been studied as a nanocarrier of probes for positron emission tomography (PET) imaging [10], tumor targeting [11], and intraoperative imaging [12].

However, the AB-type Lactosome has a drawback of alteration of *in vivo* disposition on multiple administrations [13]. At the first administration, the AB-type Lactosome showed a long lifetime in the bloodstream, but at the second administration, the AB-type Lactosome accumulated immediately in liver resulted in the rapid clearance from the bloodstream. The similar phenomenon has been already reported with PEGylated liposome and named as the accelerated blood clearance (ABC) phenomenon [14-16]. The ABC phenomenon was reported with other polymeric micelle using PEG

as a hydrophilic block.[17] It is therefore suggested that the ABC phenomenon may be commonly observed with nanoparticles. The ABC phenomenon has been clarified due to the production of anti-nanoparticle IgM upon the first administration of nanoparticles. The hydrophilic polymer chains surrounding on the nanoparticles are found to be T-independent antigens to induce the immune reaction [16]. In the case of Lactosome, poly(sarcosine) covering the surface was identified as an antigen by the competitive inhibition assay [13].

In order to suppress the immune reaction against nanoparticles, there have been several reports that were recently reviewed by Ishida et al. [18] So far, factors of nanoparticle size, different kinds of hydrophilic chains and core structure of the core-shell micelle, and the administration regimen have been discussed [19, 20]. The author is here to discuss another factor, that of the surface density of the hydrophilic polymer chains on polymeric micelle. This study is in the same line of the surface modification with polymer brushes in the field of polymer materials [21, 22]. When the surface modifying polymer chains become a high-density polymer brush state corresponding to a surface density of 0.3 chains/nm² or over, the surface shows a lubricating surface and also an anti-fouling property, etc. The author designed a novel amphiphilic polydepsipeptide having three hydrophilic branch chains connected to one hydrophobic poly(lactic acid) chain (A₃B-type). The A₃B-type polymeric micelle size went down to ca. 22 nm from the AB-type of ca. 36 nm [23]. However, the *in vivo* clearance time of the A₃B-type Lactosome became shorter than that of the AB-type Lactosome, which this factor was taken into consideration later. Further, the author found that the hydrodynamic diameters of these Lactosomes can be precisely adjustable with loading benzyloxycarbonyl-poly(L-lactic acid)₃₀ (Z-PLLA₃₀). Therefore, the author

could prepare the AB- and the A₃B-type Lactosomes having the same hydrodynamic diameter, which enabled them to evaluate separately the two factors of the nanoparticle size and the surface density of the poly(sarcosine) chains influencing the ABC phenomenon.

Materials and methods

Instruments

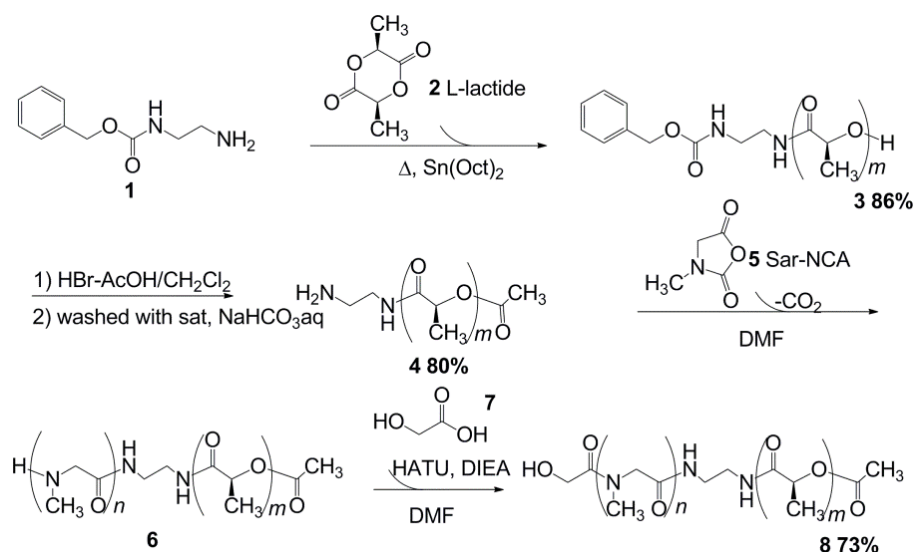
Hydrodynamic diameter and molecular weight of Lactosome were measured by dynamic light scattering (DLS) and static light scattering (SLS) on a Nano-ZS (Malvern, UK). Transmission electron microscope (TEM) was carried out on a JEM-3100FEF (JEOL Ltd., Japan). dn/dc was measured by DRM-3000 (Otsuka Electronics, Japan). ELISA assay for anti-Lactosome antibody was performed with using Multiscan Spectrum Microplate Photometer (Thermo Scientific, USA). Near-infrared fluorescence (NIRF) images were taken by Clairvivo OPT (Shimadzu Corp., Japan, *ex.* 785 nm/*em.* 845 nm). The pseudo images were constructed from the photon counts. The fluorescence intensity of solubilized pyrene and ICG was measured with using an RF-5300 Fluorescence Spectrophotometer (Shimadzu Corp. Japan). γ -Counter used was COBRA II (Packard Instrument, USA).

Synthesis of Lactosome (AB-type)

Lactosome (AB-type) was composed of amphiphilic block polydepsipeptide, poly(sarcosine)₆₄-*block*-poly(L-lactic acid)₃₀. Synthesis of the amphiphilic block poly-depsipeptide was performed as previously reported.[6, 9] Briefly, initiating from amino group of *N*-Cbz-1,2-ethylenediamine, hydrophobic poly(L-lactic acid) block was

polymerized using 15 equivalent of L-lactide monomer against the initiator. As catalyst for the polymerization, 0.2 wt% tin(IV) octanoate against L-lactide was added, and the polymerization was performed at 120 °C for 12 h. After construction of the PLLA block, Cbz group located at terminal end of the PLLA block was deprotected by 25% HBr-AcOH. The exposed terminal amino group was further used as initiator for polymerization of sarcosine *N*-carboxyanhydride (NCA). The monomer feed ratio against the macroinitiator was set to be 100 eq, and N-terminal end of poly(sarcosine) block was consecutively endcapped with glycolic acid. The scheme of AB-type polymer shows in Scheme 1. The unnecessary homo polymer of poly(Sar) was removed by dialysis after be formed particle. The degree of polymerization of the poly(Sar) and poly(L-lactic acid) blocks were determined to be 64 and 30 by ¹H NMR spectrum from the relative area of sarcosine *N*-CH₃ and lactic acid CH₃ peaks against terminal acetyl COCH₃ peak, re-spectively.

¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.32-1.70 (m, 90H, PLLA CCH₃), 2.10 (s, 3H, CH₃CO), 2.70-3.25 (m, 196H, Sar N-CH₃), 3.75-4.48 (m, 128H, Sar NCH₂CO), 5.02-5.28 (m, 30H, PLLA CH).



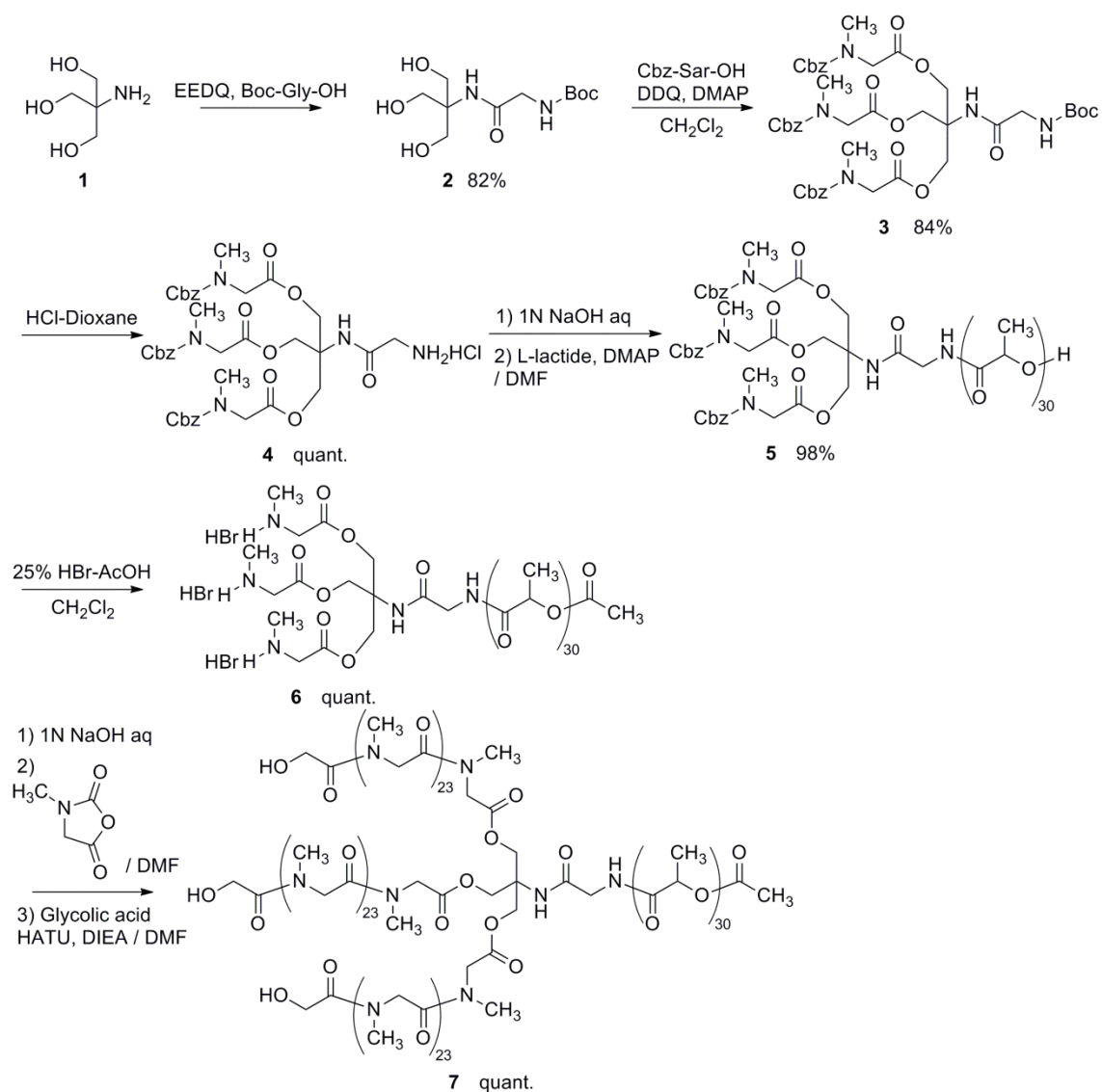
Scheme 1. Scheme for the synthesis of AB polymer.

Synthesis of Lactosome (A₃B-type)

Lactosome (A₃B-type) was composed of amphiphilic block polydepsipeptide, (poly(sarcosine)₂₃)₃-*block*-poly(L-lactic acid)₃₀. Synthetic details of the A₃B-type amphiphilic block polydepsipeptide was written in the previous paper [23]. Branched type initiator for the L-lactide polymerization was designed and synthesized from tris(hydroxymethyl)aminoethane. Initiating from one amino group in the branched-type initiator, L-lactide (15 eq.) was polymerized in DMF solution at 55 °C, using *N,N*-dimethyl-4-aminopyridine (DMAP) as a catalyst. After the polymerization, three Cbz groups, which were introduced as protective groups for the other three amino groups in the initiator, were removed by 25% HBr-AcOH. Initiating from the amino groups, three poly(sarcosine) blocks were polymerized using *N*-carboxyanhydride (NCA) as monomer. The monomer feed ratio against the macroinitiator was 70. Then, N-terminal end of the poly(sarcosine) blocks were sequentially endcapped by glycolic acid. The scheme of A₃B-type polymer shows in Scheme 2. The unnecessary homo polymer of poly(Sar) was removed by dialysis after be formed particle.

The degree of polymerization was determined by the same method with AB-type amphiphilic polydepsipeptide, and those of each poly(Sar) and poly(L-lactic acid) blocks were determined to be 23 (total 69) and 30, respectively.

¹H NMR (400 MHz, DMSO): 5.2 (30H, q, CHCH₃ of PLLA), 4.4-4.0 (6 + 144H, m, -C-(CH₂O-)₃, -CH₂- of Sar), 3.0-2.7 (209H, m, N-CH₃ of Sar × 3), 2.1 (3H, s, acetyl group located at polymer terminal end), 1.5 (99H, d, CHCH₃ of PLLA)



Scheme 2. Scheme for the synthesis of A₃B polymer.

Synthesis of ICG-PLLA

Indocyanin green (ICG) labeled PLLA 30-mer (ICG-PLLA₃₀) were performed as pre-viously reported [6, 9]. ICG-PLLA₃₀ was obtained by labeling the amino group in PLLA block with ICG-Sulfo-OSu.

Synthesis of ¹²⁵I-BzPLLA

To the solution of ^{125}I SIB (59.3 MBq) in acetonitrile (300 μL), $\text{H}_2\text{N-PLLA}_{30}$ (1.5 mg) in DMSO (150 μL) and triethylamine (0.20 μL) were added. The reaction mixture was stirred at 100 °C for 20 min, and then purified by gel permeation chromatography using Shodex Asahipak GF310-HQ column (7.5 $\times$ 300 mm) eluted with acetonitrile 0.5 mL/min to obtain ^{125}I -BzPLLA₃₀ (28 MBq) in acetonitrile.

Lactosome and ICG-Lactosome preparation (AB-type and A₃B-type)

The typical micelle preparation method is as follows. AB-type (1 mg) or A₃B-type (1 mg) polydepsipeptide in a test tube was dissolved in chloroform, and evaporated to form a polymer thin film on the test tube wall. Water was added to the tube so that the concentration of the amphiphilic polymer became 1 mg/ml. The dispersion was heated at 85 °C for 20 min. After freeze-drying the solution, saline (1 ml) was added, and the solution was filtered with using a syringe filter (pore size 0.20 μm) just before administration. For the preparation of indocyanine-labeled Lactosome (ICG-Lactosome), AB-type (1 mg) or A₃B-type (1 mg) polydepsipeptide and ICG-PLLA₃₀ (2 nmol) were dissolved together in chloroform, and the molecular assemblies were prepared similarly to lactosome. For NIRF imaging, the ICG amount injected to a mouse was set to be 10 nmol/kg. Lactosome loaded with benzyloxycarbonyl-poly(L-lactic acid)₃₀ (Z-PLLA₃₀) was prepared from a mixture of AB-type (1 mg) or A₃B-type (1 mg) polydepsipeptide and Z-PLLA₃₀ (0–150 mol% against each polydepsipeptide) by the similar procedures to other Lactosomes.

^{125}I -Lactosome preparation

AB-type (9.0 mg) or A₃B-type (18.0 mg) was mixed in an acetonitrile solution of

^{125}I -BzPLLA₃₀ (1.5 mg, 28 MBq). The solution was evaporated using Soltra mini (Biotech Lab, Japan) to form polymer film on a glass tube wall. Saline (1.0 mL) was added to the test tube, and the dispersion was sonicated at 85 °C for 2 min to form ^{125}I -Lactosome.

TEM images

A drop of dispersion was mounted on a carboncoated Cu grid and stained negatively with 2% phosphor tungstic acid, followed by suction of the excess fluid with a filter paper. TEM images were obtained at an accelerating voltage of 100 keV.

SLS measurement

Molecular weights of Lactosome (AB-type and A₃B-type) were determined by SLS measurements. Saline was used as a solvent and for a dilution of the stock solution (1.0 mg/ml). Sphere model ($R_g = 0.774 \times R_h$), was applied for a shape correction.

Critical association concentrations (CAC)

To estimate CAC values for Lactosome, the method was used based on the solubilization of waterinsoluble fluorescent dye, pyrene, in micelles.[24] In case of Lactosome, 100 μl of pyrene solution in chloroform (10 mg/ml) and 200 μl of serial dilutions of lactosome in test tubes were dried under vacuum. Then, 2 ml of saline was added to each tube with the dried pyrene. The dispersion was heated at 85 °C for 20 min and the solution was filtered with using a syringe filter (pore size: 0.20 μm) to remove the non-solubilized pyrene. Final concentration of Lactosome solutions were $1 \times 10^5 - 1 \times 10^9$ M. The fluorescence intensity of solubilized pyrene was measured at an

excitation wavelength of 339 nm and emission wavelength of 390 nm using an RF-5300 Fluorescence Spectrophotometer.

Serum preparation

Lactosome (AB-type, A₃B-type, AB-type + Z-PLLA₃₀, and A₃B-type + Z-PLLA₃₀ : 100 µg/body) was injected to 8-week-old BALB/c nu/nu mice which were purchased from Japan SLC, Inc. (Japan) (n = 3 per group). Mice blood were collected in Microstrainer[®] tube (BD Corp., USA) from inferior vena cava under anesthesia condition at 1 week after the administration of Lactosome. The serum was obtained by centrifugation (10 min, 3000 rpm) and saved at -40 °C.

For triplicate administration experiments, Lactosome (A₃B-type: 100 µg/body) was injected to 8-week-old BALB/c mice which were purchased from Shimizu Laboratory Supplies Co., Ltd (Japan) (n = 3 per group). The A₃B-type Lactosomes were injected to the mice at 4 h, and 8 h after the first administration. Sera were collected similarly to the method described above.

ELISA

Lactosome (AB-type: 0.5 µg/well) in 50 µL distilled water was added to 96-well plates and air dried completely for 1 day. To each well, 150 µL of blocking buffer (2% BSA/PBS) was added and incubated for 2 h. The wells were washed four times with washing buffer (PBS-T: 0.05% Tween 20 in PBS). The serum after appropriate dilution was added to the wells and incubated for 2 h. After the incubation, the wells were washed four times by PBS-T. Peroxidase conjugated goat-anti-mouse IgM in 0.1% BSA/PBS (50 µl, Southern Biotech, USA) was added as the secondary antibody. After

incubation for 2 h, the wells were washed again four times with PBS-T. o-Phenylenediamine (0.5 mg/ml, Sigma, St. Louis, MO), which was dissolved in 0.0003% H₂O₂-0.1 M citrate phosphate buffer (pH 5.0), was added to the microplate. At 10 minutes after the o-phenylenediamine addition, optical density (OD) was determined from UV absorbance at 490 nm/ reference at 620 nm. The actual values of anti-Lactosome IgM upon AB-type Lactosome administration were ca 0.51, 0.45, 0.51 and 0.44 in Figure 5, Figure 7, Figure 8a and Figure 8b, respectively.

Cell culture

Pancreatic carcinoma (SUIT-2/pEF/LUC) cell line was maintained at 37 °C with 5% FBS (Nacalai Tesque, Inc., Kyoto, Japan) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen Corp. USA) supplemented with GlutaMAX™ -I Supplement (2 mmol/l, Gibco, Invitrogen Corp., USA), Plasmocin™ prophylactic (5 µg/ml, Nacalai Tesque, Inc., Kyoto, Japan), penicillin (100 U/ml), and streptomycin (100 µg/ml).

Preparation of tumor-bearing mice

SUIT-2/pEF/LUC cells (5×10^5 cells) were dissolved in phosphate-buffered saline (PBS, 20 µl), and subcutaneously inoculated into right femoral region of 7-week-old BALB/c nu/nu mice (n = 3 per group). ICG-Lactosome (AB-type or A₃B-type polydepsipeptide of 100 µg with ICG of 10 nmol/kg) was injected to the mice after 2 weeks from the tumor transplantation. NIRF images were taken at 1.5, 3, 4.5, 6, 9, 24, 48, and 168 h after the administration. At 7 days after first administration, ICG-Lactosome (AB-type and A₃B-type polydepsipeptide of 100 µg with ICG amount

of 10 nmol/kg) was injected again. NIRF images were taken at 15 min, 6 h and 24 h after second administration. During the imaging period, mice were held on the imaging stage under anesthetized condition with 2.5% of isoflurane gas in an air flow (1.5 l/min).

Biodistribution with ^{125}I -Lactosome preparation

SUIT-2/pEF/luc cells (1×10^6 cells) were dissolved in phosphate-buffered saline (PBS, 40 μL), and subcutaneously inoculated into right femoral region of 7-week-old male nude mice (n=4 per group). ^{125}I Lactosome (AB-type: 436 kBq / A₃B-type: 30 kBq/body) was injected to the mice. The mice were dissected at 2 h, 24 h, 48 h, 72 h 96 h and 168 h from the injection and the collected bloods were counted with using γ -counter.

Fluorescence intensity of ICG-Lactosome

ICG-Lactosome (AB-type, A₃B-type: 100 μg /body) was injected to 8-week-old BALB/c mice which were purchased from Shimizu Laboratory Supplies Co., Ltd (Japan) (n = 3 per group). In case of A₃B triplicate administration group, ICG-Lactosome (A₃B-type) was injected to the mice at 4 h, and 8 h after first administration. Sera were collected at 15 min, 2 h, 6 h, 12 h, and 24 h after first administration. The fluorescence intensity of ICG was measured at an excitation wavelength of 730 nm and emission wavelength of 812 nm using an RF-5300 Fluorescence Spectrophotometer.

Statistical analysis

All results are expressed as mean $\pm$ SD. Differences between groups in IgM production and NIRF imaging studies were assessed by the t test for independent samples. P values of p value < 0.05 and p value < 0.01 are considered statistically significant and designated by * and **, respectively.

Ethics

All of her *in vivo* animal experiments were approved by the Animal Research Committee of Kyoto University. Animals were treated humanely.

Results

Properties of AB-type and A₃B-type Lactosome

The AB-type Lactosome is composed of poly(sarcosine)₆₄-*block*-poly(L-lactic acid)₃₀ and the hydrodynamic diameter was ca. 36 nm (Figure 1a and 1c). However, the A₃B-type Lactosome prepared from (poly(sarcosine)₂₃)₃-*block*-poly(L-lactic acid)₃₀ shows a smaller hydrodynamic diameter of ca. 22 nm (Figure 1b and 1d).

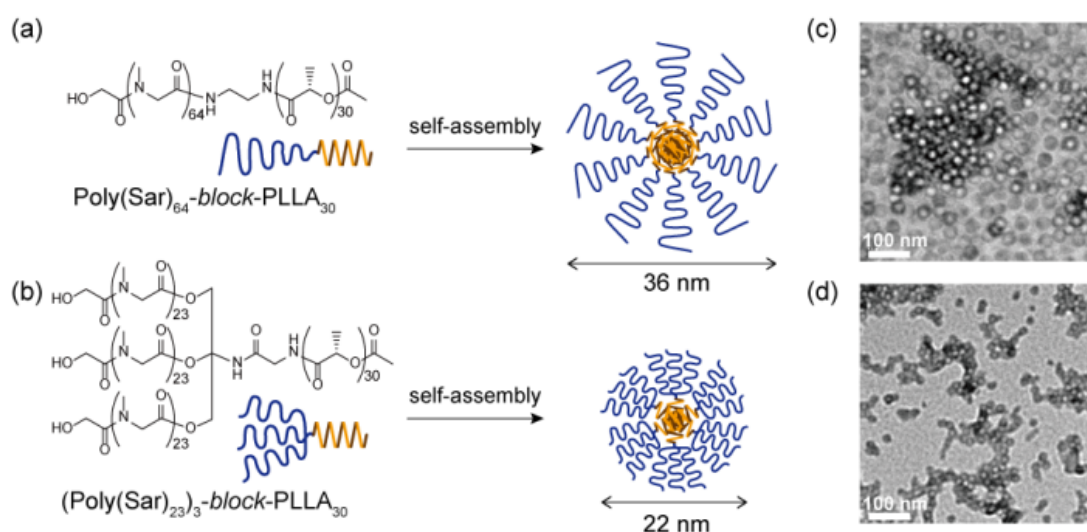


Figure 1. Schematic presentation of the AB-type and A₃B-type polymeric micelles (a, b). TEM images of AB-type(c) and A₃B- type (d).

TEM images also showed the spherical polymeric micelles of the AB-type and the A₃B-type polymers having diameters of ca. 34 and 21 nm, respectively [23]. The magnified views are shown in Figure 2.

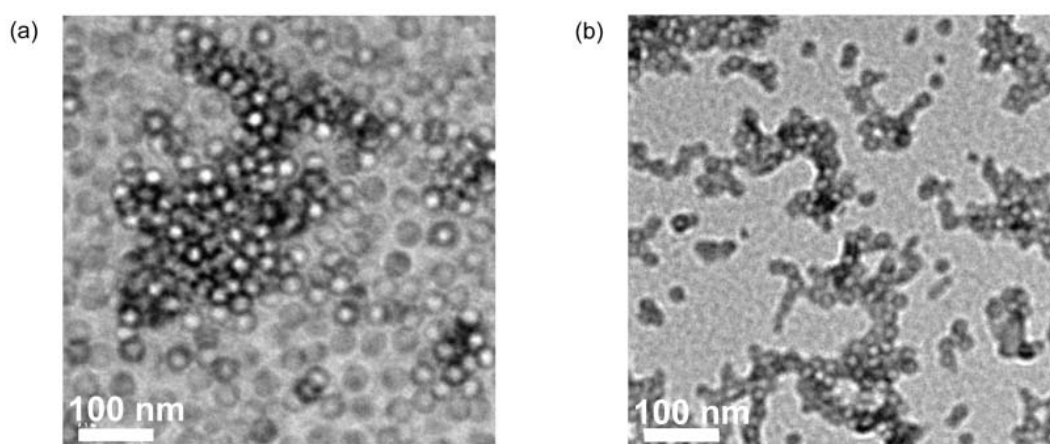


Figure 2. The TEM images of the AB-type(a) and A₃B- type (b). The scale bar indicates 100 nm.

Accordingly, the surface density of poly(sarcosine) chains on the A₃B-type Lactosome became 4 times higher than that on the AB-type Lactosome (Table 1). However, the half-life time in a bloodstream of the A₃B-type Lactosome was found to be 4.3 h, which was much shorter than the AB-type Lactosome of 17.2 h, suggesting that the *in vivo* stability of the A₃B-type Lactosome was impaired probably by accommodating so many hydrophilic chains into the shell layer of the polymeric micelle. The critical association concentrations (CAC) of the AB-Lactosome and the A₃B-Lactosome were found to be as low as 1.3×10^{-8} M and 1.9×10^{-9} M, respectively (on the basis of the general evaluation method using a fluorescent probe of pyrene (Figure 4). Although the *in vivo* stability of the A₃B-type Lactosome was impaired, the

self-assembling propensity remains high.

Table 1. Characterizations of the AB-type and A₃B-type lactotomes.

	hydrodynamic diameter (nm)*a	PDI	poly(Sar) density (chain/nm ²)*b	blood half-time (h)*c
AB-type	36.0 ± 0.5	0.05	0.07	17.2
A ₃ B-type	22.3 ± 0.3	0.05	0.30	4.3

*a The hydrodynamic diameter obtained by DLS measurement (n = 3).

*b The details are shown in Table 2.

*c The decays of the Lactosomes in the bloodstream are shown in Figure 3.

Table 2. Properties of AB-type and A₃B-type

	AB-type	A ₃ B-type
diameter/diagonal length (pdi)	36 nm (0.05) *a	22 nm (0.05) *a
molecular weight of one nanoparticle	1895 kDa *b	1064 kDa *b
molecular weight of amphiphilic block polymer	6872 Da	7299 Da
number of amphiphilic block polymers in one nanoparticle	280	150 (sarcosine chain 450)
surface area	4069 nm ²	1520 nm ²
poly(sarcosine) density	0.07 chain/nm ²	0.30 chain/nm ²

*a The hydrodynamic diameter obtained by DLS measurement.

*b SLS measurement.

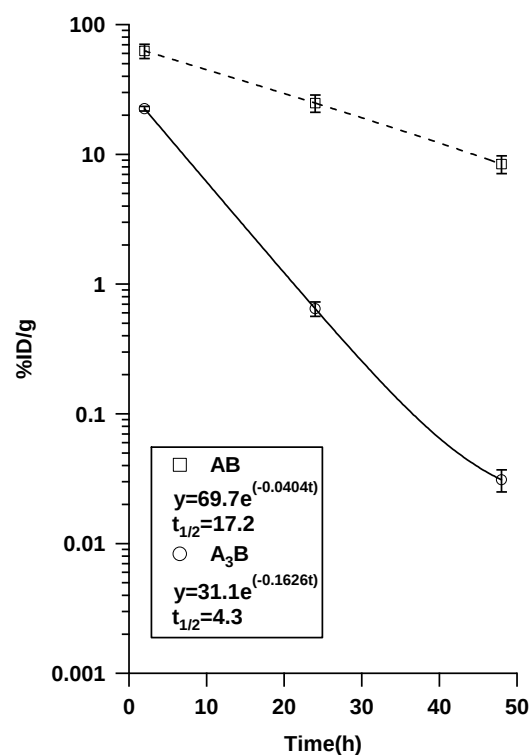
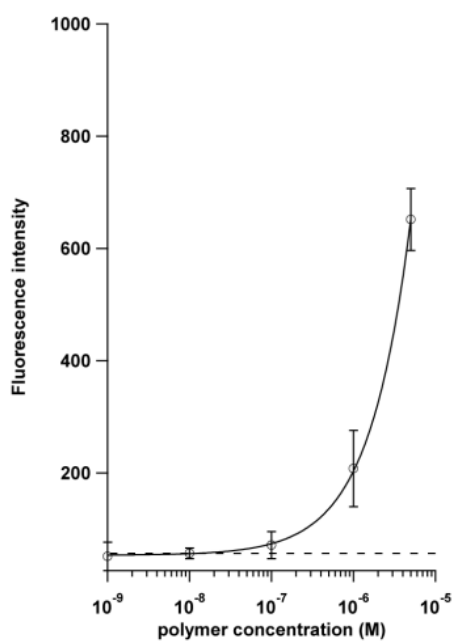


Figure 3. Time course of the residual amount of ^{125}I -Lactosome in plasma. Biodistribution data was fitted by IGOR Pro 4.09A, and the blood half-life times of ^{125}I -Lactosome (AB-type and A_3B -type) were calculated by the single component decay.

(a)



(b)

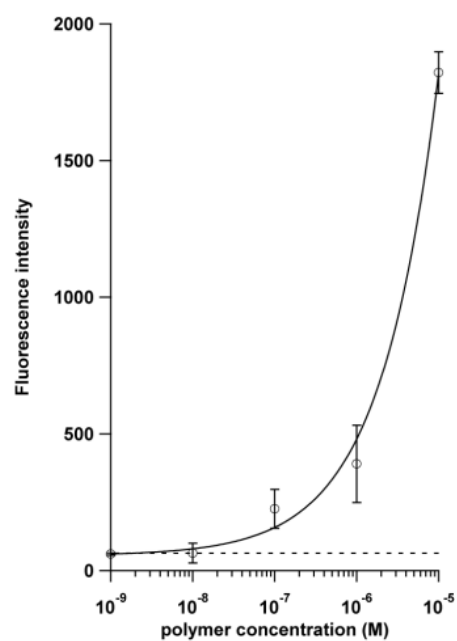


Figure 4. CAC determinations of the AB-type Lactosome (a) and the A₃B-type Lactosome (b).

The ABC Phenomenon

Production of anti-Lactosome-IgM at 7 days after the AB-type or A₃B-type Lactosome administration was evaluated by ELISA (Figure 5a). The production of anti-Lactosome-IgM was lower with the A₃B-type Lactosome than the AB-type Lactosome, indicating the lower immunogenicity of the A₃B-type Lactosome than the AB-type lactosome.

In vivo dispositions of the Lactosomes were analyzed by NIRF imaging. The AB-type Lactosome at the first administration spread over the whole body and gradually accumulated in the tumor region due to the EPR effect (Figure 5b). However the AB-type Lactosome on the second administration at 7 days after the first administration accumulated immediately in liver as previously reported (Figure 5d) [13]. However, the A₃B-type Lactosome spread over the whole body to accumulate in the tumor region at either the first or the second administration (Figure 5c and 5e).

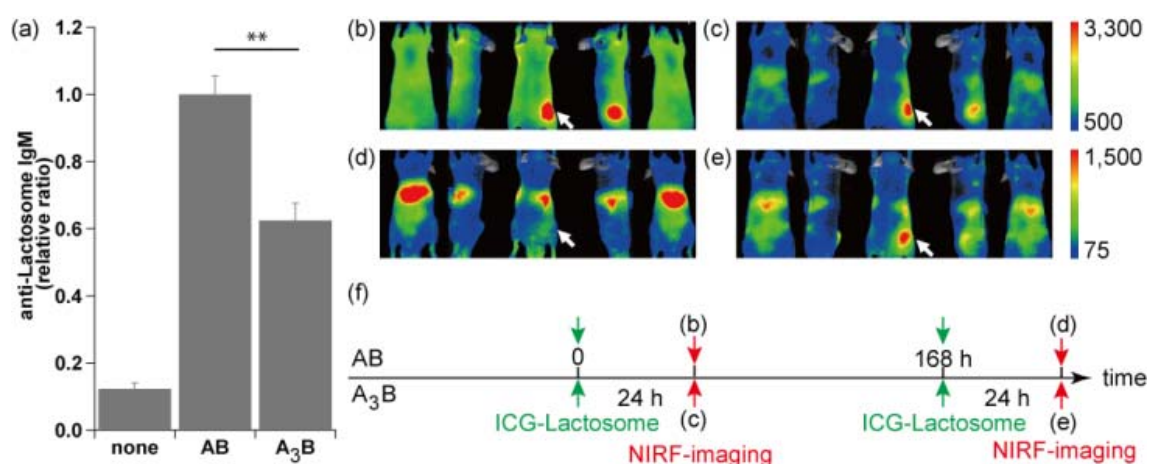


Figure 5. IgM productions upon the administrations of the AB-type and the A₃B-type Lactosomes (a) (n = 3 per group). Anti-Lactosome IgM productions are normalized by

taking the anti-Lactosome IgM production with the AB-type Lactosome as a reference, 1.0. Pharmacokinetic changes (NIRF images) upon multiple doses of the AB-type (b, d) and the A₃B-type (c, e) Lactosomes. The images (d, e) were taken at 7 days af-ter the first administration of the AB-type and A₃B-type Lactosomes. The time schedule is shown in panel f.

Region-of-interest (ROI) analyses also support that the accumulation amount of the A₃B-type Lactosome in the tumor region (the right femoral region, Figure 5e) was 3–5 times higher than that at the healthy left femoral region at the second administration (Figure 6a). The accumulation ratios of liver against background by ROI analyses also show that the liver accumulation of the A₃B-type Lactosome was significantly low just after the second administration followed by a moderate increase, while the AB-type Lactosome accumulated strongly in liver even right after the second administration (Figure 6b).

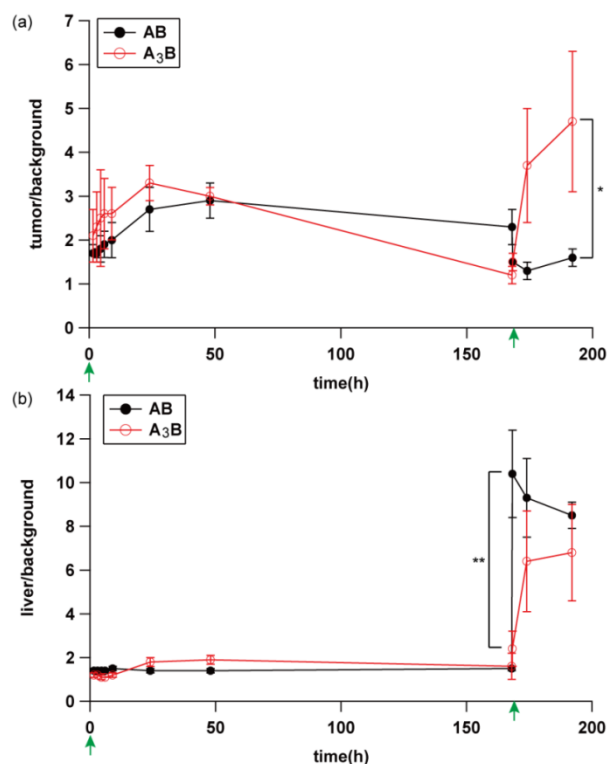


Figure 6. Time profiles of the ratio of ROI at tumor against background (tumor/background) (a). The time profiles of the ratio of ROI at liver against

background (liver/background) (b). The p value: not significant (NS), * $p < 0.05$, ** $p < 0.01$.

The AB-type and the A₃B-type Lactosomes showed the different life times in bloodstream. In order to look into the effect of this difference on the ABC phenomenon, the author maintained the concentration of the A₃B-type Lactosome in blood by the repeated administrations at 4 h intervals (0, 4, and 8 h). With the repeated administrations, the concentration in blood was kept high (Figure 7a), but the production of anti-Lactosome IgM was found to be remained as low as the single administration of the A₃B-type Lactosome (Figure 7b).

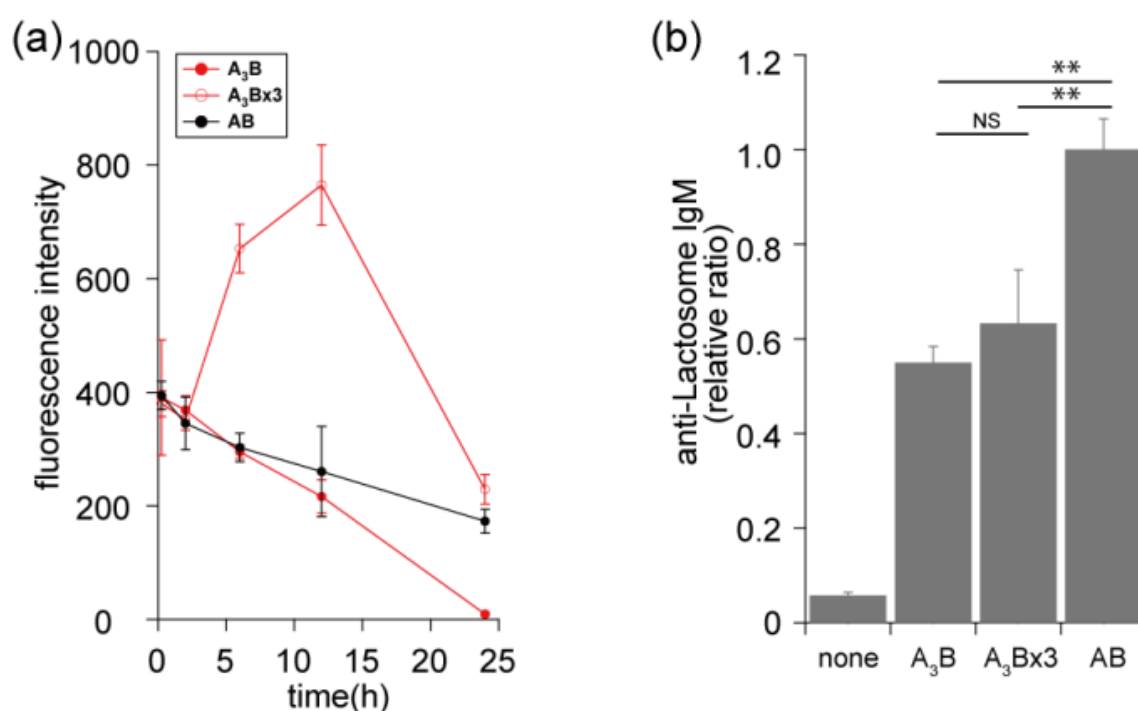


Figure 7. Time profiles of the Lactosome concentrations in blood using the ICG-labeled AB-type and the A₃B-type Lactosomes (a). The productions of the anti-Lactosome IgM with the single dose, the triplicate doses of the A₃B-type Lactosome, and the AB-type Lactosome (b). The anti-Lactosome IgM productions are normalized by taking that with the AB-type Lactosome as 1.0. A₃B×3 represents the triplicate doses of the A₃B-type Lactosome at 4 hour intervals.

The nanoparticle size of the Lactosomes can be increased by loading Z-PLLA₃₀ in the hydrophobic core of the polymeric micelle [23]. The A₃B-type Lactosome increased the hydrodynamic diameter gradually with loading Z-PLLA₃₀ up to 39 nm from 22 nm (PDI = 0.038–0.070) (Figure 8a). Similarly, the AB-type Lactosome is variable in size from 38 nm to 52 nm (PDI = 0.052–0.123) with loading Z-PLLA₃₀ (Figure 8b). The production amounts of anti-Lactosome-IgM were evaluated by ELISA with using these Lactosomes of different nanosizes (Figure 8a and 8b). It is clearly shown that the anti-A₃B-type-Lactosome-IgM productions were lower than the anti-AB-type-Lactosome-IgM production (Figure 8a). However, the size dependences of the anti-Lactosome-IgM productions were not obvious within the each group of the A₃B-type and the AB-type Lactosomes (Figure 8a and 8b).

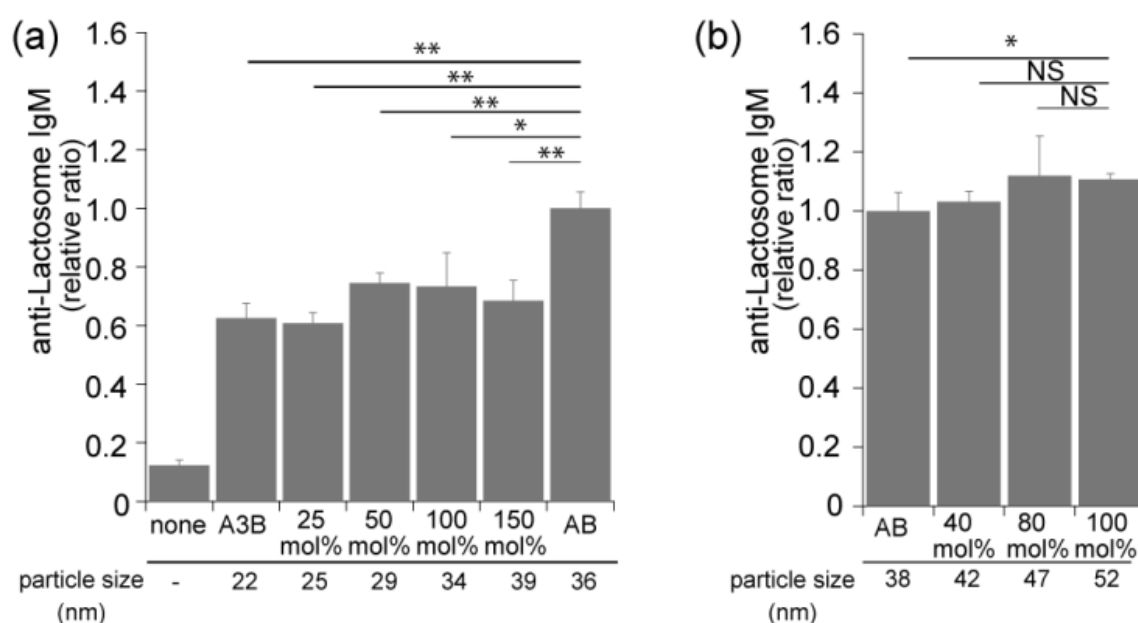


Figure 8. The productions of anti-Lactosome IgM with Lactosomes varying the particle sizes. The A₃B-type Lactosomes increased the size from 22 nm to 39 nm with the addition of Z-PLLA₃₀ from 0 mol% to 150 mol% (a). The AB-type Lactosomes increased the size from 38 nm to 52 nm with the addition of Z-PLLA₃₀ from 0 mol% to

100 mol% (b). The anti-Lactosome IgM productions are normalized by taking that with the AB-type Lactosome as 1.0. The p value: not significant (NS), * $p < 0.05$, ** $p < 0.01$

However, a difference of anti-Lactosome-IgM productions between two groups of the A₃B-type and the AB-type Lactosomes was significant in the whole range of micelle sizes examined here (Figure 9). Notably, when the anti-Lactosome-IgM amounts were compared between the A₃B-type Lactosome and the AB-type Lactosome of the similar size of ca. 40 nm, the former became lower than the latter, suggesting that the size of the polymeric micelle should not be critical for the anti-Lactosome-IgM production.

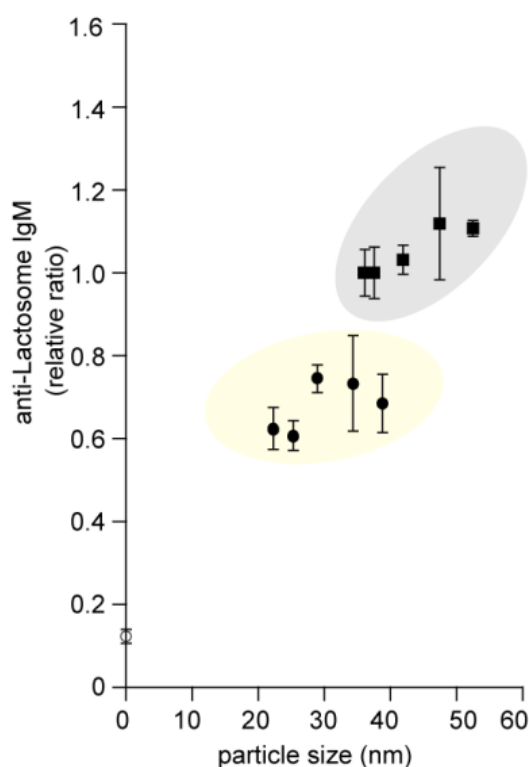


Figure 9. Size dependences of the anti-Lactosome-IgM productions with administrations of the AB-type and the A₃B-type Lactosomes. The graph is reconstructed from data of Figure 8. Anti-Lactosome IgM productions are normalized by taking that with the AB-type Lactosome as 1.0. (n = 3 per group).

In regard to the ABC phenomenon of the AB-type Lactosome, the level of

anti-Lactosome IgM started to increase from 3 days after the first administration up to a saturation level at 5 days and later [13]. The AB-type Lactosome on the following administration at later than 5 days from the first administration accumulated immediately in liver due to the recognition by anti-Lactosome IgM, which was confirmed by NIRF imaging using the NIRF-labeled Lactosome. In the present study, we have evaluated the A₃B-type Lactosome on the ABC phenomenon. The A₃B-type Lactosome is composed of the same hydrophilic and hydrophobic blocks as the AB-type Lactosome, but the hydrophilic block number in one molecule of the A₃B-type Lactosome is three times higher than the AB-type Lactosome, which raises the local density of poly(sarcosine) chains on the surface. Notably, the A₃B-type Lactosome showed the attenuated ABC phenomenon compared with the AB-type Lactosome as shown by NIRF imaging and the anti-Lactosome IgM production.

As far as the attenuation of the ABC phenomenon is concerned, there have been already several reports; 1) the PEGylated polymeric micelles by decreasing the particle size less than 30 nm [25], 2) coating of nanoparticles with poly(*N*-vinyl-2-pyrrolidone) [26], 3) modification of liposome surface with a polyglycerol-derived lipid [18], 4) a PEG-shell-possessing polymeric micelle with hydrophilic inner core (PEG-P(Lys-DOTA-Gd) [27], and 5) changing the administration regimen of high doses and the prolongation of a time interval between injections [18]. Actually, the A₃B-type Lactosome with a 22 nm diameter attenuated the ABC phenomenon, which may be categorized into the group 1) described above. However, there are some controversies here against the interpretation that the particle size is crucial for the ABC phenomenon. The A₃B-type Lactosome with loading Z-PLLA₃₀ to increase the particle size up to 40 nm still showed the suppression effect on the anti-Lactosome IgM production as much

as the A₃B-type Lactosome of 22 nm (Figure 8a). Even though the two kinds of Lactosome have the similar particle size of 40 nm, the A₃B-type Lactosome suppressed the anti-Lactosome IgM production but the AB-type Lactosome triggered the IgM production significantly (Figure 9). Small particle size should be favorable for suppression of the anti-Lactosome IgM production, but the size is not simply all the trigger for the ABC phenomenon.

The author can now raise two factors for the ABC phenomenon. One is the blood clearance time and the other is the local density defined by the micelle size, the chain length of the hydrophilic blocks, and the molecular shape. The half-life time in the bloodstream of the A₃B-type Lactosome was 4.3 h, which is considerably shorter than the AB-type lactosome of 17.2 h (Table 1). The shorter life time of nanoparticles should result in less chance to trigger the immune system. Indeed, Ishihara et al. reported that the ABC phenomenon was not induced upon the repeated injections of the PVP-coated nanoparticles which have a short life time in the bloodstream [26]. Here, the triplicate doses of the A₃B-type Lactosome at 4 h intervals with keeping the high concentration in blood did not increase the IgM production from that with the single dose. The shorter half-time is therefore not the major factor for the reduction of the ABC phenomenon (Figure 7).

The surface density of the hydrophilic blocks differs significantly between the two kinds of Lactosomes. The surface density reaches to 0.30 chain/nm² for the A₃B-type Lactosome, which is 4 times higher than 0.07 chain/nm² for the AB-type Lactosome (Table 1). Even though the high surface density should decrease with incorporation of Z-PLLA₃₀ into the A₃B-type Lactosomes, the three poly(sarcosine) chains are bundled at the C-terminal resulting in locally a high density of poly(sarcosine) chains (the

molecular shape effect). However, in the case of the AB-type micelle, a long poly(sarcosine) block of 64-mer should generate a loose space at the peripheral region of the shell layer of the polymeric micelle. The short poly(sarcosine) block of 23-mer employed for the A₃B-type Lactosome might be another reason for raise the local high density of poly(sarcosine) chains because the shorter chains can avoid the curvature effect.

Then, the author currently has been trying to study the chain length effect on the ABC phenomenon. Two other A₃B-type Lactosomes having longer poly(sarcosine) chains of 33-mer and 50-mer, were prepared. These A₃B-type Lactosomes also formed polymeric micelles with a diameter less than 30 nm. Interestingly, these Lactosomes did not show the ABC phenomenon either because the *in vivo* imaging showed that the liver capture right after the second administration was not significant (Figure 10). Further, a long poly(sarcosine) block of 60-mer did not induce the ABC phenomenon when the poly(sarcosine) block was expressed densely at the surface of peptide-nanosheets prepared by self-assembling of an amphiphilic polypeptide, poly(sarcosine)₆₀-*block*-(L-Leu-Aib)₆ [28]. All these results support the interpretation that the high local density of poly(sarcosine) chains rather than the poly(sarcosine) chain length should be a major cause for suppression of the ABC phenomenon. It is considered that the locally packed poly(sarcosine) chains may exclude other molecules from penetration into the surface region, and prevent the polymeric micelle from interaction with B-cell receptors.

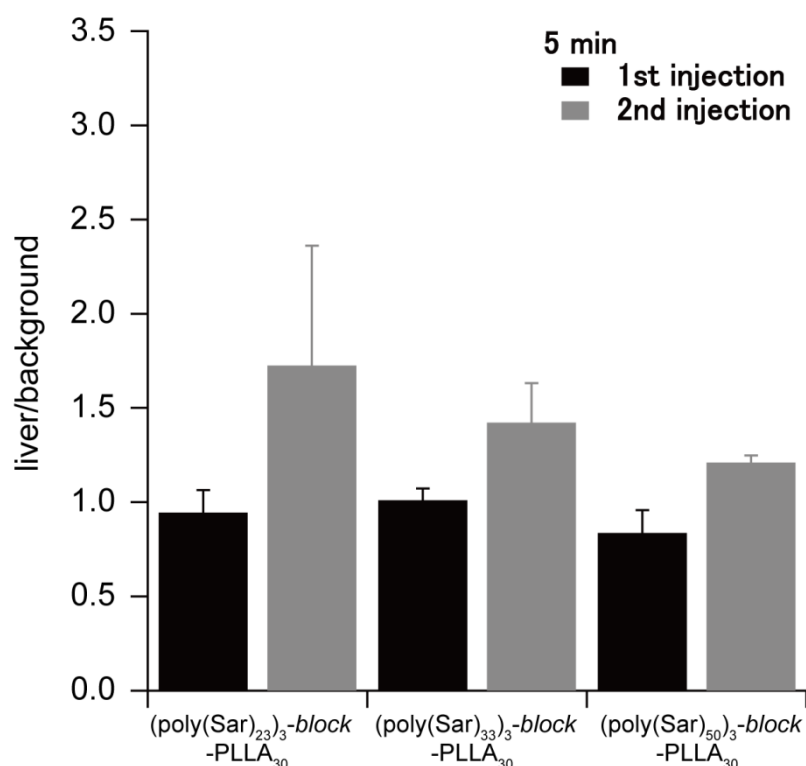


Figure 10. Influence of sarcosine chains. The ratio of ROI at liver against background (liver/background) at 5 min after the A₃B-type Lactosomes incorporated ICG-PDLA. The A₃B-type Lactosomes composed of (poly(Sar)₂₃)₃-block-PLLA₃₀, (poly(Sar)₃₃)₃-block-PLLA₃₀, and (poly(Sar)₅₀)₃-block-PLLA₃₀, respectively.

One drawback of the A₃B-type Lactosome is, however, the decrease of the tumor accumulation due to the shorter life time in the bloodstream (Figure 11). The author is now finding out the optimal chain length of poly(sarcosine) and composition of Lactosome to stabilize the polymeric micelle structure to improve the accumulation in tumor with the attenuated ABC phenomenon.

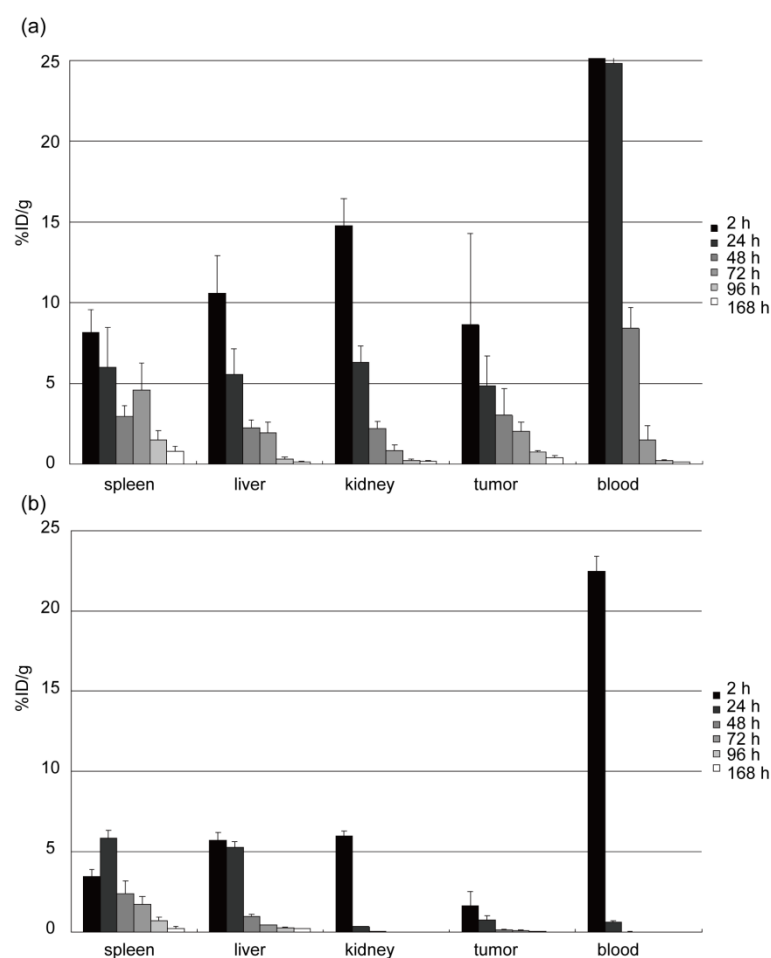


Figure 11. Biodistribution of Iodine-131 labeled particles. The AB-type Lactosome (a) and the A₃B-type Lactosome (b).

Applications of nanoparticles for diagnosis and therapy are currently appealing. However, the ABC phenomenon with nanoparticles should be solved out before putting the nanoparticles on clinical researches. So far, there have been several ways reported to attenuate the ABC phenomenon, but the detailed mechanism remains to be solved. Here, the local density of poly(sarcosine) chains of Lactosomes was shown to influence the ABC phenomenon. The author think that the higher local density of the surrounding hydrophilic polymer chains should be related with prevention of the interaction between micelles and B cell receptors, but further studies are needed to make it clear.

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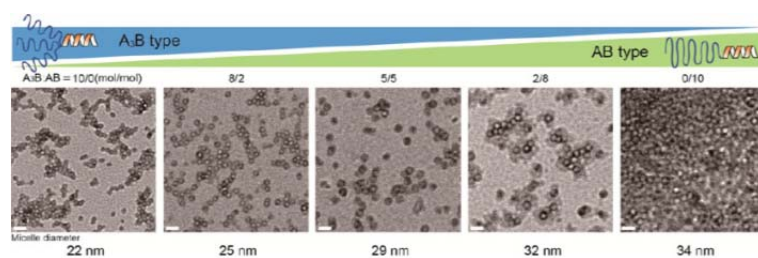
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Chapter 5

Size Control of Core–Shell-type Polymeric Micelle with a Nanometer Precision



Introduction

Recently, amphiphilic polymer-based nanomaterials have been attracting much attention, for example, as carriers of the diagnostic and the therapeutic agents, which are anticipated to be applied for theranostics [1-8]. These nanoparticles have advantages of high *in vivo* stability due to their relatively strong intermolecular hydrophobic interactions and selective delivery of encapsulated drugs and/or imaging agents to the targeted region under a controlled way. Especially tumor targeted nanoparticles have been frequently reported because of the enhanced permeation and retention (EPR) effect, which is a phenomenon that nanoparticles in a certain size range have a strong tendency to accumulate and retain in the tumor region [9-11]. In tumor tissues, submicrometer sized defects exist on the vascular wall due to the rapid angiogenesis, allowing permeation of macromolecules through the wall, and further the lymph system around tumor is not well developed enough to exclude foreign compounds of nanoparticles.

For utilization of nanoparticles to medicinal purposes, especially in tumor imaging, morphological control of the nanoparticles is considered to be essential in terms of their biodistribution. Especially, the nanoparticle size is critical for the leakage from blood vessels and the remaining property in the tumor regions [12, 13]. The dimension control of polymeric assemblies has been so far achieved primarily by changing the molecular packing parameter of the hydrophilic and hydrophobic balance of amphiphilic block polymers via variation of components and/or block lengths [14-16]. However, as far as polymeric micelles are concerned, the constituent amphiphilic polymers are considered to take random structure in the assemblies, resulting in relatively a wide size distribution of the molecular assembly. The dimensions of molecular assemblies, therefore, cannot be precisely predictable.

Association of small molecules in water can be driven generally by hydrophobic interaction, electrostatic interaction, hydrogen bonding, and π - π stacking, etc., to generate molecular assemblies with a defined morphology and dimensions [17-19]. On the other hand, amphiphilic polymers usually result in a wide size distribution of molecular assemblies due to the random-coil conformation of the constituent polymers. However, the semicrystalline block copolymers, which have at least one crystallizable block in the sequence, have been pointed out to be effective to control the dimensions of the molecular assemblies, because the crystallizable blocks could form a nanoconfined environment leading to the regular polymer-chain alignment in the molecular assemblies [20].

The author has focused her attention on helical blocks as the crystallizable block to generate well-defined molecular assemblies. So far, the author has prepared molecular assemblies from amphiphilic block polypeptides and polydepsipeptides where the hydrophobic blocks of polypeptide and poly(lactic acid) adopted helical structures [1, 2, 21-24]. Indeed, the authors successfully prepared polymeric micelles, nanotubes, and vesicles with homogeneous dimensions. Further, combination of righthanded and left-handed helices could yield the round-bottom type molecular assembly due to the stereocomplex formation among the hydrophobic helical blocks, showing that helix packing is highly influential in the determination of dimensions of the molecular assemblies [22-24].

Amphiphilic polydepsipeptides, which are composed of a hydrophilic poly(sarcosine) (poly(Sar)) block ranging from 60 to 90 mer and a hydrophobic poly(L-lactic acid) (PLLA) block of 30-mer, poly(sarcosine)₆₀₋₉₀-*block*-poly(L-lactic acid)₃₀ (poly(Sar)₆₀₋₉₀-*block*-(PLLA)₃₀), afforded polymeric micelles with a diameter of ca. 34

nm, which was named as “Lactosome” [2]. In this Article, the size control of the polymeric micelle is challenged by following two approaches. The first approach is to incorporate the hydrophobic block, poly(L-lactic acid), into the hydrophobic core of the AB-type polymeric micelle, leading to swelling the polymeric micelles. The second approach is to shrink the AB-type polymeric micelle with incorporation of the A₃B-type polydepsipeptide, (poly(Sar)₂₃)₃-*block*-(PLLA)₃₀, because the bulky hydrophilic moiety (the A₃ moiety) should favor the larger curvature of the polymeric micelle. As a result, size control of the micelle with a nanometer precision can be achieved.

Material and methods

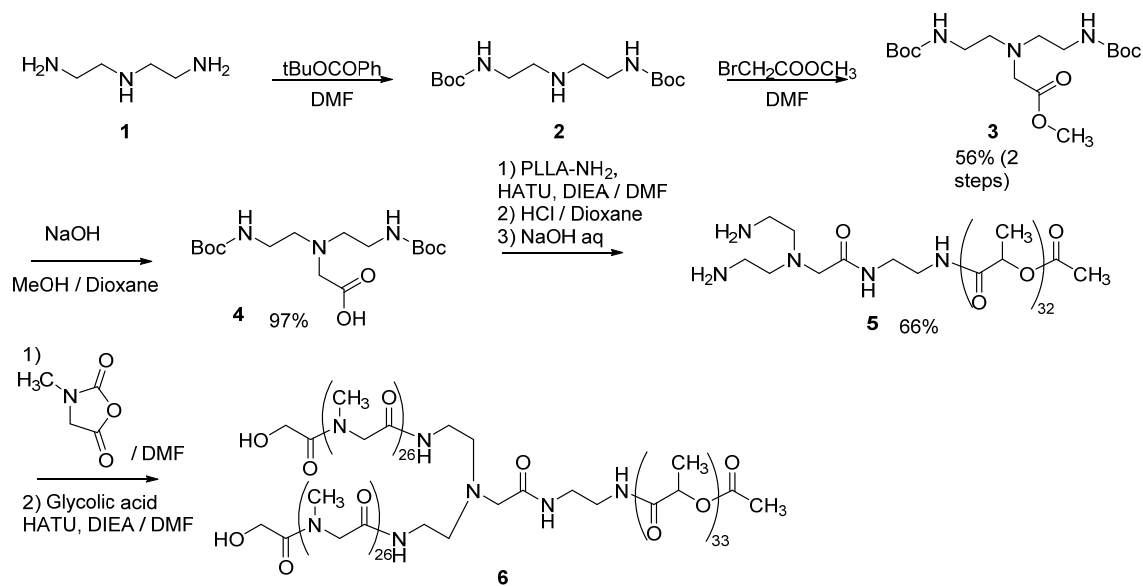
Materials

All reagents and solvents were purchased commercially and were used as received unless otherwise noted.

Synthetic route for three types of AB, A₂B, and A₃B type amphiphilic polydepsipeptide

All reagents and solvents purchased were used without further purification. NMR spectra were recorded with a Bruker DPX-400 spectrometer. All assignments were based on a correlation spectroscopy (COSY) experiment. AB type polymer was synthesized as written in a previous paper [2, 25]. Synthetic details of A₂B and A₃B type polymers were summarized as follows.

A₂B type



To the solution of 1 (0.50g, 4.85 mmol) in DMF (5.0 mL), *tert*-butylphenylcarbonate (2.07 g, 10.7 mmol) was added, and the reaction mixture was stirred overnight at room temperature under dry atmosphere. DMF was evaporated under reduced pressure, and the residue was washed with 4% KHSO₄, 1N NaOH aq, and brine. Compound 2 was extracted by chloroform, and was used for the next reaction after evaporation.

After the crude compound 2 was dissolved into DMF (5.0 mL), methyl bromoacetate (0.93 g, 6.06 mmol) and diisopropylethylamine (0.63 g, 4.85 mmol) were added to the solution. The reaction mixture was stirred at 0 °C for 30 min, and then at room temperature for 24 h under dry atmosphere. After evaporation, the residue was dissolved into chloroform, and washed with 4% KHSO₄, 1N NaOH aq, and brine. The organic layer was dehydrated with anhydrous Na₂SO₄, filtrated through cotton, and evaporated. The residue was purified by silica-gel column chromatography eluting with n-hexane/ethyl acetate = 3/1, 3/2 (v/v), to obtain compound 3 (1.01 g, 2.71 mmol) in 56% yield (2 steps).

^1H NMR (400 MHz, CDCl_3): 5.1 (2H, br, NHBoc), 3.7 (3H, s, $-\text{OCH}_3$), 3.4 (2H, s, $\text{N}-\text{CH}_2-\text{COOCH}_3$), 3.2 (4H, $\text{N}-\text{CH}_2-\text{CH}_2-\text{NHBoc}$), 2.7 (4H, $\text{N}-\text{CH}_2-\text{CH}_2-\text{NHBoc}$), 1.4 (18H, s, Boc)

Compound 3 (0.48 g, 1.29 mmol) was dissolved into the mixed solution of MeOH (5.2 mL) and 1,4-dioxane (5.2 mL). To the solution, 2.6 mL of 1 N NaOH aq was added, and the reaction mixture was stirred at room temperature for 2 h, and evaporated. The residue was dissolved into MeOH, and white precipitate was removed by filtration. The filtrate was purified by size column chromatography of Sephadex LH-20 column using MeOH as eluent to obtain pure 4 (0.45 g, 1.25 mmol) in 97% yield.

^1H NMR (400 MHz, $\text{CDCl}_3+\text{MeOD}$): 3.4 (2H, s, $\text{N}-\text{CH}_2-\text{COOCH}_3$), 3.1 (4H, $\text{N}-\text{CH}_2-\text{CH}_2-\text{NHBoc}$), 2.6 (4H, $\text{N}-\text{CH}_2-\text{CH}_2-\text{NHBoc}$), 1.4 (18H, s, Boc)

As previously reported, poly(L-lactic acid) (PLLA) block was synthesized using N-Cbz-ethylenediamine as an initiator. By deprotection of the Cbz group, $\text{PLLA}_{32}-\text{NH}_2$ was obtained. Mixture of $\text{PLLA}_{32}-\text{NH}_2$ (0.33 g, 0.14 mmol) and 4 (0.10 g, 0.28 mmol) was dissolved into DMF (3.0 mL), and then, HATU (0.16 g, 0.42 mmol) and N,N-diisopropylethylamine (DIEA, 0.10 g, 0.76 mmol) were added at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, and then at room temperature for 2 days under dry atmosphere. After evaporation of the solvent, the residue was washed by 4% KHSO_4aq , sat. NaHCO_3aq , and brine, and then extracted by chloroform. After dehydration of the organic layer by anhydrous Na_2SO_4 , obtained polymer was purified by Sephadex LH-20 column using DMF as eluent.

The purified polymer was dissolved into 4 N HCl/dioxane solution (3.0 mL), and

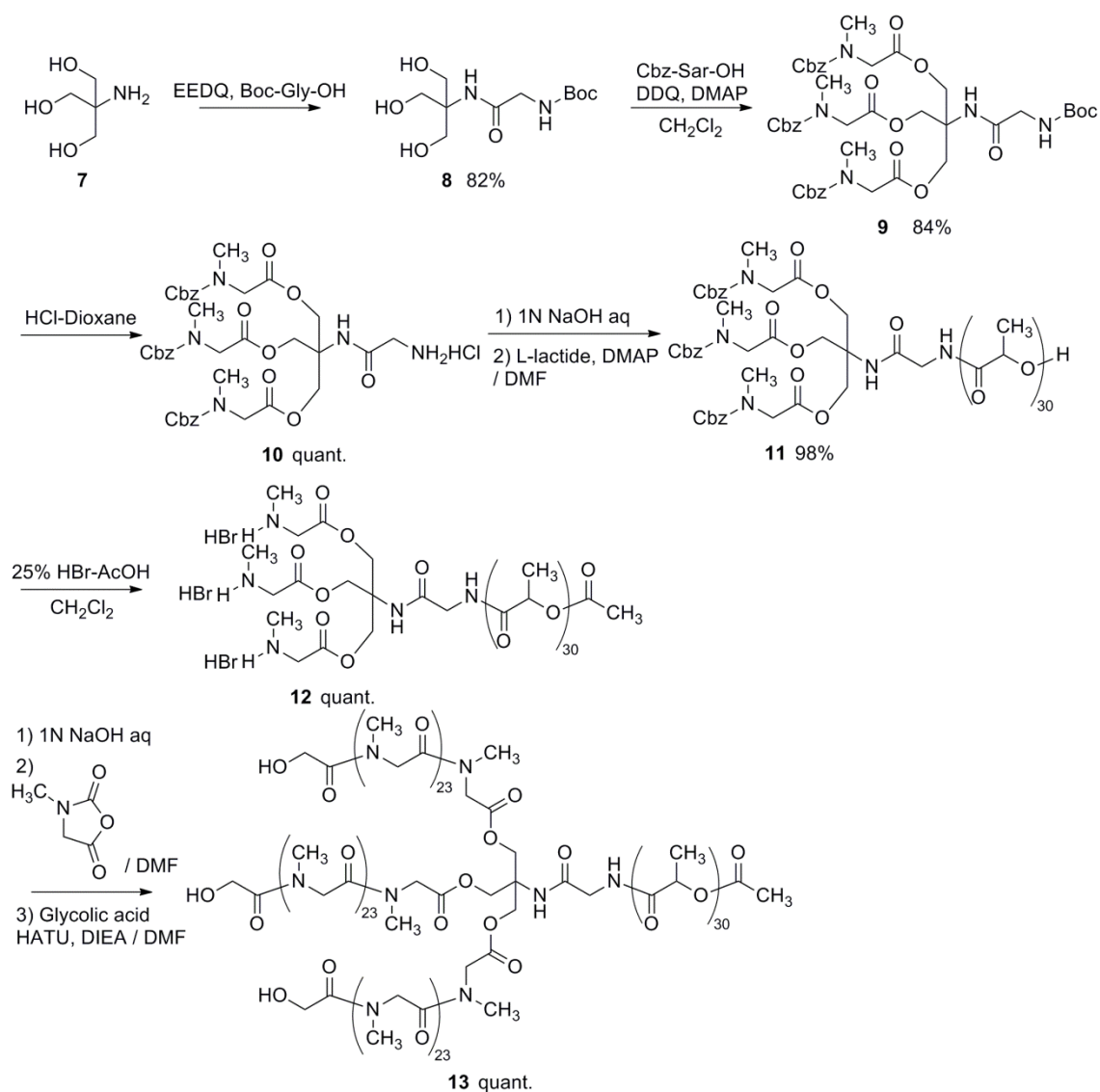
the reaction mixture was stirred at room temperature for 3 h. After evaporation of the solution, the obtained white residue was washed by 1 N NaOH aq and brine, and extracted by chloroform. The organic layer was dehydrated by anhydrous Na₂SO₄, filtrated through cotton, and evaporated to obtain polymer 5 (0.23 g, 0.092 mmol) in 66% yield.

¹H NMR (400 MHz, CDCl₃): 5.2 (32H, q, CHCH₃ of PLLA), 3.1 (4H, N-CH₂-CH₂-NHBoc), 2.5 (4H, N-CH₂-CH₂-NHBoc), 2.1 (3H, s, acetyl group located at polymer terminal end), 1.6 (98H, d, CHCH₃ of PLLA), 1.4 (18H, s, Boc)

Polymer 5 was used as a macroinitiator for polymerization of sarcosine block via N-carboxyanhydride (NCA) method. To the solution of polymer 5 (0.23 g, 0.092 mmol) in DMF (18 mL), Sar-NCA (1.05 g, 9.16 mmol) was added, and the reaction mixture was stirred at room temperature under Ar. After 24 h from the reaction started, glycolic acid (35 mg, 0.46 mmol), HATU (0.17 g, 0.46 mmol), and DIEA (88 mg, 0.69 mmol) were added at 0 °C, and stirred more for 24 h at room temperature. The final solution was purified by Sephadex LH20 column using DMF as eluent to obtain polymer 6. Final chain length of polymer 6 was determined to be (poly(Sar)₂₆)₂-*block*- PLLA₃₃ using integral value of ¹H NMR spectrum.

¹H NMR (400 MHz, DMF-*d*₇): 5.1 (33H, q, CHCH₃ of PLLA), 4.4-3.9 (104H, m, N-CH₂-CO of poly(Sar)), 3.0-2.7 (m, N-CH₃ of poly(Sar)), 2.0 (3H, s, acetyl group located at polymer terminal end), 1.5 (100H, d, CHCH₃ of PLLA)

A₃B type



To the reaction mixture of tris(hydroxymethyl)aminoethane 7 (1.9 g, 0.016 mol) and Boc-glycine-OH (2.5 g, 0.014 mol) in ethanol (75 mL), EEDQ (6.0 g, 0.024 mol) was added, and the solution was refluxed for 4.5 h. After removal of ethanol by evaporation, the residue was purified by silica-gel column chromatography eluting with chloroform/methanol = 1/0, 40/1, 10/1 (v/v) to obtain purified compound 8 (3.2 g, 0.012 mol) in 82% yield.

^1H NMR (400 MHz, CDCl_3): 7.2 (1H, br, NHCO), 5.2 (1H, br, $\text{tBuO}(\text{C}=\text{O})\text{NH}$), 3.8 (5H, m, $\text{N}-\text{CH}_2-\text{CO}$, $-\text{CH}_2-\text{OH} \times 3$), 3.7 (6H, s, $-\text{CH}_2-\text{OH} \times 3$), 1.4 (9H, s, Boc)

Compound 8 (0.28 g, 1.0 mmol) and Cbz-sarcosine-OH (0.80 g, 3.6 mmol) were dissolved into dichloromethane (10 mL). To the reaction mixture, dicyclohexyl carbodiimide (DCC, 0.89 g, 4.3 mmol) and 4-dimethylamino pyridine (DMAP, 26 mg, 0.21 mmol) were added at 0 °C. The reaction mixture was stirred at room temperature under dry atom overnight and then purified by silica gel column chromatography eluting with *n*-hexane/ethyl acetate = 2/1, 1/1, 1/2 (v/v) to obtain 9 (0.72 g, 0.84 mmol) in 84% yield.

¹H NMR (400 MHz, CDCl₃): 7.3 (15H, m, aromatics of Cbz × 3), 6.4 (1H, br, *t*BuO(C=O)NH), 5.1 (6H, d, -CH₂- of Cbz × 3), 4.5-4.3 (6H, m, -CH₂- of Sar × 3), 4.1-4.0 (6H, m, -C-(CH₂O-)₃), 3.7 (2H, br, N-CH₂-CO of Gly), 3.0 (9H, s, N-CH₃ of Sar × 3), 1.4 (9H, s, Boc)

To the solution of compound 9 (0.71 g, 0.79 mmol) in dioxane (1.0 mL), 4 N hydrogen chloride in dioxane solution (7.0 mL) was added. The reaction mixture was stirred at room temperature for 5 min, and then evaporated under reduced pressure. The residue was purified by silica gel column chromatography eluting with chloform/methanol = 10/1 (v/v) to obtain pure 10 (0.66 g, 0.79 mmol) in quantitative yield.

¹H NMR (400 MHz, CDCl₃): 7.8 (1H, br, -NHCO-), 7.3 (15H, m, aromatics of Cbz × 3), 5.1 (6H, d, -CH₂- of Cbz × 3), 4.5-4.3 (6H, m, -CH₂- of Sar × 3), 4.0 (6H, m, -C-(CH₂O-)₃), 3.4-3.3 (2H, br, N-CH₂-CO of Gly), 3.0 (9H, s, N-CH₃ of Sar × 3)

Compound 10 was washed with 1N NaOH aq, and extracted by chloroform. After

dehydration of the organic layer with anhydrous MgSO_4 , chloroform was removed by evaporation to obtain white precipitate (0.49 g, 0.61 mmol) as an initiator for the polymerization. To the solution of the initiator in DMF (2.0 mL), L-lactide (1.32 g, 9.20 mmol) and 4,4-dimethylaminopyridine (DMAP, 75 mg, 0.61 mmol) was added, and the reaction mixture was stirred under dry atmosphere at 55 °C for 3 days, and then dropped into ice-cooled methanol (200 mL). The resulting white precipitate was collected by centrifugation to obtain compound 11 in 98% yield.

^1H NMR (400 MHz, CDCl_3): 7.2 (15H, m, aromatics of Cbz $\times$ 3), 5.1 (6 + 28H, m, $-\text{CH}_2-$ of Cbz $\times$ 3, CHCH_3 of PLLA), 4.5-4.3 (6H, m, $-\text{CH}_2-$ of Sar $\times$ 3), 4.0-3.8 (6H, m, $-\text{C}-(\text{CH}_2\text{O}-)_3$), 3.6 (2H, m, N- CH_2 -CO of Gly), 2.9 (9H, s, N- CH_3 of Sar $\times$ 3), 1.5 (90H, d, CHCH_3 of PLLA)

Compound 11 was dissolved into the mixed solution of dichloromethane (10 mL) and 25% HBr/AcOH solution, and the reaction mixture was stirred at room temperature under dry atmosphere for 18 h. The reaction mixture was dropped into ice-cooled MeOH (200 mL) after concentration by evaporation, and polymer 12 was collected by centrifugation as a white precipitate.

^1H NMR (400 MHz, CDCl_3): 5.3 (32H, q, CHCH_3 of PLLA), 4.7 (6H, m, $-\text{CH}_2-$ of Sar $\times$ 3), 4.3 (6H, m, $-\text{C}-(\text{CH}_2\text{O}-)_3$), 2.9 (9H, s, N- CH_3 of Sar $\times$ 3), 2.1 (3H, s, acetyl group located at polymer terminal end), 1.5 (90H, d, CHCH_3 of PLLA)

The chloroform solution of compound 12 was washed by 1 N NaOH aq and brine. The organic layer was dehydrated with anhydrous MgSO_4 , filtrated through celite, and dried over under reduced pressure. To the macroinitiator (0.43 g, 0.078 mmol),

Sar-NCA (0.54 g, 4.69 mmol) and DMF (9.5 mL) were added, The reaction mixture was stirred at room temperature under Ar for 24 h, and then for 2 days after addition of glycolic acid (30 mg, 0.38 mmol), HATU (0.15 g, 0.39 mmol), and DIEA (0.10 mL, 74 mg, 0.57 mmol). Finally, polymer 13 was purified by size column chromatography of Sephadex LH-20 column using DMF as eluent. Chain length of polymer 13 was determined to be poly((Sar)₂₃)₃-*block*-PLLA₃₀ using integral value of ¹H NMR spectrum.

¹H NMR (400 MHz, DMSO): 5.2 (30H, q, CHCH₃ of PLLA), 4.4-4.0 (6 + 144H, m, -C-(CH₂O-)₃, -CH₂- of Sar), 3.0-2.7 (209H, m, N-CH₃ of Sar × 3), 2.1 (3H, s, acetyl group located at polymer terminal end), 1.5 (99H, d, CHCH₃ of PLLA)

Typical Molecular Assembly Preparation Method

Polymer mixture (1.0 mg) dissolved in chloroform was added to the test tube. After the solution was removed by evaporation, the sample was completely dried under reduced pressure to form a thin polymer film on the test tube. To the test tube was added saline (1.0 mL) at the final polymer concentration of 1.0 mg/mL, and the solution was sonicated at 28, 45, 100 kHz using a bath-type sonicator (W-113, Honda Electronics Co., Ltd., Japan) under heating condition (85–90 °C) for 1 min each to assist formation of thermodynamically stable self-assembled nanoparticles. The obtained dispersion of the molecular assembly was used without further purification.

Evaluation of Prepared Molecular Assemblies

Hydrodynamic diameter of the molecular assembly was determined by dynamic light scattering (DLS) measurements using a Malvern Zetasizer nano ZS. TEM images

were taken using a JEOL JEM-3100FEF at an accelerating voltage of 100 keV. Dispersion of the molecular assembly was applied on a carbon-coated Cu grid, and the samples were negatively stained with 2% phosphor tungstic acid. For the histogram evaluation of the TEM image, 25 points were randomly selected from each TEM image, and horizontal and vertical diameters of the selected nanoparticle were manually measured by the gauging function of Adobe Photoshop CS 14.0 Extended. The evaluation was repeated two times using different samples, and the average diameters and standard errors were calculated.

RESULTS

Polymer Syntheses

The linear AB-type polymer of poly(Sar)₇₀-*block*-PLLA₃₀ was synthesized as previously reported (Figure 1a). Chemical structures of the branched A₂B- and A₃B-type polymers are illustrated in Figure 1b and c. The poly(L-lactic acid) (PLLA) block was synthesized via melt or solution polymerization methods, and the poly(sarcosine) block was via the *N*-carboxy-aminoacidanhydride (NCA) polymerization method. The *N*-terminal of poly(sarcosine) block was converted to a noncharged hydroxyl group with capping by glycolic acid. Lengths of synthesized polymers were determined by ¹H NMR spectra utilizing the acetyl group as the normalizing value, which is located at the PLLA terminal end. The branched A₂B- and A₃B-type amphiphilic polydepsipeptides synthesized in this study were determined to be (poly(sarcosine)₂₆)₂-*block*-poly(L-lactic acid)₃₃ ((poly(Sar)₂₆)₂-*block*-PLLA₃₃) and (poly(sarcosine)₂₃)₃-*block*-poly(L-lactic acid)₃₀ ((poly(Sar)₂₃)₃-*block*-PLLA₃₀), respectively.

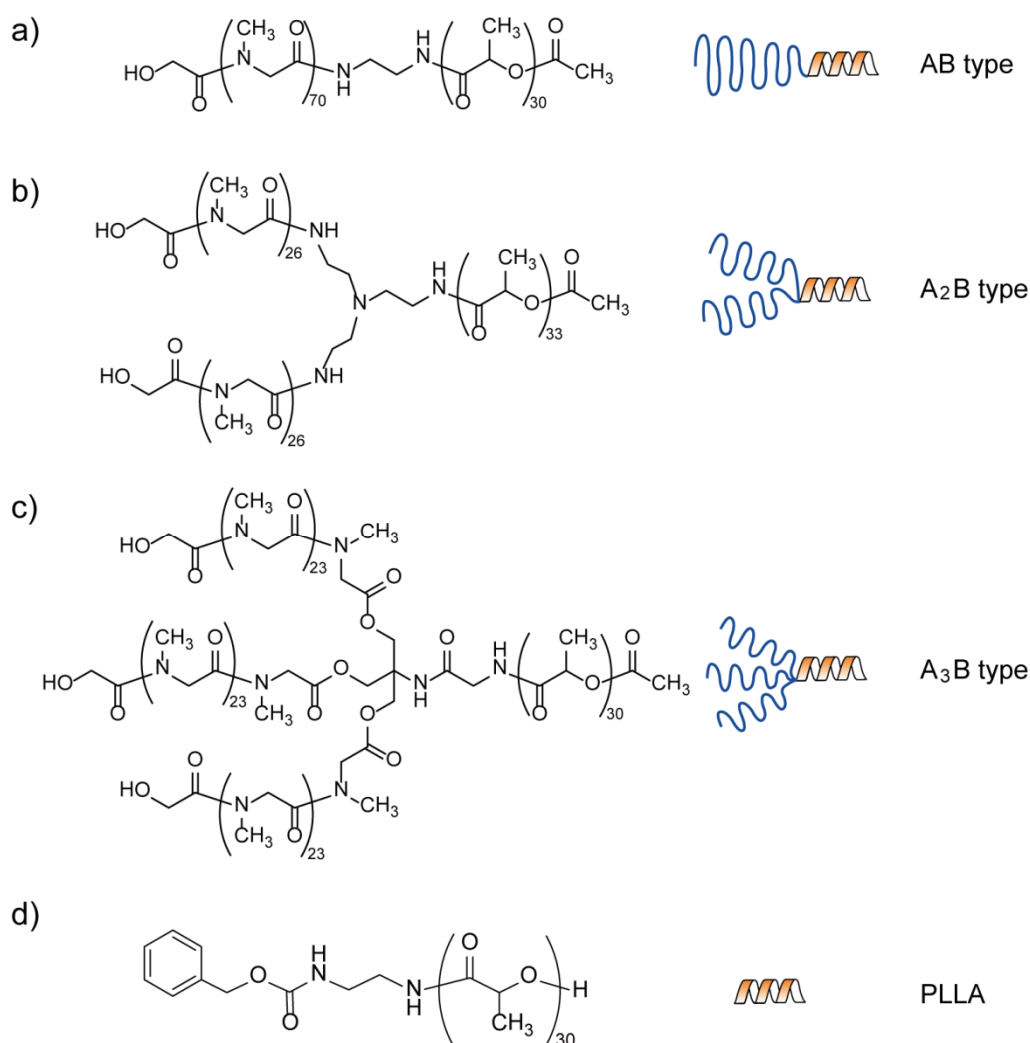


Figure 1. Chemical structures of (a) AB-, (b) A₂B-, (c) A₃B-type amphiphilic poly-depsipeptides, and (d) PLLA. Hydrophilic and hydrophobic polymer lengths were determined by ¹H NMR spectra by using acetyl protons at the PLLA O-terminal end for normalization.

Size Control of Lactosome by Mixing PLLA

As compared to the conventional molecular assembly preparation method using organic solvent, the film rehydration method has advantages of the simple purification process and avoiding the risk for the residual organic solvent in the assembly. Therefore, the film rehydration method was adopted in this study. As previously reported, poly(Sar)₇₀-*block*-PLLA₃₀ formed a polymeric micelle whose diameter is ca. 34 nm. On

preparation of the polymer film on test tube surface, 0–300 mol % of PLLA 30mer against the polydepsipeptide was mixed, and polymeric micelles were prepared. The hydrodynamic diameters of the polymeric micelles were determined by the dynamic light scattering (DLS) measurements and are summarized in Table 1. The hydrodynamic diameters (Z-average value) became larger in accordance with the PLLA mixing ratio, and reached up to 96 nm when the PLLA mixing ratio was 300 mol %. The homogeneity of the polymeric micelles was high with the mixing ratios below 50 mol % indicated by the polydispersity indices (PDI) being less than ca. 0.1. At higher mixing ratios, the polydispersity indices still were less than 0.15, suggesting relatively high homogeneity.

Table 1. Hydrodynamic diameters of Lactosome prepared from linear AB-type amphiphile and PLLA mixture

PLLA mixing amount (mol% against amphiphilic polydepsipeptide)	Hydrodynamic diameter (Z-average value, nm)	Polydispersity index
0	32.1	0.068
5	35.2	0.087
10	35.2	0.079
25	38.9	0.103
50	44.5	0.140
100	58.6	0.127
200	78.7	0.146
300	95.8	0.101

Preparation of Molecular Assembly from Branchedtype Amphiphilic Polydepsipeptides

Utilizing the A₂B and A₃B-type branched amphiphilic polydepsipeptides, which were

newly synthesized in this study, molecular assembly was prepared under the same condition with linear AB-type polymer. The results of the DLS measurements of the polymer dispersions are summarized in Table 2. Hydrodynamic diameters of the molecular assemblies prepared from the AB and A₃B-type polymers were 34 and 21 nm, respectively. Polydispersity indices were 0.095 and 0.12, respectively, indicating a narrow size distribution. On the other hand, the A₂B-type polymer afforded a larger molecular assembly of 210 nm.

Table 2. The diameters of the AB-, A₂B-, and the A₃B-type polydepsipeptide assemblies determined by DLS and TEM.

Polymer	DLS		TEM
	Hydrodynamic diameter (Z-average value, nm)	Polydispersity index	Diameter (nm)
AB	33.8	0.095	34.3 ± 1.8
A ₂ B	2.1 × 10 ²	0.073	-
A ₃ B	20.7	0.12	20.5 ± 1.4

To confirm the morphologies of these molecular assemblies, images of transmission electron microscopy (TEM) were taken (Figure 2). In all cases, the core region of the assemblies was not stained by phosphor tungstic acid, denying the vesicular structure. Therefore, the spherical polymeric micelle formation prepared from the AB- and A₃B-polymer but the worm-like micelle formation from the A₂B-type polymer were observed. The micelle sizes were measured in the TEM images giving the diameters of 34.3 ± 1.8 and 20.5 ± 1.4 nm, respectively, for the AB- and A₃B-type polymeric micelles, which are agreeable with the results of the DLS measurements. The TEM images also indicate the high homogeneity of the polymeric micelles as shown by the DLS measurements.

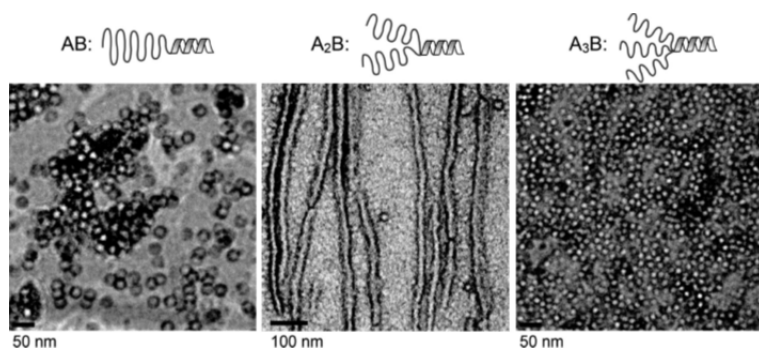


Figure 2. TEM images of three types of amphiphilic polydepsipeptide. Polymer dispersion was negatively stained by 2% phosphor tungstic acid

Size Control with Mixing AB- and A₃B-type Polydepsipeptides

Both the AB- and the A₃B-type polymers afforded spherical micelles but with different diameters. Next, preparation of molecular assembly was performed from the mixtures of the AB- and A₃B-type polymers with varying mixing ratios from 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, to 0:10 (mol/mol). Those mixed polymer films were treated at 90 °C with sonication in the same manner as the previous preparation. Molecular assemblies prepared were observed by DLS and TEM, and the effect of the mixing ratio on the morphology of the molecular assemblies was examined (Table 3). In accordance with increasing the A₃B-type polymer mixing ratio, the hydrodynamic diameters of the molecular assemblies declined from 34 nm (A₃B/AB = 0/10) to 22 nm (A₃B/AB = 10/0). The spherical polymeric micelle morphology was retained in these ratios based on TEM observations (Figure 3 and Figure 4). Further, the diameters determined by DLS and TEM are consistent, and also show high size homogeneity of the polymeric micelles (Table 3).

Table 3. The diameters of the mixed assemblies of the AB- and the A₃B-type polydepsipeptides determined by DLS and TEM.

Polymer (A ₃ B/AB, mol/mol)	DLS		TEM
	Hydrodynamic diameter (Z-average value, nm)	Polydispersity index	Diameter (nm)
10/0	21.5	0.11	20.5 ± 1.4
9/1	25.1	0.23	-
8/2	25.2	0.22	23.3 ± 1.1
7/3	26.6	0.18	-
6/4	28.0	0.15	-
5/5	29.2	0.10	29.3 ± 1.9
4/6	30.2	0.088	-
3/7	31.5	0.11	-
2/8	32.8	0.080	31.0 ± 1.6
1/9	32.9	0.069	-
0/10	33.9	0.097	34.3 ± 1.8

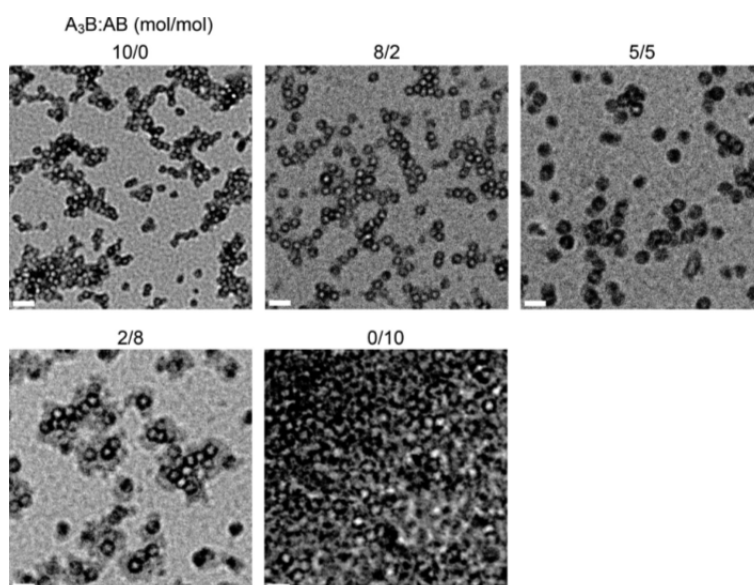


Figure 3. TEM images of the polymeric micelles prepared from the AB- and the A₃B-type polydepsipeptides with varying mixing ratios. Polymer dispersions were negatively stained by 2% phosphor tungstic acid. Scale bar: 50 nm.

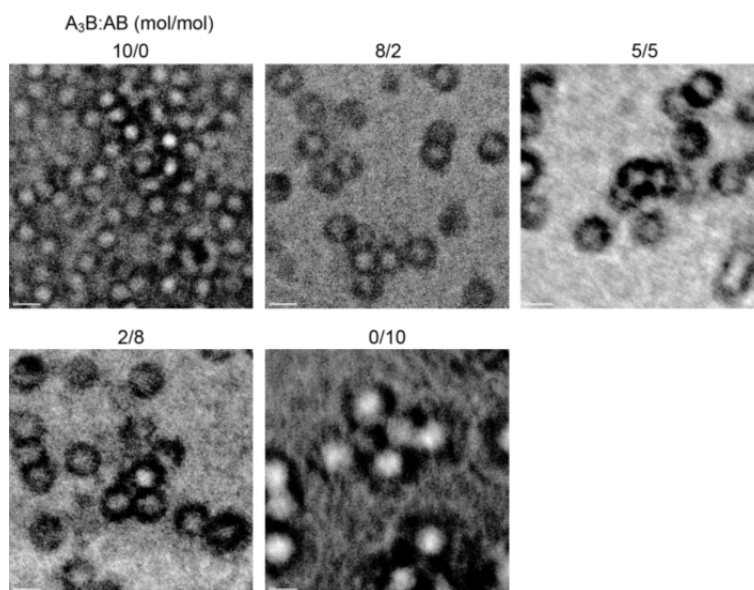


Figure 4. Enlarged TEM images of the polymeric micelles prepared from the AB- and the A₃B-type polydepsipeptides with varying the mixing ratios. Polymer dispersions were negatively stained by 2% phosphor tungstic acid. Scale bar: 20 nm

Discussion

Branched Structure and Molecular Assembly Morphology

The molecular assembly morphology drastically changed from the spherical polymeric micelle of the AB-type and the A₃B-type polymers to the worm-like micelles of the A₂B-type polymer. As previously reported, the block of PLLA 30 mer takes a 310 helical structure,¹⁵ whose chain length amounts to be ca. 6 nm. The extended chain lengths of poly(sarcosine) 70-mer and 23-mer are estimated to be about 17 and 6 nm, respectively. The observed micelle sizes prepared from the AB- and the A₃B-type polymers (34 and 20 nm) are therefore agreeable roughly with the estimated sizes using these block lengths under the assumption of taking the normal micelle structure.

Static light scattering (SLS) analyses revealed that the polymer aggregation number of the A₃B-type micelle was decreased by 29% from that of the AB-type micelle. On the

other hand, the micelle volume of the A₃B-type micelle was decreased by 80% from that of the AB-type micelle. The surface density of the poly(sarcosine) chains of the A₃B-type micelle was therefore about 6 times higher than that of the AB-type micelle. These drastic changes of the micelle dimensions should be due to the differences in the polymer architecture, AB or A₃B.

The morphology prepared from the A₂B-type polymer that is different from others should be due to the differences in the molecular shapes. In Figure 5, the conformational analysis was carried out by using molecular mechanics 2 (MM2) program. The A₂B-type polymer is branched in three ways at the nitrogen atom, and takes a fanlike geometry. To shield the hydrophobic molecular surface, the planar-like A₂B polymer is considered to be favorably stacked into the worm-like morphology as illustrated in Figure 6. On the other hand, the hydrophilic three poly(sarcosine) chains in the A₃B-type polymer are presumed to take a three-dimensionally spread configuration in solution. As a result, the A₃B-type polymer should form the spherical poly-meric micelle due to the isotropic shape of the hydrophilic moiety.

The A₂B-type polymer formed the worm-like micelles. From the TEM images, the diameter of the worm-like micelles was found to be ca. 25 nm. The curvature of the molecular assemblies therefore increases in the order of AB-type < A₂B-type < A₃B-type, which is ascribable to the bulkiness of the hydrophilic moiety of these amphiphilic polydepsipeptides.

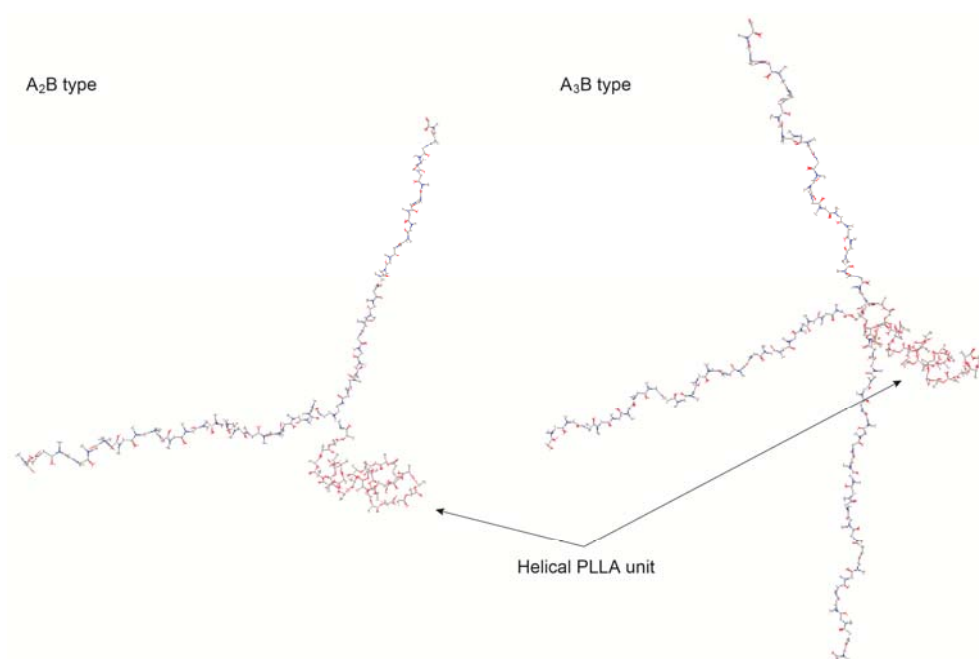


Figure 5. Roughly calculated conformation of A_2B and A_3B type amphiphilic polydepsiptides. The calculation was performed by PerkinElmer Chem3D Pro 13.0 using molecular mechanics program 2 (MM2).

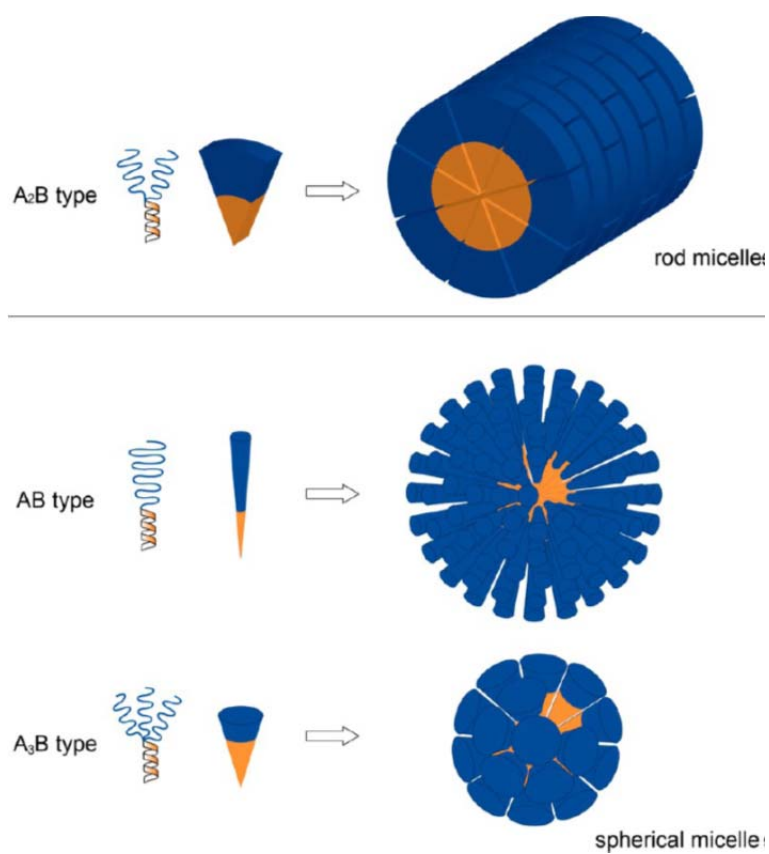


Figure 6. Illustration of the spherical polymeric micelle and the worm-like micelle prepared from three types of the amphiphilic polydepsipeptides.

Micelle Size Increase with Incorporation of PLLA

The hydrodynamic diameters of the polymeric micelles prepared from the AB-type polymer increased in accordance with the mixed amount of the hydrophobic PLLA. Similarly, the polymeric micelle sizes prepared from the A₃B-type polymer increased with mixing PLLA, but the degree of increase was smaller than that of the AB-type polymeric micelles at the same PLLA mixing ratios. The relationship between the diameter and the PLLA amount seems to be linear, but the author assumes that more void spaces in the hydrophobic core should be generated with increasing amount of PLLA. Further, as the micelle core increases in volume, the regularity of the helical PLLA packing is considered to be loosened. Indeed, the incorporation of hydrophobic drugs into the AB-type polymeric micelles seems to be enhanced with the addition of PLLA due to these two factors. However, this point is currently under investigation.

Micelle Size Decrease with Incorporation of the A₃BtypePolymer

The hydrodynamic diameters of the AB-type polymeric micelles decreased the size with incorporation of the A₃B-type polymer. The diameters were linearly correlated with the mixing ratio of the A₃B-type polymer (Table 3). The R² value on the linear approximation was as high as 0.99 (Figure 7). Further, the polydispersity indices of the polymeric micelle sizes in the range from 22 to 34 nm were maintained at a low level, suggesting that the polymeric micelles are highly homogeneous in their sizes. When the AB- and the A₃B-type polydepsipeptides were mixed together on micelle formation, helical PLLA blocks of both polymers are considered to be regularly aligned in the

molecular assembly due to the rigid and regular structure of the helical PLLA. On the other hand, the hydrophilic poly(sarcosine) chain density at the polymeric micelle surface gets higher with incorporation of the A₃B-type polymer into the AB-type polymer.

As a result, the polymeric micelle sizes should decrease with the incorporation of more A₃B-type polymers. Notably, the polymer association is based on the regular molecular packing of the helical PLLA blocks; these polymeric micelles can be highly homogeneous in their sizes while keeping the polydispersity indices low.

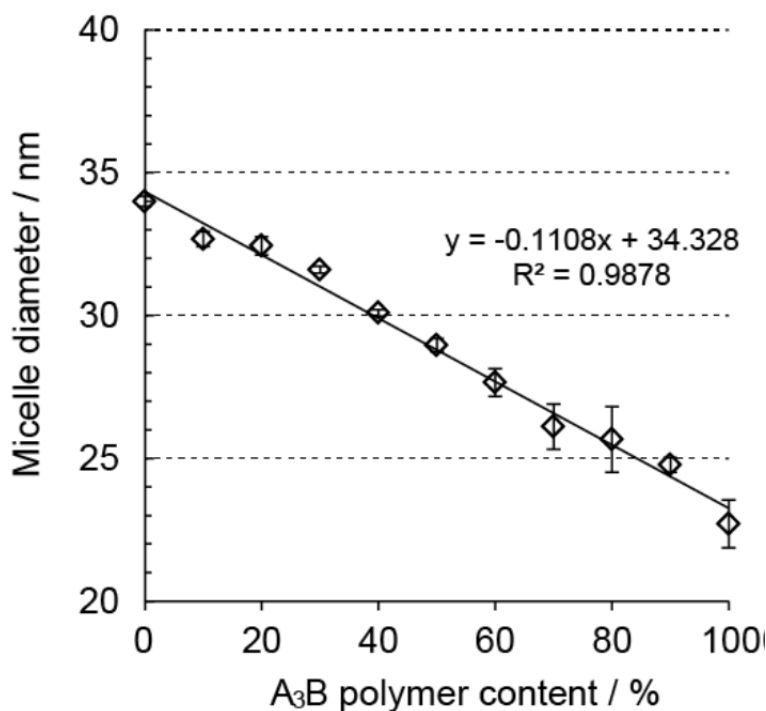


Figure 7. Relationship between diameter of polymeric micelle and mixture ratio of AB and A₃B type amphiphilic polydepsipeptides. On each polymer mixing ratio, three samples were prepared, and DLS measurements were performed. Size of the micelle is linearly correlated with the mixing ratio ($R^2 = 0.99$). Hydrodynamic diameter of prepared nanoparticle was analyzed by dynamic light scattering (DLS) measurement by using Malvern Nano-ZS (United Kingdom). DLS results were expressed in terms of the Z-average, which is the intensity based harmonic mean.

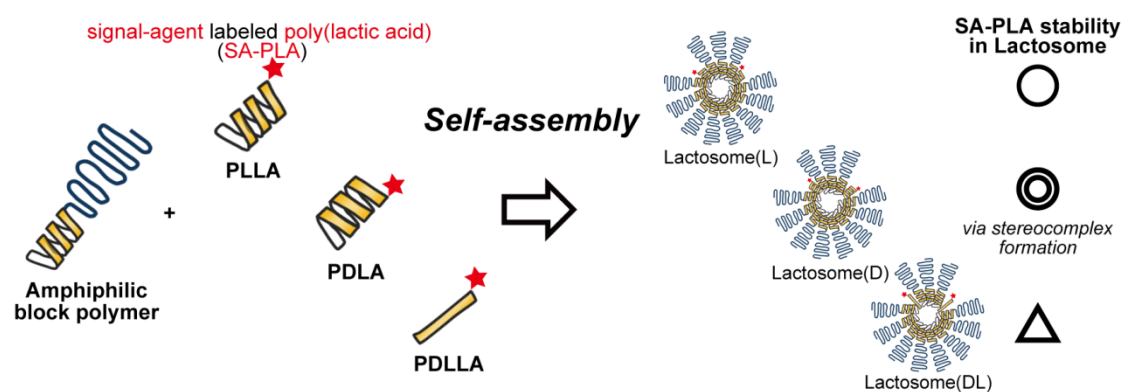
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Chapter 6

Control of *in Vivo* Blood Clearance Time of Polymeric Micelle by Stereochemistry of Amphiphilic Polydepsipeptides



Introduction

Core-shell type polymeric micelle, “Lactosome,” is composed of amphiphilic polydepsipeptide with a hydrophobic block of helical poly(L-lactic acid) (PLLA) and a hydrophilic block of poly(sarcosine) [1]. Sarcosine, *N*-methylated glycine, is a kind of natural amino acids found in muscles and other body tissues, and it is metabolized endogenously by sarcosine dehydrogenase [2]. PLLA is a well-known biocompatible and biodegradable polymer [3, 4]. Lactosome is therefore considered to be biocompatible materials, which is confirmed by no acute toxicity of Lactosome by a single-dose toxicity test with using mice.

Lactosome has been examined on application for imaging probe of solid tumors. A characteristic point of Lactosome about *in vivo* disposition is a significantly low level of accumulation in liver resulting in a long *in vivo* blood clearance time [5, 6]. Lactosome therefore accumulates selectively in solid tumors due to the enhanced permeability and retention (EPR) effect [7, 8]. In the view point of imaging probe, however, nanoparticles with the long *in vivo* blood clearance time have a drawback of remaining signal at background for a long period, leading to insufficient contrast in images of tissues with large blood volume [9-11]. In the present study, the author tries to improve the signal ratio at tumor against background by controlling the *in vivo* blood clearance time of Lactosome.

Near-infrared fluorescence (NIRF) image is taken by using Lactosome containing NIRF-labeled poly(L-lactic acid) (PLLA) of 1.5 mol% [5]. It is known that stability of polymeric micelles *in vivo* is influenced by compounds entrapped in the hydrophobic core of the micelle [12-14]. The hydrophobic core of Lactosome polymeric micelles is constructed by dense molecular packing of helical PLLA blocks, where NIRF-labeled

PLLA at the low content can be easily incorporated without changing the diameter of polymeric micelles. PLLA takes a left-handed helical structure and is known to form stereocomplex with right-handed helical poly(D-lactic acid) (PDLA) providing materials with high mechanical strength [15-17]. Physical stability of Lactosome in the blood stream is therefore considered to increase with the addition of PDLA through stereocomplex formation. On the contrary, Lactosome may be destabilized by the incorporation of poly(D,L-lactic acid) (PDLLA) into the hydrophobic core through disturbing the helix molecular packing. The authors recently revealed that enantiopure amphiphilic blockpeptides having a hydrophobic block with a helix structure self-assembled into nanotubes [18]. On the other hand, a mixture of amphiphilic blockpeptides having right-handed and left-handed helices as the hydrophobic block yielded vesicles due to stereocomplex formation [19-21]. Stereochemistry in the hydrophobic core of self-assemblies is therefore an important factor for determining properties of selfassemblies. Lactosome with mixing PLLA, PDLA, and PDLLA is examined here on stability in the blood stream and consequently on tumor imaging.

Material and methods

Lactosome preparation

Synthetic method for ICG-labeled poly(lactic acid) is summarized in the previous paper [1, 5] (Scheme 1). Lactosome dispersion in saline was prepared as described in the previous paper [1, 5].

Measurements

Circular dichroism (CD) spectroscopic data were taken using a JASCO J600

spectropolarimeter at room temperature with an optical cell of 0.1 cm optical path length. Malvern Zetasizer-Nano was used for dynamic light scattering (DLS) measurements. All measurements were performed at 25 °C.

***In vivo* imaging using tumor-bearing mice**

Tumor-bearing BALB/c nu/nu nude mouse was prepared by transplantation of human pancreatic cell line of SUI-2/pEF-Luc at right femoral region as previously reported.

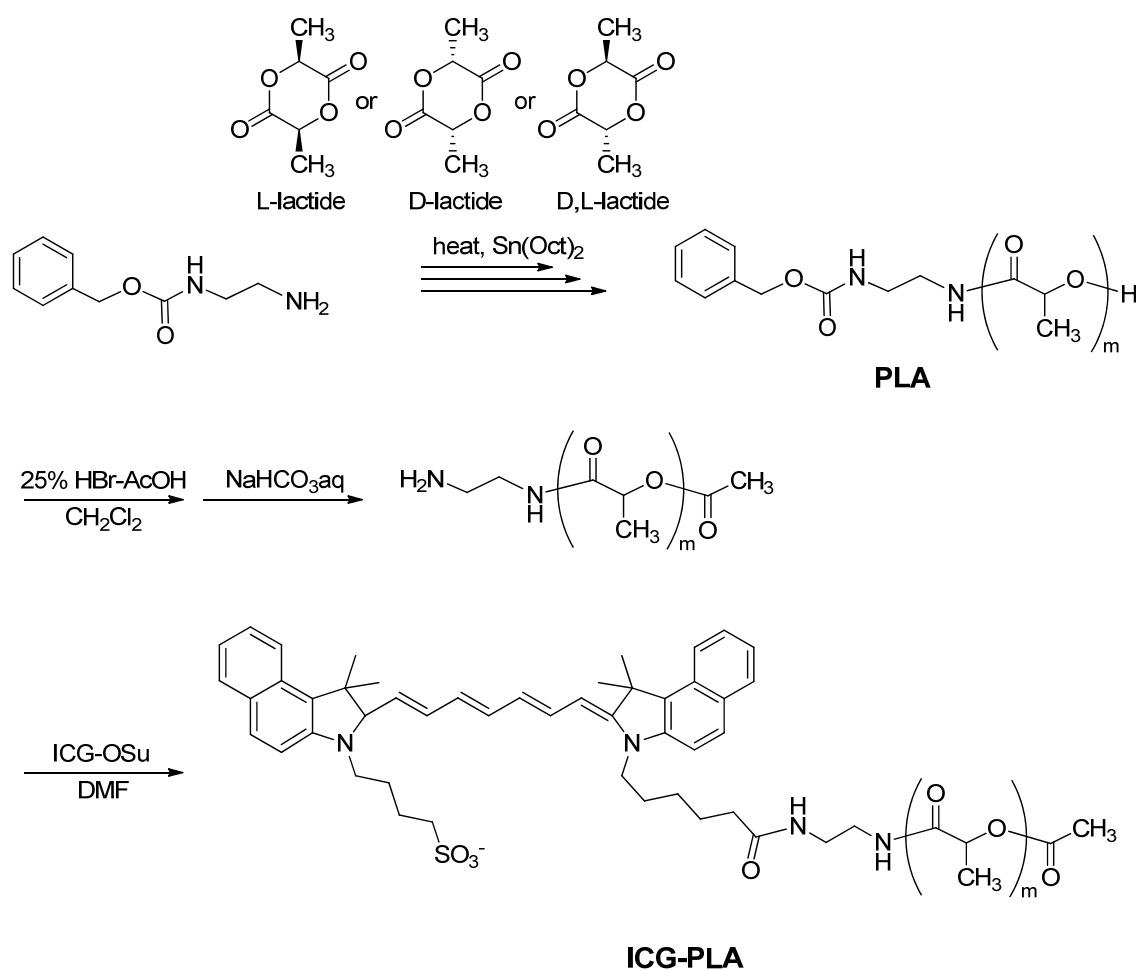
To the tumor-bearing mice, a saline solution of ICG-labeled Lactosome (Lactosome containing PLLA, PDLA, or PDLLA with indocyanine green (ICG)) (1.0 mg/mL, 50 μ L) was intravenously administrated from the tail vein. Concentration of ICG-labeled Lactosome was normalized by UV absorption intensity at 795 nm dissolved in DMSO. *In vivo* NIRF image was taken by Clairvivo OPT (Shimadzu Corp.) with using a filter set of excitation of 785 nm and emission of 845 nm. During the imaging process, the mouse was held on the stage under anesthetized condition with 2.5% of isoflurane gas in air flow (1.5 L/min).

Results and discussion

Polymer synthesis

ICG-labeled poly(lactic acid) was chemically synthesized as illustrated in Scheme 1. Initiating from mono-*N*-Cbz-ethylenediamine, poly(lactic acid) was synthesized by the melt polymerization method with using three types of the lactide monomers, L,L-lactide, D,D-lactide, and D,L-lactide, to yield PLLA, PDLA, and PDLLA, respectively. After the polymerization, PLLA and PDLA were purified by precipitation in methanol.

PDLLA was purified by size exclusion chromatography (SEC) of Sephadex LH-20 column on methanol. The Cbz group was deprotected by 25% HBr-AcOH, and to the amino group, ICG was covalently attached via amide linkage. The degree of polymerization of the poly(lactic acid)s was set at ca. 30, which is a critical value for enantiopure poly(lactic acid)s to take a helical structure. PLLA and PDLA used here are noted with the determined residue numbers as PLLA-28 and PDLA-31, respectively, which were confirmed to take a left-handed and a right-handed helical structure, respectively, by CD measurements (Figure. 1). On the other hand, PDLLA (PDLLA-30) took a disordered structure based on no signal at CD spectrum.



Scheme 1. Synthetic scheme for ICG-labeled poly(lactic acid)s

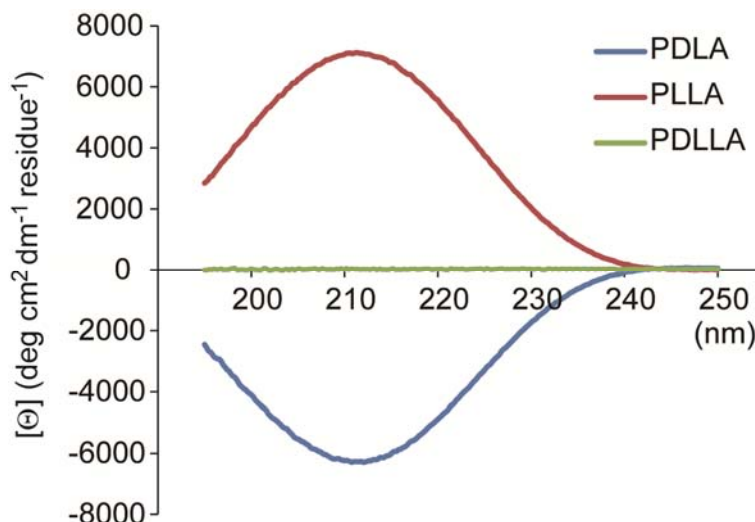


Figure 1. Circular dichroism spectra of PLLA-28, PDLA-31, PDLLA-30 in CH_3CN . The concentrations were set at 1.2 mg/mL for all the samples.

Molecular assembly containing PLLA, PDLA, and PDLLA

The amphiphilic polydepsipeptide composed of a hydrophobic 30mer block of helical PLLA and a hydrophilic 70mer block of poly(sarcosine) self-assembled into core-shell type polymeric micelles with 35 nm diameter. The effect of PLLA-28 addition to the amphiphilic polydepsipeptide on the self-assembly morphology was examined by DLS measurements. With increase of the mixing PLLA-28 amount, the hydrodynamic diameters of Lactosome remained within 20% increase in the range of less than 25 mol% addition, and became significantly larger beyond 50 mol% addition (Figure. 2). PDLLA-30 also showed the same effect on the hydrodynamic diameters as PLLA-28 (Figure. 2). On the other hand, PDLA-31 induced drastic increase of the hydrodynamic diameters, which became over 100 nm with the addition of 25 mol% (Figure. 2). Further, white precipitation was observed with 75 mol% addition.

PLLA is known to form hexagonal crystalline structure. In the hydrophobic core of Lactosome, PLLA blocks are considered to be regularly aligned and molecularly packed to form locally the crystalline structure. The crystalline domains should be confined into the small space of the polymeric micelle core however with many structural defects. When PLLA-28 or PDLA-30 was added to the Lactosome, these molecules may fill the defects in the polymeric micelle core, and thus the assembly sizes remained to be similar values within 25 mol% addition. On the other hand, PDLA-31 forms stereocomplex with the matrix polydepsiptide of Lactosome. The addition of PDLA therefore directly increases the size of the polymeric micelle.

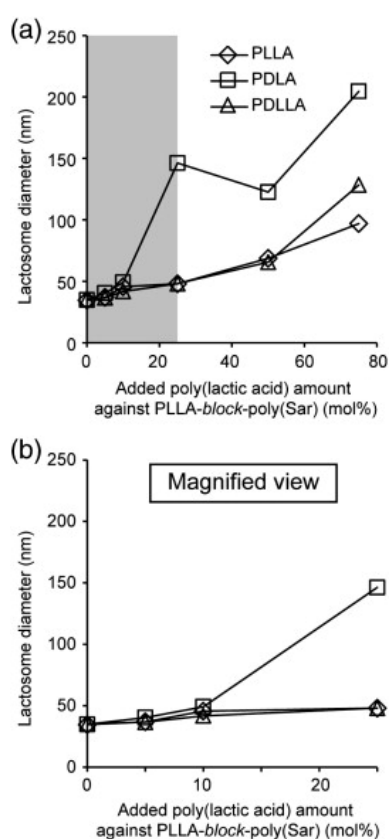


Figure 2. Lactosome diameter containing 0–75 mol% three types of poly(lactic acid)s. Figure 2b is a magnified view of the highlighted region in Figure 2a.

The T_g value of poly(lactic acid) is reported to be 50–60 °C. However, no peaks were

observed by differential scanning calorimetry (DSC) measurements of PLLA and PDLA (5 mol%) (Figure. 3). On the other hand, the poly(lactic acid)s T_g peak was observed with the Lactosome dispersion when it was prepared at 0 °C. These data also support that PLLA-28 or PDLA-31 should be nicely packed in the hydrophobic core of the Lactosome constituents due to the heat treatment at 90 °C at the micelle preparation.

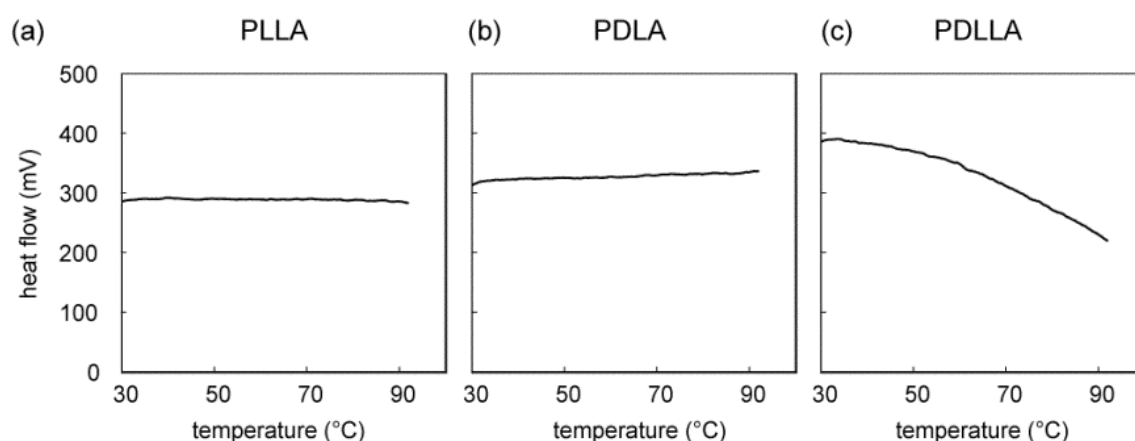


Figure 3. Differential scanning calorimetry (DSC) measurement of Lactosome dispersions containing three types of 5 mol% poly(lactic acid)s. Scanning range was set to be 30–90 °C.

Effect of poly(lactic acid) stereochemistry on NIRF *in vivo* imaging

PLLA-28, PDLA-31, and PDLLA-30 were labeled with indocyanine green (ICG) to obtain ICG-PLLA, ICG-PDLA, and ICG-PDLLA, respectively. ICG-PLLA, ICG-PDLA, and ICG-PDLLA were incorporated into Lactosome at 1.5 mol%, which scarcely changes the size of Lactosome. Lactosomes containing ICG-PLLA, ICG-PDLA, and ICG-PDLLA are abbreviated as ICG-Lactosome(L), ICG-Lactosome(D), and ICG-Lactosome(DL), respectively, which were administered

intravenously to tumor-bearing mice. *In vivo* dispositions of these Lactosomes are quite different in time courses of NIRF imaging (Figure. 4).

In the case of ICG-Lactosome(L), ICG fluorescence signal spread over the whole body soon after the administration, and gradually accumulated at the tumor region as previously reported. ICG fluorescence intensity at the tumor region became maximum at 24 h after the administration. In the case of ICG-Lactosome(D), the ICG fluorescence signal at the tumor region became maximum at 24 h similar to the case of ICG-Lactosome(L), but the signal intensity remained constant for another 24 h, which makes a contrast with a gradual decrease of ICG-Lactosome(L) at 48 h. ICG-Lactosome(D) thus shows a longer retention property at the tumor region than ICG-Lactosome (L). On the other hand, ICG-Lactosome(DL) spread quickly over the whole body, and diminished the fluorescence intensity at the background monotonously with time after the administration. The fluorescence intensity at the tumor region remained nearly the same level from 4 h to 24 h after the administration, suggesting the less EPR effect on the accumulation of ICG-Lactosome(DL) at the tumor region. These results suggest the stability of these Lactosomes in the blood stream changes depending on the stereochemistry in the polymeric micelle core.

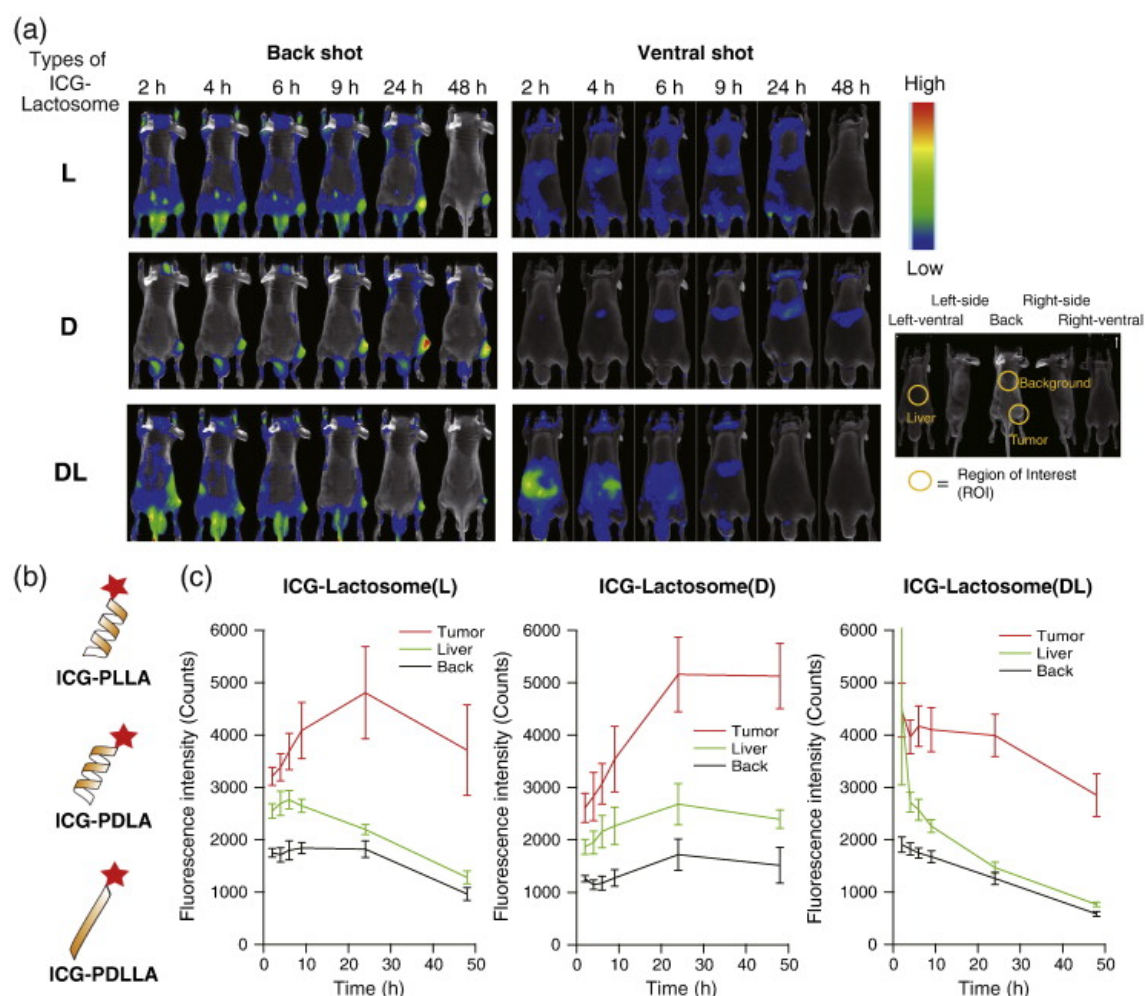


Figure. 4. (a) *In vivo* imaging of subcutaneous pancreatic cancer-bearing mouse using three types of ICG-Lactosomes (n=5). Images were taken at 48 h after the Lactosome administration. For the NIRF imaging, Clairvivo OPT system, which can take images from five different directions at one time, was used. (b) Graphical image of three types of ICG-poly(lactic acid)s used for Lactosome labeling. (c) Time-courses of NIRF signal intensities detected at three ROIs as illustrated in Figure. 4a.

Interestingly, the amounts of Lactosomes accumulated at the tumor region in the time range from 2 h to 9 h after the administration become the highest with ICG-Lactosome(DL) and decrease in the order of ICG-Lactosome(DL) > ICG-Lactosome(L) > ICG-Lactosome(D). However, at 48 h after the administration, the amount with ICG-Lactosome(D) is the highest and decreases

reversely in the order of ICG-Lactosome(D)> ICG-Lactosome(L)>ICG-Lactosome(DL) (Figure. 5).

In vivo blood clearance times of these Lactosomes were studied. Blood serums were collected from mice at 2, 8, 24, and 48 h after the ICG-Lactosome administration. The decays of ICG fluorescein intensities in the serums were analyzed and fitted with a single component exponential decay curves (Figure. 5).

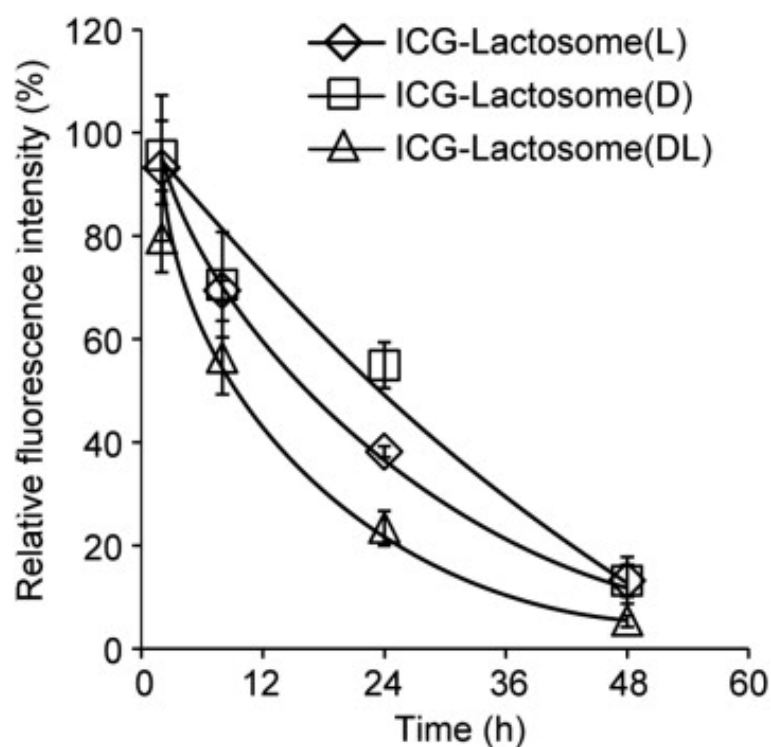


Figure. 5. Time courses of ICG amounts of three kinds of Lactosomes remained in plasma. NIRF Intensities were analyzed at 2, 8, 24, 48 h after the ICG-Lactosome administrations (n=3–4).

The attenuation constants (half-life time in the blood stream) of ICG-Lactosome(L), ICG-Lactosome(D), and ICG-Lactosome(DL) are calculated to be 0.042 h⁻¹ (24 h), 0.018 h⁻¹ (54 h), and 0.070 h⁻¹ (14 h), respectively, suggesting that the stability of

Lactosomes in the blood stream decreases in the order of ICG-Lactosome(D)>ICG-Lactosome(L)>ICG-Lactosome(DL). The amounts of Lactosome accumulated at the tumor region at 48 h after the administration, which is a far longer period than the half-life times of Lactosomes, decrease reasonably in the order of the stability decrease in the blood stream. The accumulation at the tumor region therefore increases reasonably with high concentrations in the blood stream for a long period. On the other hand, in the initial period after the administration, the reversed order of the accumulation amounts suggests that there should be another factor for determining the accumulation at the tumor region. Indeed, in the initial period, the concentrations of Lactosomes in the blood stream are not so different among Lactosomes because the time periods are shorter than the half-life times of Lactosomes. For another factor, for example, flexibility of Lactosomes may determine the easiness of Lactosome leakage from blood vessel walls at the tumor region. ICG-Lactosome(DL) micelles are considered to be flexible by less crystalline structure with incorporation of PDLLA-30 than other Lactosomes. The flexible micelles therefore may tolerate distortion for Lactosome leakage through the defects of the blood vessel walls, however, which is remained to be studied.

ICG-Lactosome(D) shows the highest stability in the blood stream, and its accumulation at the targeted tumor region is the largest among the three types of Lactosomes. However, from the viewpoint of tumor imaging, the tumor/background signal contrast is important. In order to check this point, the tumor/background and the tumor/liver ICG signal intensity ratios are studied (Figure. 6). The signal contrasts become higher with time after the administration to reach to the tumor/background ratio of ca. 5 and the tumor/liver ratio of 3.6 with using ICG-Lactosome(DL) at 48 h after the

administration, which are the highest values among Lactosomes. These data suggest that the signal disappearance from the background is influential on the signal contrast, and ICG-Lactosome(DL) of the short blood clearance time but without impairing the EPR effect is favorable for tumor imaging.

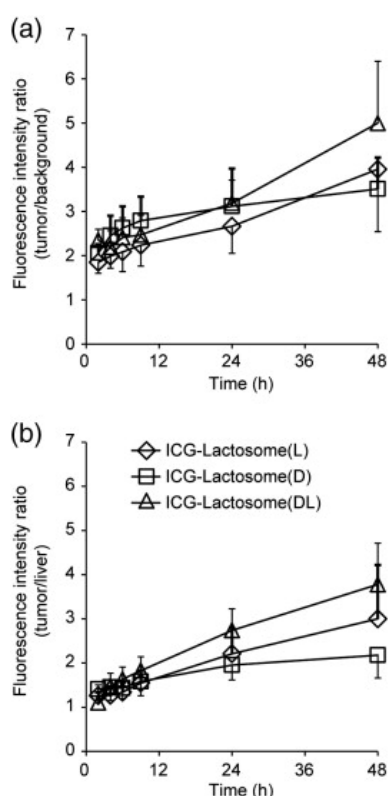


Figure. 6. Time courses of fluorescence intensity ratio at tumor against (a) background and (b) liver.

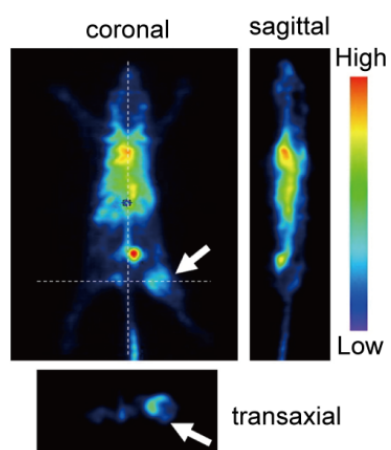
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Chapter 7

Radiosynthesis and Initial Evaluation of ^{18}F Labeled Nanocarrier
Composed of Poly(L-lactic acid)-*block*-poly(sarcosine) Amphiphilic
Polydepsipeptide



Introduction

Cancer has been the leading cause of death since 1980's in Japan with currently more than 300000 people dying from it every year, and presents an important public health challenge to people in terms of incidence, mortality and economic burden [1]. In order to reduce mortality rates, diagnosis of tumors at the early stages is quite important in evaluation of the metastatic potential and potential response to therapy as well as the detection of tumor itself.

Positron emission tomography (PET) is one of the popular molecular imaging techniques of normal and/or abnormal biologic processes at the cellular and molecular level which has attracted attention recently as a tool for diagnosis, therapeutic evaluation, and prognostic evaluation of tumors [2-4]. 2-Deoxy-2-[^{18}F]fluoro-Dglucose (FDG), which allows the evaluation of glucose metabolism, is one of the most commonly used radiopharmaceuticals in oncology. FDG-PET imaging, however, is rather poor at detection of brain tumors, liver cancer, gastric cancer, prostate cancer, renal carcinoma and bladder cancer due to the accumulative property into organs or urination of FDG [5, 6]. To avoid these problems, many novel post FDG radiopharmaceuticals including nanomedicines such as liposomal nanoparticle drugs are currently developed for the diagnosis and detection of tumors [7-10].

Nanocarriers with diameters from a few tens of nm to 200 nm for cancer imaging probes and anti-cancer reagents are expected to accumulate in solid tumors due to the enhanced permeability and retention (EPR) effect [11-17]. The advantages of using polymers for the nanocarriers are the physical stability compared with liposome made of lipids or other metal [18], and their facile size adjustment in the range from a few ten nm to a hundred nm. Furthermore, polymeric nanoparticles composed of polypeptides

are considered to be excellent in terms of biocompatibility and biodegradability for molecular imaging studies [19-21].

Core-shell-type micelle was already prepared from di- and triblock polymers composed of PLLA and poly-(ethylene glycol) (PEG) [22]. Instead of using PEG, which is pointed out to cause allergic reactions [23], poly(sarcosine) (PS) was used here for the hydrophilic segment. Sarcosine is a naturally occurring amino acid and highly water-soluble. Copolymers composed of PLLA and PS are thus considered to be biodegradable and biocompatible. The author especially focuses on and have developed polymeric micelles composed of amphiphilic polydepsipeptides, poly(L-lactic acid)-*block*-poly(sarcosine), named as “Lactosome” [24], as a novel nanocarrier which possesses a tumor accumulation property due to the EPR effect. The author has examined nanoparticles labeled with near-infrared fluorescence chromophores as molecular probes for solid tumor imaging [25-27]. Lactosomes are designed to show a prolonged *in vivo* blood clearance time. Two characteristics of Lactosomes prevented them from being trapped by the reticuloendothelial system, such as the liver and spleen and/or excreted through liver and kidney. First, the diameter of the polymeric micelles is adjusted to be 35 nm with a narrow size distribution. Second, the surface of the micelles is densely covered by hydrophilic poly(sarcosine) chains. Further, Lactosomes are composed of the biocompatible polydepsipeptide. Indeed, a near-infrared fluorescent (NIRF) imaging of tumor-bearing mouse using NIRF labeled Lactosomes was successful for imaging liver tumors resulting from orthotopic implantation [25].

If Lactosomes labeled with ^{18}F instead of NIRF agents are developed, they have the potential to be useful radiopharmaceuticals for tumor diagnosis by PET. In this study, with the aim of developing radiotracers for *in vivo* solid tumor imaging by PET, ^{18}F

labeled Lactosomes were synthesized and evaluated.

Materials and methods

General

Unless otherwise stated, all chemical reagents and solvents were obtained from commercial sources and were used directly. Gel filtration column chromatography was performed on Sephacryl S- 100 High Resolution (GE Healthcare, Little Chalfont, UK), and analytical TLC and radio-TLC were carried out on Silica gel 60F 254 (Merck & Co., Inc., Whitehouse Station, NJ, USA). In the synthetic procedures, organic extracts were routinely dried over anhydrous Na_2SO_4 and evaporated with a rotary evaporator under reduced pressure. Fluorine-18 was produced by an ultracompact cyclotron (CYPRIS model 325R; Sumitomo Heavy Industry Ltd., Tokyo, Japan) at Kyoto University Hospital or a cyclotron (HM-18, Sumitomo Heavy Industry Ltd., Tokyo, Japan) at Hamamatsu Photonics PET Center from $[\text{}^{18}\text{O}]\text{H}_2\text{O}$ by the $^{18}\text{O}(\text{p,n})^{18}\text{F}$ reaction. Radiochemical yields were expressed at the end-of bombardment (corrected for decay), relative to the amount of the $[\text{}^{18}\text{F}]$ fluorinating agent measured as total radioactivity present in the reaction vial.

^1H - or ^{19}F -NMR spectra were recorded with a DPX400 (Bruker, Billerica, MA, USA), and the chemical shifts are reported in parts per million downfield from tetramethylsilane or CFCl_3 . UV spectra were recorded with a DU530 Life Science UV/Vis Spectrometer (Beckman Coulter Inc, Fullerton, CA, USA) or a BioPhotometer plus 6132 (Eppendorf, Hamburg, Germany). The hydrodynamic diameters of the nanocarriers were measured by a dynamic light scattering spectrophotometer, Photall DLS-8000KS (Otsuka Electronics Corp., Osaka, Japan) or Zetasizer Nano-ZS (Malvern

Instruments Ltd., Worcestershire, UK). NIRF images were taken by Clairvivo OPT (Shimadzu Corp., Kyoto, Japan). Animal PET images were taken by Clairvivo PET (Shimadzu Corp., Kyoto, Japan). HPLC was done using a LaChrom Elite system (Hitachi, Tokyo, Japan) fitted with a Cosmosil 5C18MS-II (10×250 mm, Nacalai Tesque, Kyoto, Japan) or TSKgel Super AW6000 (6.0×150 mm, Tosoh Corp., Tokyo, Japan) with monitoring of the radioactivity as well as UV absorption (at 254 nm or 210 nm). The radioactivity was also quantified with a CRC-30 radioisotope calibrator (Capintec Inc., Ramsey, NJ, USA) or ARC-2000 gamma-counter (Aloka, Tokyo, Japan). The identity of radiolabeled compounds was supported by HPLC co-injection studies.

All animal experiments were carried out in accordance with the regulations on animal experiments of the Kyoto University.

Synthesis of amphiphilic polymers of poly(Sar)₇₅-*block*-PLLA₃₀

Amphiphilic polymer of poly(Sar)₇₅-*block*-PLLA₃₀ was synthesized as previously reported [24]. The synthesis of the compound was confirmed by ¹H-NMR measurement.

Preparation of aminopolyether-supported potassium [¹⁸F]fluoride (K¹⁸F/Kryptofix) complex

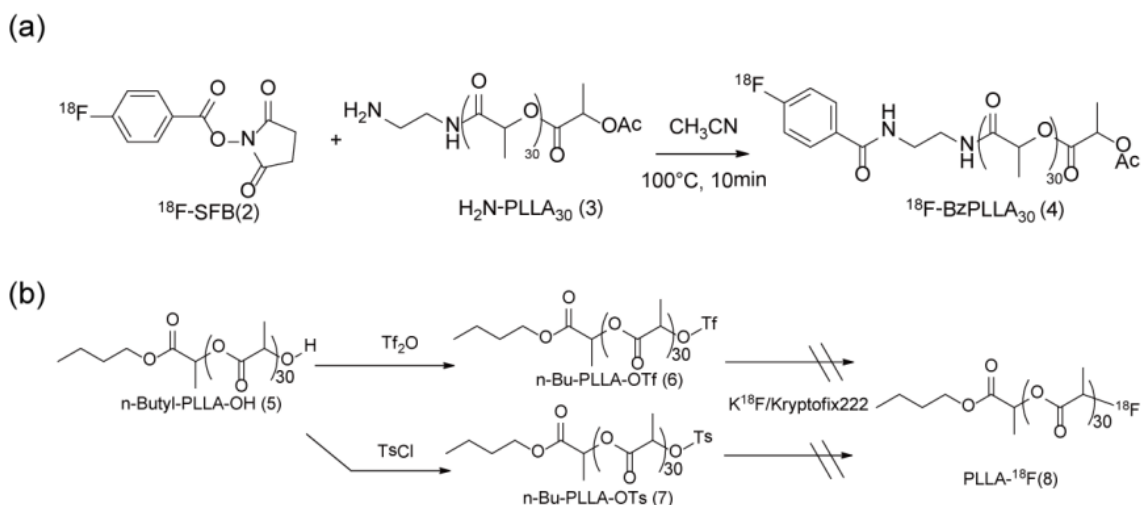
Aminopolyether (Kryptofix 2.2.2)-supported potassium [¹⁸F]fluoride(K¹⁸F/Kryptofix) complex was obtained by a slight modification of the method reported previously [28-30]. In brief, K₂CO₃·1.5H₂O (1.2 mg) and Kryptofix 2.2.2 (3.3 mg) were added to irradiated water which included [¹⁸F]fluoride in a screw capped glass vial, and by subsequent removal of the water under a stream of argon at 110 °C by azeotropic distillation with dry CH₃CN (1 mL). Addition of CH₃CN (1 mL) to the residue and

azeotropic distillation were repeated twice. Finally, the reaction vessel was cooled to room temperature and used for further labeling.

Attempts to preparation of ^{18}F -labeled PLLA

The following are typical procedures of Scheme 1b and the results are summarized in the discussion part. (1) PLLA-OH was supplied by Technology Research Laboratory at Shimadzu Corp. Trifluoromethanesulfonic anhydride (72.4 mg, 257 μmol) and N,N-dimethyl-4-aminopyridine (20.2 mg, 257 μmol) were added to a solution of PLLA-OH (300 mg, 128 μmol) in dichloromethane (4.5 mL). The mixture was stirred at room temperature for 18 h. The reaction mixture was poured into a cooled methanol (35 mL) and centrifuged at 20 °C with 2000 rpm for 10 min. After filtered off a white precipitate, the precipitate was washed with cold methanol and dried under reduced pressure to obtain PLLA-OTf (282 mg, 114 μmol , 89%) as a white powder. ^1H -NMR (CDCl_3) δ : 0.93 (m, 3H), 1.55-1.70 (m, 110H), 1.70-1.80 (m, 8H), 4.08-4.22 (m, 2H), 5.10-5.35 (m, 33H), ^{19}F -NMR (CDCl_3) $\delta(\text{ppm})$: -75.5 (s). PLLA-OTf was dried by co-evaporation with dry CH_3CN under reduced pressure. PLLA-OTf (1.0 mg) was added to a solution of K^{18}F /Kryptofix (113 MBq) in acetonitrile (0.3 mL). The mixture was stirred at 100 °C for 10 min. The reaction mixture was analyzed with gel permeability chromatography by Shodex Asahipak GF-310HQ (7.6 $\times$ 300 mm, flow rate 0.5 mL/min, Showa Denko K.K., Tokyo, Japan) using acetonitrile as the mobile phase. Although UV absorbance peak for the starting material at 15 min as a retention time was disappeared, no peak of radioactivity for the target product PLLA- ^{18}F was observed at 15 min on the HPLC chromatogram. However multi-peaks of radioactivity for decomposition products at 20–25 min as a retention time were observed.

(2) A solution of p-toluenesulfonyl chloride (192.2 mg, 1.01 mmol), N,N-dimethyl-4-aminopyridine (10.3 mg, 0.084 mmol) and triethylamine (102.0 mg, 1.008 mmol) in dichloromethane (3 mL) was added to a solution of PLLA-OH at 0 °C. The mixture was stirred at 0 °C under argon atmosphere for 22 h. The reaction mixture was poured into cooled methanol (50 mL) and centrifuged at 20 °C with 2000 rpm for 10 min. After filtering off a white precipitate, the precipitate was washed with cold methanol and dried under reduced pressure to obtain PLLA-OTs (299.7 mg, 82%) as a white powder. ¹H-NMR (CDCl₃) δ: 0.93 (m, 3H), 1.32-1.80 (m, 119H), 2.45 (s, 3H), 4.08-4.22 (m, 2H), 5.01-5.30 (m, 33H), 7.31-7.35 (d, 2H), 7.82-7.86 (d, 2H). PLLA-OTs (1.0 mg) was added to a solution of K¹⁸F/Kryptofix (52 MBq) in acetonitrile (0.3 mL). The mixture was stirred at 50 °C for 10 min. The reaction mixture was analyzed with gel permeability chromatography by Shodex Asahipak GF-310HQ (7.6×300 mm, flow rate 0.5 mL/min) using acetonitrile as the mobile phase. Although UV absorbance peak for the starting material at 15 min as a retention time disappeared, no peak of radioactivity for the target product PLLA-¹⁸F was observed at 15 min on the HPLC chromatogram. However multi-peaks of radioactivity for decomposition products at 21–28 min as a retention time were observed.



Scheme 1. a) Synthesis of ^{18}F -BzPLLA₃₀. b) Attempted synthesis of PLLA- ^{18}F .

Preparation of succinimidyl 4- ^{18}F fluorobenzoate (^{18}F -SFB)

Succinimidyl 4- ^{18}F fluorobenzoate(^{18}F -SFB) was prepared according to the one-pot procedure with some modifications [31, 32]. In this study, two kinds of precursors, ethyl-(4-trimethylammmonium) benzoate trifluoromethane-sulfonate or t-butyl-(4-trimethylammmonium)benzoate trifluoromethane-sulfonate, were used for ^{18}F -SFB preparation. Ethyl-(4-trimethylammmonium)benzoate trifluoromethane-sulfonate was supplied from Hamamatsu Photonics PET Center laboratory. t-Butyl-(4-trimethylammmonium) benzoate trifluoromethane-sulfonate was supplied from Professor Hideo Saji at Kyoto University Graduated School of Pharmaceutical Sciences. The precursor (1.0 mg) in 0.5 mL of CH_3CN was added to the dried K^{18}F /Kryptofix complex mentioned above and reacted at 100 °C for 15 min. After the reaction mixture had been cooled to room temperature, 50 μL of 10% Bu_4NOH in methanol or a mixture of 20 μL of 1 M Pr_4NOH in methanol and 0.5 mL of CH_3CN was added; and hydrolysis was carried out at 100 °C for 5 min.

Then, N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU,

10 mg) in 0.3 mL of CH₃CN was added to the reaction mixture and converted to ¹⁸F-SFB at 100 °C for 5 min. The reaction mixture was diluted with 10 mL of 5% CH₃COOH and passed through a Sep-Pak C18 cartridge (Waters, Corp., Milford, MA, USA). ¹⁸F-SFB retained on the cartridge was washed with water (1 mL) and then released with 0.3 mL of CH₃CN. Then, the ¹⁸F-SFB was concentrated by argon at 100 °C and used for labeling. The radiochemical yield of ¹⁸F-SFB was 10.2%–13.4% at the end of bombardment (EOB).

For PET study using ¹⁸F-SFB with large amount of radioactivity, the ¹⁸F-SFB was synthesized by using a modified CUPID system (Sumitomo Heavy Industries, Tokyo, Japan). The HPLC column used for the purification of ¹⁸F-SFB was an Inertsil ODS-3 (10×250 mm, 5 μm, GL Sciences Inc., Tokyo, JAPAN).

Preparation of 4-¹⁸F-benzoyl-PLLA₃₀

Free amino group bearing poly(L-lactic acid) (H₂N-PLLA₃₀) was obtained as an initiator for the sarcosine NCA polymerization in the synthesis of amphiphilic poly(Sar)₇₅-*block*-PLLA₃₀ polymers. The coupling reaction of the polymerNH₂ terminal end with ¹⁸F-SFB was carried out as follows. A solution of ¹⁸F-SFB (92.5 MBq-1.94 GBq) in acetonitrile (300 μL) was added to a solution of H₂N-PLLA₃₀ (1.5-2.9 mg) in DMSO (300 μL). The mixture was stirred at 100 °C for 10 min. The reaction mixture was purified with gel permeability chromatography by Shodex Asahipak GF-310HQ (7.6×300 mm, flow rate 0.5 mL/min) using acetonitrile as the mobile phase. 4-¹⁸F-benzoyl-PLLA₃₀ (¹⁸FBzPLLA₃₀) was collected as a radioactive peak fraction eluted at 17.6 min as a retention time. The radiochemical yield of ¹⁸F-BzPLLA₃₀ was 22.0%–36.6% EOB. The specific activity was 17.0–644.0 MBq/μmol.

Preparation of ^{18}F -labeled Lactosome

A dichloromethane solution (1.5 mL) of poly(Sar)₇₅-*block*-PLLA₃₀ polymer (9 mg) was poured into a glass test tube. The solvent was removed under the reduced pressure and a thin film was formed on wall surface of the tube. Then, a solution of ^{18}F -BzPLLA₃₀ in acetonitrile (1 mL) was added to the glass tube including a thin film of the amphiphilic block polymer and the solvent was removed under a nitrogen stream at 110 °C. Saline (2 mL) was added to the test tube, and the solution was treated by a bath-type ultrasonic processor for 15 min at 50 °C. The obtained radioactive solution was used without further purification for the biodistribution and PET studies using tumor bearing mice.

To confirm the purity and formation of ^{18}F -labeled Lactosome, a part of the resulting aqueous solution was chromatographed on Sephacryl S-100 HR with 1/15 mol/L phosphate buffer (pH 7.4).

Preparation of NIRF-labeled Lactosome

NIRF-labeled Lactosome was prepared as the previously reported method [25]. Briefly, 1.0 mg of ICG (indocyanine green)-OSu was added to the DMF solution of 2.46 mg of poly(L-lactic acid) (PLLA), whose amino group was designed as an initiator for the sarcosine NCA polymerization at the synthesis of amphiphilic PLLA-*block*-PS polymers. The reaction mixture was stirred at room temperature overnight under light shielding conditions. The reaction mixture was purified with size exclusion chromatography using a Sephadex LH-20 column using DMF as eluent to obtain ICG-PLLA.

A chloroform solution of the poly(Sar)₇₅-*block*-PLLA₃₀ polymer (3 mg) containing 1.5 mol% of the ICG-PLLA was evaporated under the reduced pressure to remove the solvent, and a thin film was formed on wall surface of the tube. Distilled water (3 mL) was added to the test tube, and the solution was treated by a bath-type ultrasonic processor for 10 min at 55 °C. After freeze-drying of the obtained solution to remove water, the residue was dissolved in saline.

Tumor bearing mice

HeLa-K cells (JCRB Cell Bank, National Institute of Biomedical Innovation, Osaka, Japan) were subcutaneously inoculated at 1×10^6 cells in 40 μ L of phosphate-buffered saline (PBS) into the right femur of 6-week-old female nude mice (BALB/c nu/nu; Japan SLC Inc., Hamamatsu, Japan). For the biodistribution and imaging study, the tumor bearing mice were used 13 days after the transplantation. The size of all tumors was a diameter of 5.0–6.0 mm.

Biodistribution study

Tumor-bearing mice were injected with ¹⁸F-labeled Lactosome (10 MBq/mouse) via a tail vein. At 10 min~6 h after the injection, the mice were sacrificed, and blood and organs (heart, lung, liver, kidney, spleen, muscle, bone, tumor, small intestine, stomach, colon, brain, pancreas) were dissected. The weight and radioactivity of blood and of each tissue were measured. Distribution data were presented as percent dose per gram of wet tissue (%ID/g).

***In vivo* small-animal PET cancer imaging**

A saline solution of ^{18}F -labeled Lactosome (5.0–13.6 MBq/mouse, 200 μL) was injected to tumor-bearing mice, and their PET images were taken by Clairvivo PET. Scans were conducted at 0, 2, 4, and 6 h after injection of ^{18}F -labeled Lactosome. Until each measuring time point, the animals were kept under unanesthetized condition. During measurement, the mice were kept on the imaging stage under anesthetized conditions by an intraperitoneal injection of chloral hydrate at 300 mg/kg, followed by continuous infusion of the anesthetic at 100 mg/kg per hour through a cannula placed into the peritoneal cavity. The data were obtained with a list mode data acquisition every 1 s for 15 min at each time point. Reconstruction was made by list-mode DRAMA (two iterations, $\gamma=0.1$) with resolution modeling. The regions of interest were drawn on the tumor and the corresponding area in the left femur.

***In vivo* near-infrared fluorescence (NIRF) cancer imaging**

After the PET analysis, NIRF imaging of the same mice was performed. A saline solution of NIRF-labeled Lactosome (2 $\mu\text{mol/L}$, 50 μL) was injected into tumor-bearing mice, and their NIRF images were taken by Clairvivo OPT. For the measurement, a filter set (*ex.* at 785 nm and *em.* at 845 nm) was used for the measurement of NIRF-labeled Lactosome. The mice were kept on the imaging stage under anesthetized conditions with 2.5% of isoflurane gas in air flow (1.5 L/min). The intensity ratio of fluorescence of the tumor versus the liver was calculated from the photon counts as the average of three mice. The regions of interest were drawn on the tumor and the corresponding area in the left femur.

Results

Preparation of ^{18}F -labeled Lactosome

The ^{18}F -labeled Lactosome for PET studies was prepared by coassembling of ^{18}F -BzPLLA₃₀ with the amphiphilic polymer poly(Sar)₇₅-*block*-PLLA₃₀ (1) (Figure. 1a) [27]. The number-average molecular weight of the hydrophobic PLLA and the hydrophilic PS blocks were determined to be 30-mer and 75-mer, respectively, by ^1H -NMR analysis. The molecular weight of an amphiphilic polymer PS₇₅-*block*-PLLA₃₀ (1) was 7.7 kDa.

^{18}F -BzPLLA₃₀ (4) was prepared by the coupling reaction of H₂N-PLLA₃₀ (3) with ^{18}F -SFB (2) (Scheme 1a). The H₂N-PLLA₃₀ (3) was also used as an initiator for the sarcosine-NCA polymerization for the synthesis of the amphiphilic polymer PS₇₅-*block*-PLLA₃₀ (1). ^{18}F -SFB was prepared according to the one-pot procedure with some modifications to the reported method [31, 32]. In this study, two kinds of precursors, ethyl-(4-trimethylammonium)benzoate trifluoromethane- sulfonate or t-butyl-(4-trimethylammonium)benzoate trifluoromethane-sulfonate, were used for ^{18}F -SFB (2) preparation. The decay-corrected radiochemical yield of ^{18}F -SFB (2) was 11%–13%. The coupling reaction of H₂N-PLLA₃₀ (3) with ^{18}F -SFB was investigated under several conditions as summarized in Table 1. The production of desired ^{18}F -BzPLLA₃₀ (4) during the reaction was followed by the radio-HPLC analysis using a size exclusion column. The best isolated radiochemical yield of ^{18}F -BzPLLA₃₀ was 37% from ^{18}F -SFB (2) with radiochemical purity of over 99%.

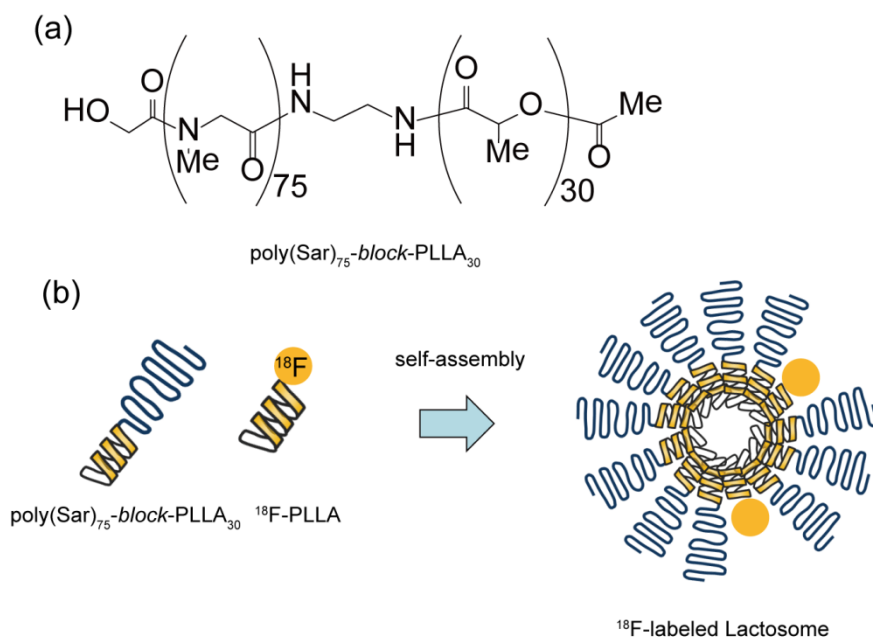


Figure 1. a) Molecular structure of amphiphilic block polymer. b) Schematic representation of preparation of ¹⁸F-labeled Lactosome.

Table 1. Preparation of ¹⁸F-Bz-PLLA by coupling reaction of H₂N-PLLA and ¹⁸F-SFB

	Amount of run substrate(3) (mg)	solvent	¹⁸ F-SFB(2) (MBq)	Reaction temperature (°C)	Reaction rate ^a (radio-HPLC) (%)	Yield (%) ^b	Specific activity (MBq/μmol)
1	1.5	CH ₃ CN	40.7	50	0.0	—	—
2	1.5	CH ₃ CN	40.7	100	8.9	5.9	1.3
3	1.0	CH ₃ CN	44.4	100	30.1	20.0	8.6
4	1.5	CH ₃ CN/DMSO	40.7	100	4.6	1.2	0.2
5	2.9	CH ₃ N/DMSO	92.5	100	67.1	36.6	17.0
6	1.5	CH ₃ CN/DMSO	1579.9	90	26.1	22.0	399.9
7	1.6	CH ₃ CN/DMSO	1935.1	100	42.8	30.1	644.0

a Analyzed by radio-HPLC.

b End of bombardment(EOB)

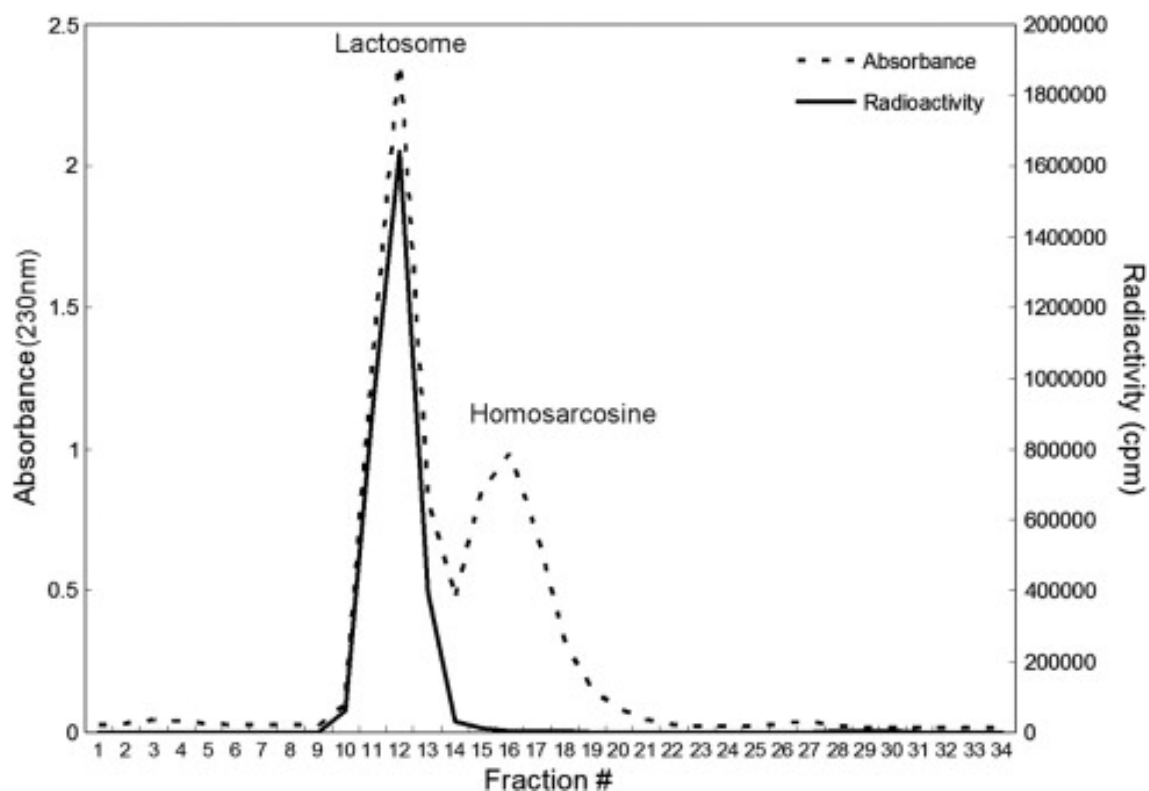


Figure 2. Size exclusion chromatograph of micelle assemblies prepared from a mixture of ^{18}F -BzPLLA₃₀ and the amphiphilic polymer by Sephacryl S-100 eluting with 1/15 M Phosphate buffer (pH 7.4).

Micelle assemblies were prepared from a mixture of ^{18}F -BzPLLA₃₀ and the amphiphilic polydepsipeptide by a film hydration method. A dichloromethane solution of poly(Sar)₇₅-*block*-PLLA₃₀ in a glass test tube was gently evaporated to form a thin film on the wall surface of the glass tube. A solution of ^{18}F -BzPLLA₃₀ in acetonitrile was added into the glass tube to remove the solvent under a nitrogen stream at 110 °C. Saline was added to the test tube, and the dispersion was sonicated at 50 °C. Usually, 222–420 MBq of ^{18}F -labeled Lactosomes was obtained. Over 99% of ^{18}F -BzPLLA₃₀ was integrated into the molecular assemblies (Figure. 2). Total synthesis time was 200–220 min from start of the ^{18}F -SFB preparation.

Biodistribution

^{18}F -labeled Lactosome was injected via the tail vein of BALB/cAJclnu/nu mice bearing HeLa cells in the femur region, and the distribution of ^{18}F activity in the various tissues was analyzed (Figure. 3). Among the tissues examined, the highest concentration of radioactivity was found in the blood fraction. ^{18}F level in the blood appeared to retain a higher level than any other tissues over a 6 h period, but showed continuous slow clearance of the radiotracer with time. The ^{18}F concentration in the liver, kidney and spleen was not so high compared with that in the blood. However, the concentration in the liver and spleen showed a continuous increase of the radiotracer with time. Radioactivity in the kidney remained essentially constant for the time period up to 6 h. The accumulations of radioactivity were quite low in the small intestine, stomach, colon and brain compared to other tissues. The extent of defluorination for ^{18}F -labeled Lactosome, indicated by bone radioactivity, was kept at a low level at 10 min, then slightly decreased to 6 h postinjection. The ^{18}F concentration in the tumor was also lower than that in the blood, but higher than that in muscle tissue (the healthy femur region) and increased continuously with time up to 6 h.

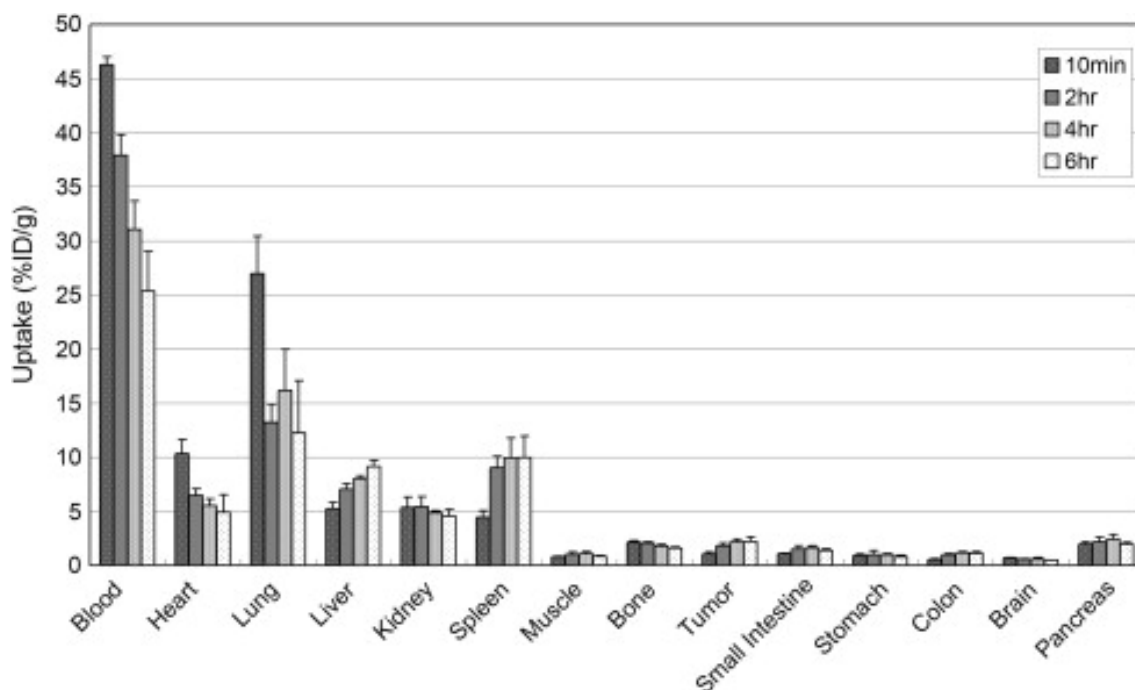


Figure 3. Tissue distribution of radioactivity after IV injection of ^{18}F labeled Lactosome in HeLa bearing mice ($n = 3$). Data points are means $\pm$ standard deviation.

***In vivo* PET imaging**

After co-injection of saline solution of ^{18}F -labeled Lactosome with NIRF-labeled Lactosome, which was prepared as previously reported [25], to tumor bearing mice via the tail vein, the PET images were initially taken by Clairvivo PET (Figure. 4). The radioactivity of ^{18}F spread over the whole body through the blood circulation. At 6 h after injection of the tracer, the PET image showed that both the heart and the bladder were strongly visualized. In addition, high level radioactivity in the trunk was observed. However, peripheral organs, such as the brain, muscle and bone, were not visualized at 6 h. The tumor in the right femur region (tumor) was clearly visualized at 6 h. The calculated tumor-to-muscle ratio was 2.2 ± 0.1 .

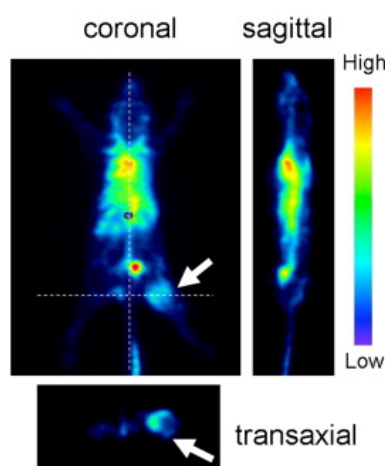


Figure 4. Typical PET images of the tumor bearing mice. The images were at 6 h after co-injection of ^{18}F -labeled Lactosome with NIRF-labeled Lactosome. Tumor can be visualized (arrow).

***In vivo* NIRF imaging**

In order to compare the tumor accumulation of ^{18}F -labeled Lactosome with that of NIRF-labeled Lactosome, NIRF images of the same tumor-bearing mice were taken by Clairvivo OPT (Figure. 5) immediately after the PET imaging. At the same position in the case of PET image of ^{18}F -labeled Lactosome, the tumor was visualized by an NIRF imaging. The accumulation was absent in the trunk, heart and bladder, which were different from those of the PET imaging. The strong signal above the tail on the left may be urinary contamination including NIRF-labeled Lactosome metabolites. The calculated tumor-to-muscle ratio was 1.5 ± 0.3 .

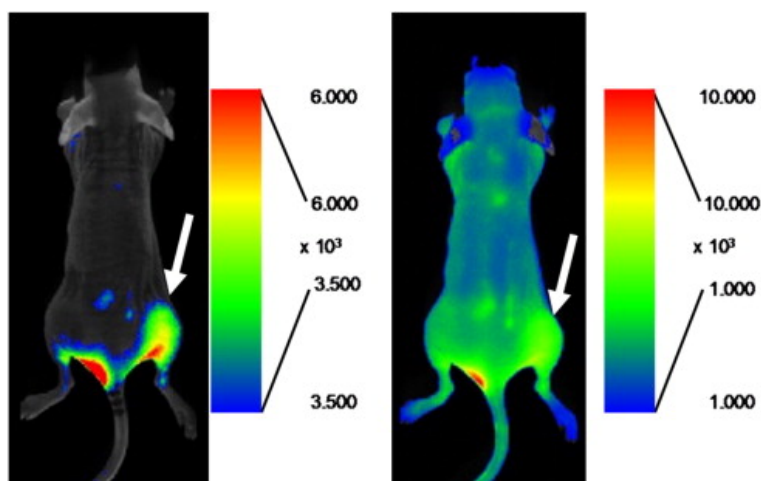


Figure 5. Typical NIRF images of the tumor bearing mice. The images were at 6 h after co-injection of ^{18}F -labeled Lactosome with NIRF-labeled Lactosome. Tumor can be visualized (arrow).

Discussions

Chemistry of ^{18}F -labeled Lactosome preparation

The hydrophobic core of Lactosome polymer micelle is constructed by dense molecular packing of helical PLLA blocks, where the labeled PLLA at low concentrations can be easily incorporated with keeping the size of polymer micelles [25]. For PET studies, the author designed Lactosome containing ^{18}F -labeled PLLA (Figure 1b). For an ^{18}F -labeled PLLA, PLLA- ^{18}F (8) was initially designed and was attempted to be prepared according to the typical ^{18}F labeling method; starting from n-butyl-PLLA-OH (5) via its triflate n-butyl-PLLA-OTf (6) or tosylated n-butyl-PLLA-OTs (7) by displacement with fluoride. However, substitution of both the triflate and the tosylate with K^{18}F /Kryptofix in acetonitrile gave multicomponent mixtures from which the desired fluorinated compound could not be isolated. This can be explained by the fact that the ^{18}F labeled poly(lactic acid) produced was easily

hydrolyzed under basic conditions (Scheme 1b).

N-Succinimidyl 4- ^{18}F fluorobenzoate (^{18}F -SFB) (2) is known as an indirect labeling reagent for protein, peptide or antibody with ^{18}F used for PET [31-33]. Another design of the incorporatable ^{18}F -labeled PLLA into the Lactosome, therefore, is ^{18}F -BzPLLA₃₀ (4), which was prepared by the coupling reaction of the H₂N-PLLA₃₀ (3) with ^{18}F -SFB (2) (Scheme 1a). As summarized in Table 1, the amount of substrate (3), reaction solvents and temperature affected the coupling yields. The best reaction conditions to obtain (4) were found to be a 10 min reaction time at 100 °C in acetonitrile/DMSO using 2.9 mg (1.3 μmol) of the substrate (3). This condition gave an isolated radiochemical yield of 37% (EOB) after a processing time of 200 min from the start of the ^{18}F -SFB (2) preparation (Table 1, run 5). The target compound (4) was also obtainable with use of CH₃CN as a reaction solvent, although the radiochemical yield was low and a long heating duration was required for completion of the reaction.

^{18}F -BzPLLA₃₀ after HPLC purification was still a mixture with the unreacted H₂N-PLLA₃₀ which was excessively added in the reaction. The author tried other purification procedures, for example, using a size exclusion column of HPLC but this was found to be less satisfactory. The effect of the presence of H₂N-PLLA₃₀ on the Lactosome assembly was checked by measuring the hydrodynamic diameter of Lactosome prepared from a mixture of 1.5 mg H₂N-PLLA₃₀ and 9 mg of poly(Sar)₇₅-*block*-PLLA₃₀. The DLS measurement showed a diameter of 41.5 nm (PDI of 0.116), which was slightly larger than Lactosome composed of pure poly(Sar)₇₅-*block*-PLLA₃₀ of 35 nm. The author thus decided to use the ^{18}F -BzPLLA₃₀ fraction containing H₂N-PLLA₃₀ where the amount of H₂N-PLLA₃₀ was less than 1.5 mg versus an amount of poly(Sar)₇₅-*block*-PLLA₃₀ of 9 mg at the point of Lactosome

preparation.

To confirm the purity and formation of ^{18}F -labeled Lactosome, a part of the resulting aqueous solution was chromatographed by size exclusion chromatography on Sephacryl S-100 (Figure 2). The elution profile showed a main peak at the fraction #11–13 with strong UV absorption at 230 nm, which is assigned to Lactosome. The small peak observed at the fraction #15–18 is due to homosarcosine, which was a contaminant of the polydepsiptide and was excluded from the molecular assembly because of its high water-solubility. ^{18}F -BzPLLA₃₀ radioactivity was also eluted in the same fraction as Lactosome. Therefore, the elution profile clearly indicated that over 99% of ^{18}F BzPLLA₃₀ was integrated in the molecular assemblies. The obtained radioactive solution of ^{18}F -labeled Lactosome was used for the biodistribution and PET studies using tumor bearing mice without further purification. Usually, 222–420 MBq of ^{18}F -labeled Lactosomes was prepared reproducibly for animal studies.

Table 2 Uptake ratio of tumor/organ of radioactivity after i.v. injection of ^{18}F -labeled Lactosome in HeLa bearing mice.

Tumor/Organ	Uptake Ratio ^a											
	10min			2hr			4hr			6hr		
Tumor/Blood	0.02	±	0.00	0.05	±	0.01	0.07	±	0.01	0.09	±	0.00
Tumor/Muscle	1.29	±	0.23	1.84	±	0.43	2.01	±	0.46	2.66	±	0.36

a) Means±SD of 3 mice

Biodistribution and Imaging studies

The tumor/muscle uptake ratio of radioactivity after ^{18}F -labeled Lactosome injection was high and increased with time (Table 2). At 6 h after the injection, a maximum ratio of 2.7 was observed. The high tumor/muscle ratio is thought to be due to the EPR effect

of Lactosome. The tumor/blood ratio was low due to the slow blood clearance during the post injection time period of up to 6 h. The mean uptake ratios of tumor/digestive tract and tumor/brain were 2.8 and 4.9, respectively.

On the basis of these results, the author performed the imaging studies by Clairvivo PET and by Clairvivo OPT using the same individual tumor bearing mice. The high level of radioactivity in the heart and trunk on the PET images reflected the high stability of Lactosome in the blood stream. In fact, the biodistribution study also showed the slow clearance from the bloodstream of the radiotracer with time over 6 h as described above. Because of the slow blood clearance, the author needed 6 h after administration to obtain clear tumor images. The tumor was visualized by an NIRF imaging at the same position in the PET image as ^{18}F -labeled Lactosome. NIRF-labeled Lactosome, which is a labeled Lactosome with fluorescent dye ICG, is known to accumulate in implanted tumor regions by the EPR effect [25]. The NIRF images in this study provided that the tumor was also clearly visualized similar to that of the PET image. In the case of an NIRF image, accumulation was absent in trunk, a heart and bladder. The difference between the two images may be explained by the fact that fluorescence from a deep part from the surface of the skin was hard to detect. Our group reported previously that the high tumor accumulation of NIRF-labeled Lactosome should be due to the EPR effect [25]. As these results of the biodistribution and the imaging in present study, the tumor accumulation of ^{18}F -labeled Lactosome might be due to the EPR effect similar to that of NIRF-labeled Lactosome.

Generally, PET images should be taken in a short period after injection of tracers. For early blood clearance of ^{18}F -labeled Lactosome radioactivity, the chemical structural modification of an amphiphilic polymer may be necessary to make it possible to be

excreted rapidly from the blood stream. Further study is ongoing for solution of this problem. In addition, the author also has further plans to evaluate of Lactosome by using long-lived radionuclide instead of ^{18}F .

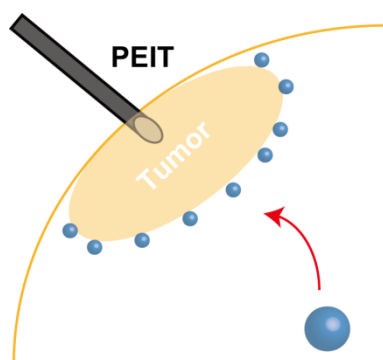
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Chapter 8

Internal Radiation Therapy Using Nanoparticle of ^{131}I -Lactosome in
Combination with Percutaneous Ethanol Injection Therapy



Introduction

Local tumor therapies like radiofrequency ablation (RFA) and percutaneous ethanol injection therapy (PEIT) have been developed for patients who could not get invasive surgery for their age and tumor region [1, 2]. RFA currently has become one of the treatments for hepatocellular carcinoma curative (HCC) [3], breast cancer [4], and Arrhythmic diseases [5]. RFA is a heat coagulation therapy, which uses ultrasound of 350–480 kHz focusing at tumor. As for of HCC treatment, PEIT is more applicable than RFA when the target tumor is located in large vessels and main bile ducts [6]. PEIT uses ethanol injection into tumor regions to induce coagulative necrosis due to dehydration and protein denaturation [7, 8]. PEIT has inherent advantages in the low cost and the safe therapy, but, in some cases, risk of relapse is high when the safety margin is not taken wide enough [8, 9]. In order to improve the therapeutic efficiency, therefore, RFA or PEIT is frequently performed in combination with anti-tumor agents such as epirubicin, cisplatin, and 5-fluorouracil [10, 11].

It has been reported that the biomarker of inflammation was slightly raised after RFA or PEIT treatment [5, 12, 13]. Nanoparticles are known to accumulate in tumor and inflammation regions by the enhanced permeability and retention (EPR) effect [14, 15]. This is because nanoparticles can leak out to tumor and inflammation regions from the leaky blood vessels and stay there for a long period due to an undeveloped lymphatic system. The accumulation of nanoparticles in solid tumors should, therefore be promoted by simultaneous treatment with RFA or PEIT.

The nanoparticle Lactosome is a polymeric micelle with diameter of 30–40 nm [16-18]. Lactosome is composed of biodegradable and biocompatible amphiphilic polymers of hydrophilic poly(sarcosine) of 60–90mer and hydrophobic poly(L-lactic

acid) of 30mer [19, 20]. The Lactosome surface is densely covered with hydrophilic poly(sarcosine) chains, and therefore, Lactosome is hardly recognized by the self-defense system of living organisms like the reticuloendothelial system. Indeed, human liver carcinoma cells orthotopically implanted on liver were successfully imaged by Lactosome carrying a near-infrared fluorescent (NIRF)-dye, indocyanine green (ICG), because of low-background signal in liver due to the escape ability of Lactosome from the healthy region of the liver (the stealth property of Lactosome) [17].

The author hypothesized that RFA or PEIT treatment induce petit inflammation in the tumor region and the marginal zone, resulting in further accumulation of the polymeric micelle on the basis of the promoted EPR effect. In this study, the effect of pretreatment with PEIT on radionuclide therapy of ^{131}I labeled Lactosome (^{131}I -Lactosome) is examined. The beta decay radioisotope of ^{131}I [21, 22] carried on Lactosome is expected to accumulate more effectively in tumor, leading to suppression of tumor proliferation with combination use of PEIT.

Experiment

Instruments

^1H -NMR spectra were recorded with a DPX400 (Bruker, Billerica, USA). Hydrodynamic diameter of Lactosome was analyzed by dynamic light scattering (DLS) on a Nano-ZS (Malvern, UK). UV assay was performed by Microplate Reader on iMark (BioRAD, USA). γ -Counter used was COBRA II (Packard Instrument, USA). Near infrared fluorescence (NIRF) images were taken by Clairvivo OPT (Shimadzu Corp. Japan).

Materials and ^{131}I -Lactosome preparation

Synthesis of poly(sarcosine)₆₄-*block*-poly(L-lactic acid)₃₀ (poly(Sar)₆₄-*block*-PLLA₃₀), and Lactosome preparation was performed as previously reported [17, 18]. The block lengths were determined by ^1H NMR measurement as poly(Sar)₆₄-*block*-PLLA₃₀. ^{131}I -labeled poly(L-lactic acid)₃₀ (^{131}I -BzPLLA₃₀) was synthesized by coupling reaction of *N*-succinimidyl 3- ^{131}I iodobenzoate (^{131}I SIB) to an amino group introduced at the PLLA terminal (Figure 1). ^{131}I SIB was synthesized with using ^{131}I NaI according to the reported method [23]. Briefly, to the solution of ^{131}I SIB (29.8 MBq) in acetonitrile (150 μL), H_2N -PLLA₃₀ (1.5 mg) in DMSO (150 μL) and triethylamine (0.20 μL) were added. The reaction mixture was stirred at 100 $^\circ\text{C}$ for 20 min, and then purified by gel permeation chromatography using Shodex Asahipak GF310-HQ column (7.5 $\times$ 300 mm) eluted with acetonitrile 0.5 mL/min to obtain ^{131}I -BzPLLA₃₀ (25.2 MBq) in acetonitrile. The decay-corrected total radiochemical yield of ^{131}I -BzPLLA was 39 % from ^{131}I NaI. A mixture of ^{131}I -PLLA₃₀ (1.5 mg) and poly(Sar)₆₄-*block*-PLLA₃₀ (9 mg) was used to prepare polymeric micelle of ^{131}I -labeled Lactosome (^{131}I -Lactosome) by the film rehydration method (Figure 2). Briefly, Poly(Sar)₆₄-*block*-PLLA₃₀ (9.0 mg) was mixed in a acetonitrile solution of ^{131}I -BzPLLA₃₀ (1.5 mg, 25.2 MBq). The solution was evaporated using Soltra mini (Biotech Lab, Japan) to form polymer film on a glass tube wall. Saline (1.0 mL) was added to the test tube, and the dispersion was sonicated at 85 $^\circ\text{C}$ for 2 min to form ^{131}I -Lactosome.

The hydrodynamic diameter of the polymeric micelle was determined to be 46 nm (PDI of 0.057) by DLS measurements, which was slightly larger than Lactosome composed of single component of poly(Sar)₆₄-*block*-PLLA₃₀ with diameter of 33 nm (PDI of 0.086). Non radioisotope labeled Lactosome was also prepared in the same

manner but without ^{131}I -PLLA₃₀.

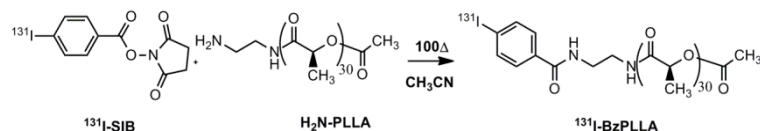


Figure 1 Synthetic route of ^{131}I -labeled poly(L-lactic acid) (^{131}I -BzPLLA)

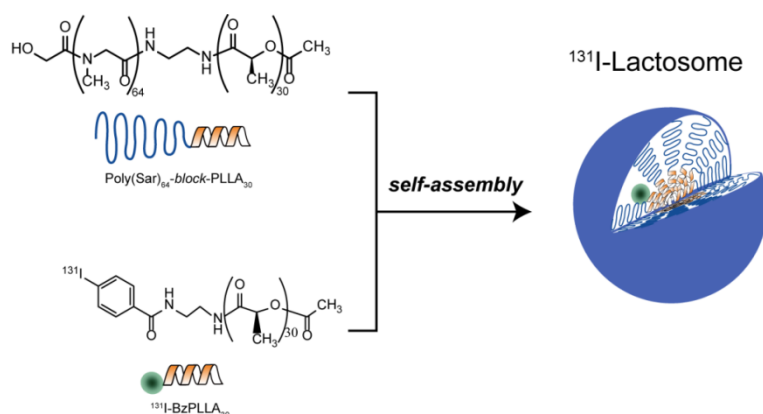


Figure 2 Illustration of the self-assembled molecular assembly, ^{131}I -Lactosome

Indocyanine green (ICG)-Lactosome preparation

ICG labeled PLLA 30-mer (ICG-PLLA₃₀) were synthesized as previously reported [17, 18, 24]. A mixture of poly(Sar)₆₄-block-PLLA₃₀ (1 mg) and ICG-PLLA₃₀ (2 nmol: 1.5 mol% ratio to poly(Sar)₆₄-block-PLLA₃₀) in chloroform was evaporated to form a thin polymer film on the test tube. Water (1 mL) was added and heated at 85 °C for 20 min. After freeze-drying the solution, saline (1 mL) was added, and the solution was filtered (pore size: 0.20 μm) just before administration to mice.

Cell culture

Murine mammary cancer (4T1) cell line was purchased from JCRB (Osaka, Japan).

4T1 cell line was maintained at 37 °C with 5% FBS (Nacalai Tesque, Inc. Kyoto, Japan) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Corp. USA) supplemented with GlutaMAX™-I Supplement (2 mmol/L, Gibco, Invitrogen Corp. USA), Plasmocin™ prophylactic (5 µg/mL, Nacalai Tesque, Inc. Kyoto, Japan), penicillin (100 U/mL), and streptomycin (100 µg/mL).

***In vitro* assay**

4T1 cells were plated in 96-well plate (500 cells, 100 µL/well). The well was incubated for 24 h, and ¹³¹I-Lactosome (15.6, 31.3, 62.5, 125, 250, 500 kBq/10 µL/well, n=3) was added. As a control, saline and non-radioisotope (non-RI) labeled Lactosome (0 kBq/10 µL/well) were added. [¹³¹I]NaI instead of ¹³¹I-Lactosome was also examined. In all experiments, the amount of Lactosome was set to be 0.2 mg/well. At 24 h, 48 h, 72 h after addition of ¹³¹I-Lactosome, the medium was aspirated and Cell Count Reagent SF (10 µL/well, Nacalai Tesque, Inc. Kyoto, Japan) and DMEM (90 µL/well) were added. UV assay (450 nm) of the well was performed at 2 h from the reagent addition with Microplate Reader on iMark (BioRAD, USA).

Preparation of tumor-bearing mice

Hairless SCID (body weight: 25 g, n=3–5 per group) was purchased from Oriental Bioservice, Inc. (Japan). 4T1 cells (5×10^5 cells) were suspended in phosphate-buffered saline (PBS, 20 µL), and inoculated into the left mammary gland region of 6–7 weeks old female mice. The mice were separated according to the tumor size after 5 days from the tumor transplantation.

Biodistribution

Hairless SCID mice implanted with 4T1 cells were divided into two groups (n=3 per group). To one group (PEIT group), dehydrated ethanol (50 μ L) was directly injected to the tumor region at 6 days after the tumor implantation. The tumor volumes were in the range of 70.8 – 140.5 mm² (diameter: 4.0 – 9.8 mm) at the ethanol injection moments. To the other group (control group), the ethanol injection was skipped. In case of human, the required ethanol volume is usually calculated according to the equation: $V \text{ (mL)} = \frac{4}{3}\pi(r + 0.5)^3$ (r = the tumor radius in cm) [25]. However, the ethanol volume injected was fixed to be 50 μ L in the current experiments, because mouse was dead over 50 μ L ethanol dose. At 24 h after the PEIT treatment, ¹³¹I-Lactosome of 3.0 MBq/kg was intravenously administrated to both groups from tail vein. The mice were dissected at 24 h, 48 h, 72 h and 168 h after the ¹³¹I-Lactosome administration and the radioactivity of organs were counted with using γ -counter. The biodistribution data were fitted using Wave Metrics IGOR Pro 4.09A, and tumor areas under curve (AUC) value was calculated.

***In vivo* near-infrared fluorescence (NIRF)-imaging with ICG-Lactosome**

Hairless SCID mice implanted with 4T1 cells were divided into two groups (n=3 per group). To one group (PEIT group), dehydrated ethanol (50 μ L) was directly injected to the tumor region at 6 days after the tumor implantation as described in the Biodistribution section. To the other group (control group), the ethanol injection was skipped. At 24 h after the PEIT treatment, ICG-Lactosome (2 mg/kg, 50 μ L) was intravenously administrated from tail vein to both groups. The amount of the injected ICG probe was set to be 4 nmol/kg. NIRF images were taken at 15 min, 1 h, 3 h, 6 h, 9

h, 24 h and 48 h after ICG-Lactosome administration with Clairvivo OPT (Shimadzu Corp. Japan. Excitation at 785 nm and emission at 845 nm for 10sec). Clairvivo OPT can take five images from different directions with one time shot. The photon counts of at the tumor region, background region (opposite side to the right mammary gland cancer region; the healthy left femoral region), and liver region in NIRF images were analyzed.

In vivo anti-tumor activity

Hairless SCID mice bearing 4T1 were divided into eight groups (n=5 per group). To four groups, PEIT was performed in the same manner as described in the Biodistribution section. As for radionuclide agents, ^{131}I -Lactosome (2.0×10^2 MBq/kg) and $[^{131}\text{I}]\text{NaI}$ (2.0×10^2 MBq/kg) were examined. Their control studies were carried out with using non-RI labeled Lactosome instead of ^{131}I -Lactosome and administration of saline (100 μL) instead of $[^{131}\text{I}]\text{NaI}$. These four kinds of treatments were combined with PEIT treatment and without. In total, there were eight groups of different combinations: (1) non-PEIT with administration of saline (100 μL) (control), (2) non-PEIT with non-RI labeled Lactosome (Lactosome), (3) non-PEIT with $[^{131}\text{I}]\text{NaI}$ ($[^{131}\text{I}]\text{NaI}$), (4) non-PEIT with ^{131}I -Lactosome (^{131}I -Lactosome), (5) PEIT with administration of saline (PEIT), (6) PEIT with non-RI labeled Lactosome (PEIT+Lactosome), (7) PEIT with $[^{131}\text{I}]\text{NaI}$ (PEIT+ $[^{131}\text{I}]\text{NaI}$), and (8) PEIT with ^{131}I -Lactosome (PEIT+ ^{131}I -Lactosome). Tumor sizes and body weights were measured every 2–3 days after the treatments. Tumor sizes were evaluated with using a pair of calipers according to $a \times b^2 \times 1/2$, where a and b are major and minor diameters of tumors, respectively. The relative tumor volume (RTV) was calculated with using the tumor volume of the injection day

as a standard. The body weight gain was the change of the weight from the injection day.

Alanine Transaminase (ALT) and Aspartate Transaminase (AST) Enzymatic Assay

Hairless SCID mice implanted with 4T1 cells were divided into two groups (n=5 per group). To one group (PEIT group), dehydrated ethanol (50 μ L) was directly injected to the tumor region at 6 days after the tumor implantation. To the other group (control group), the ethanol injection was skipped.

The next day, the whole blood of two groups were collected and incubated at 37 °C for 10 minutes. The samples were centrifuged at 10000 rpm for 10 min and the sera were collected.

ALT and AST Enzymatic Assay were performed with Enzymatic Assay kit (Bioo Scientific, Austin, TX). Collected sera (10 μ L) were added to the microplate wells and Reagent Mix (240 μ L) to the wells. The absorbance was immediately measured at 340 nm at 37 °C by Microplate Reader on SPECTRA MAX250 (Molecular Devices, USA) and remeasured after 5 min later. The concentration of ALT and AST were determined by the decrease in absorbance in 5 min by 1072.

Optical characteristics of ICG-PLLA₃₀

The fluorescence intensity of ICG-PLLA₃₀ concentration (1, 1.5, 2, 3, 4, and 8 mol%) in Lactosome were measured by Spectrofluorophotometer on RF-5300PC (Shimadzu Corp. Japan) The total concentrations of ICG and amphiphilic polydepsipeptide were adjusted to 0.48 μ M.

***In vivo* near-infrared fluorescence (NIRF)-imaging with ICG in H₂O**

ICG in H₂O (50 µL) was intravenously administrated from tail vein to BALB/c nu/nu mice implanted with Suit2 cell. The amount of the injected ICG probe was set to be 4 nmol/kg. NIRF images were taken at 5 min after administration with Clairvivo OPT (Shimadzu Corp., Japan. Excitation at 785 nm and emission at 845 nm for 10sec).

Statistical analysis

All results are expressed as mean $\pm$ SD. Differences between groups in biodistribution were assessed by the t-test for independent samples. Differences between the 2 groups against control group in NIRF-imaging and *in vivo* anti-tumor activity were examined for statistical significance by two-way ANOVA using JUMP software (SAS Institute Inc, USA). P-value < 0.05 and P-value < 0.01 were considered statistically significant.

Ethics

All of her *in vivo* animal experiments were approved by the Animal Research Committee of Kyoto University. Animals were treated humanely.

Results and discussion

***In vitro* assay**

The cytotoxicity of ¹³¹I-Lactosome to 4T1 cell was studied with using [¹³¹I]NaI as a control. As a result, inhibition of cell proliferation was observed in the cases of ¹³¹I-Lactosome or [¹³¹I]NaI over 250 kBq/well dose but was not observed in other cases

(Figure 3a and Figure 3b). The beta-decay radioisotope of ^{131}I in the polymer micelle showed therefore the same cell toxicity as $[^{131}\text{I}]\text{NaI}$ in medium.

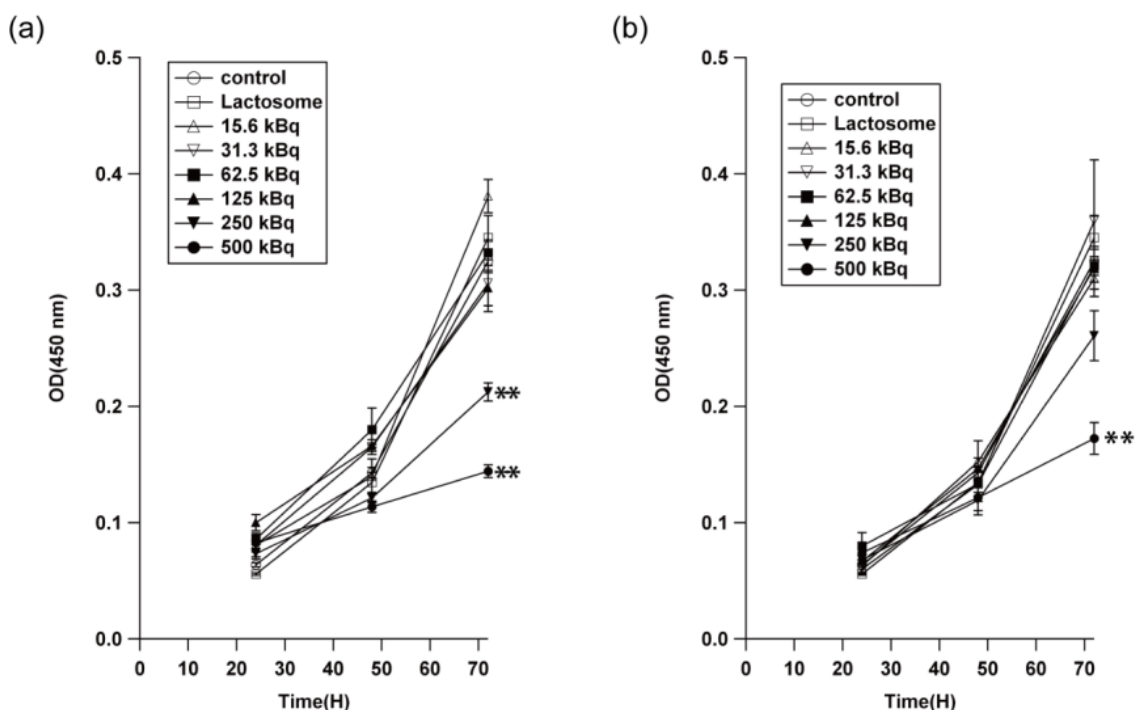


Figure 3 *In vitro* anti-tumor activity on 4T1 cell at 24 h, 48 h, 72 h after addition of ^{131}I -Lactosome (a) and $[^{131}\text{I}]\text{NaI}$ (b). ** $p < 0.01$

Effect of PEIT on ^{131}I -Lactosome biodistribution

The blood-half life time of ^{131}I -Lactosome was calculated to be 15.6 h (Figure 4). The long life time in the blood stream is due to the hydrophilic surface of polymer micelles covered densely with poly(sarcosine) chains. The long life time is favorable for accumulation of Lactosome in solid tumors due to the EPR effect.

^{131}I -Lactosome of 3.0 MBq/kg was intravenously injected both to the PEIT and the control groups, and ^{131}I biodistribution with time was examined by the organs extraction method using γ -counter (Figure 5). The amounts of ^{131}I accumulation in the tumor region became larger with PEIT at all the times after the ^{131}I -Lactosome dosage: at 24 h

9.67 ± 0.77 to 12.1 ± 1.4 , at 48 h 4.03 ± 1.20 to 11.9 ± 6.5 , at 72 h 0.70 ± 0.11 to 2.08 ± 1.43 , and at 168 h $0.04 \pm 0.01\%$ to 0.98 ± 0.64 %ID/g (the numbers represent accumulation without PEIT to that with PEIT). The standard deviations, however, were relatively large, which may be ascribable to difficulty in quantitative tumor resection. Accordingly, the statistical analysis with t-test against control were high (24 h : $p=0.06$, 48 h : $p=0.11$, 72 h : 0.17, 168 h : $p=0.06$). But, the accumulation amounts increased with PEIT at all these four measurement times, suggesting that the promotion effect of PEIT is meaningful. It should be noted that the average ^{131}I -accumulation in the implanted tumor region increased three times higher with PEIT than that without PEIT at 48 h and 72 h after ^{131}I -Lactosome administration.

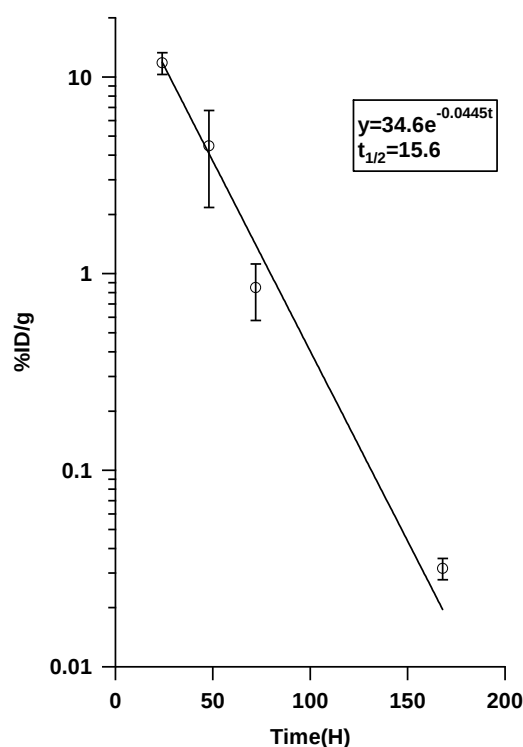


Figure 4 Time course of the residual amount of ^{131}I -Lactosome in plasma. Biodistribution data in Figure. 5 was fitted by IGOR Pro 4.09A, and blood-half life time of ^{131}I -Lactosome was calculated to be 15.6 h

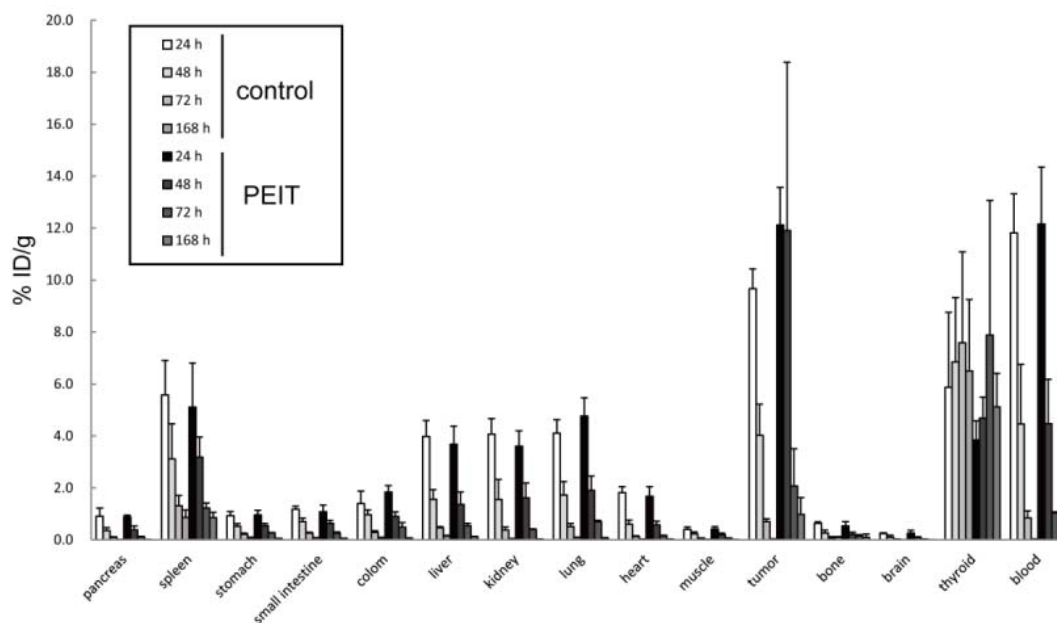


Figure 5 Biodistribution of ¹³¹I at the 24 h, 48 h, 72 h and 168 h after ¹³¹I-Lactosome administration. White and black bars indicate control (non-PEIT), and PEIT groups, respectively

Accumulation amounts of ¹³¹I-Lactosome in the tumor areas between 0–168 h (tumor areas under curve (AUC)) with and without PEIT were determined to be 27.1 and 14.4 [(%ID/g)·h₀₋₁₆₈], respectively, supporting the promotion effect of PEIT on the ¹³¹I-Lactosome accumulation in tumor as expected. On the other hand, ¹³¹I accumulations in other organs were not influenced by PEIT compared with the control group.

PEIT has been reported to raise the biomarker of inflammation [5, 12, 13]. The accumulation of ¹³¹I-Lactosome in tumor region should therefore be increased by the remodeling processes upon inflammation due to the enriched leaky neovascularity.

Combination of chemotherapy such as dose of epirubicin, cisplatin, or 5-fluorouracil with PEIT has been adopted for tumor therapy [10]. However, small molecular agents

including [^{131}I]NaI are in general rapidly excreted from blood circulation [26-28]. Moreover, there is a problem with [^{131}I]NaI, which accumulates selectively in stomach and thyroid gland [29]. On the other hand, blood half-life of ^{131}I -Lactosome was found to be 15.6 h (Figure 4). By encapsulating ^{131}I -PLLA in Lactosome, blood circulation behavior of ^{131}I can be prolonged and prevent ^{131}I from accumulation in stomach and thyroid gland.

The autopsy revealed that there were not any differences in various organs between the control group and the PEIT group. The Alanine Transaminase (ALT) and Aspartate Transaminase (AST) activities which are indices of liver damage were maintained at the same level in the two groups (Figure 6).

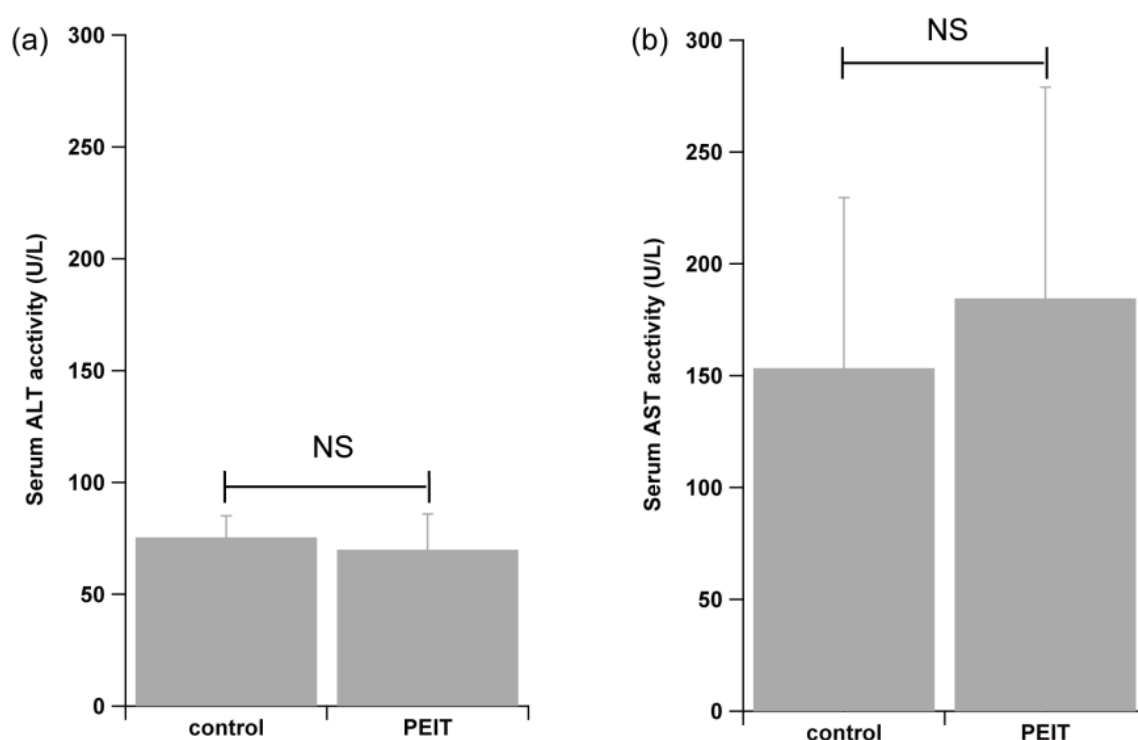


Figure 6 ALT and AST activity in plasma of control group and PEIT group (n=5 per group). Assay was performed at the 24h after PEIT. The p value: not significant (NS)

Effect of Lactosome in combination with PEIT on NIRF imaging

NIRF imaging was performed further to confirm the PEIT effect on the Lactosome accumulation in the tumor region (Figure 7). ICG-Lactosome was injected intravenously to tumor-bearing mice with and without PEIT. The contents of ICG-PLLA₃₀ in Lactosome were maintained at a low level of 1.5 mol%, at which no fluorescence quenching occurs (Figure 8). On the other hand, when the content of ICG exceeds over 2 mol%, the concentration quenching was observed. Administration of ICG alone accumulated in liver immediately (Figure 8). The values of the region of interest (ROI) on the tumor region at 48 h after administration were found to increase up to $20,480 \pm 3,010$ photon counts with PEIT from $5,530 \pm 2,050$ photon counts without PEIT (Figure 7a). Photon counts of liver and the healthy control region were not changed with PEIT (Figure 7b and Figure 7c). Taken together, the promotion effect of PEIT on the Lactosome accumulation in tumor was also supported by *in vivo* NIRF imaging. The NIRF intensity in the tumor region became 3.7 times stronger with PEIT than without (Figure 7d and Figure 7e), which is agreeable with the biodistribution result using ¹³¹I-labeled Lactosome.

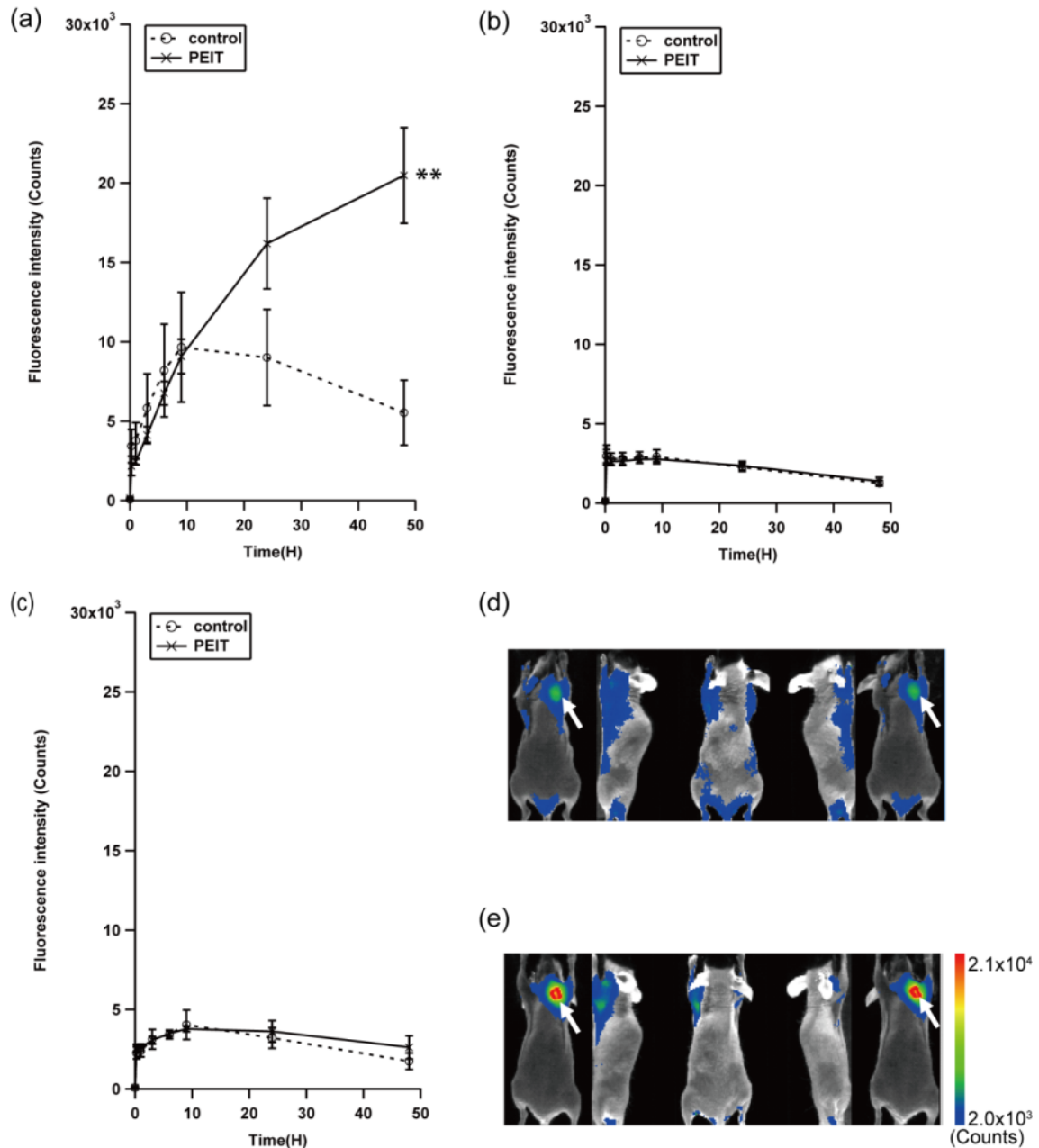


Figure 7 NIRF images of mice without or with PEIT (n=3 per group). Time-course of fluorescence intensity was detected from the NIRF imaging in tumor region (a), background region (the opposite healthy side to the tumor region) (b), and liver region (c). ○: control (non-PEIT), ×: PEIT, * * $p < 0.01$. Images were taken at 48 h after ICG-Lactosome administration; control (non-PEIT) (d) and PEIT (e). Tumor regions are indicated by white arrows. The fluorescein signal ranges were set to be from max count 21000 to min count 2000

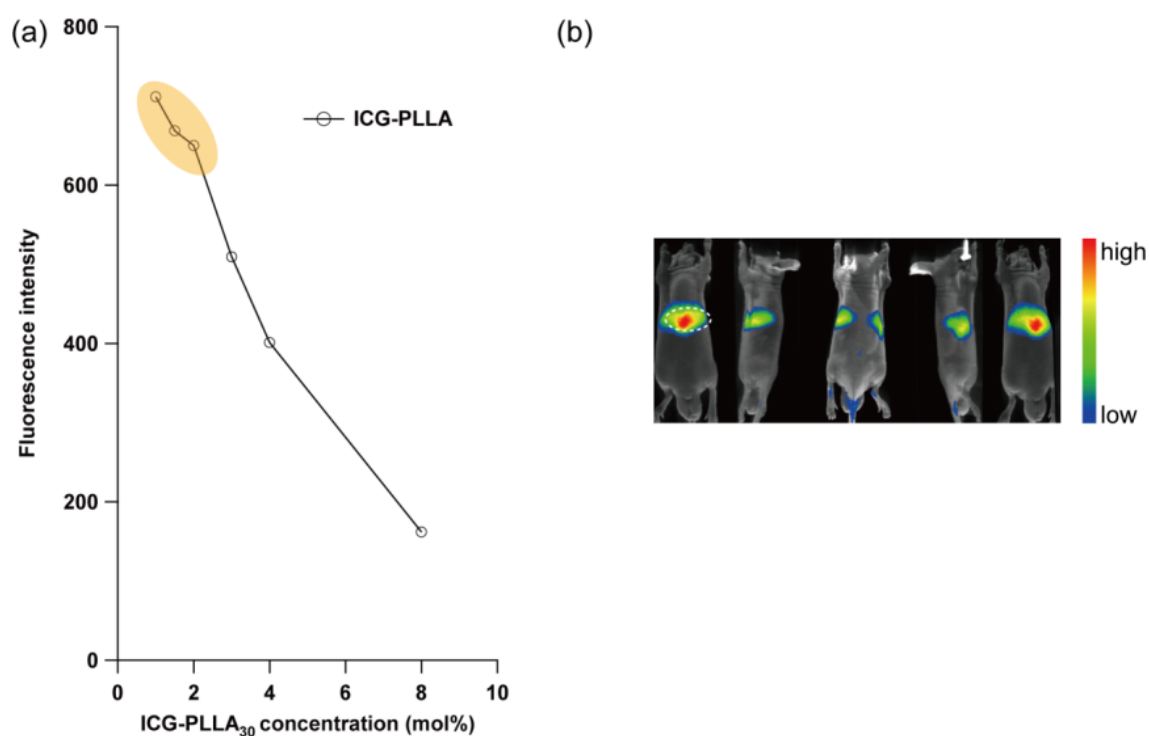


Figure 8 The fluorescence intensity of ICG-PLLA₃₀ concentration 1, 1.5, 2, 3, 4, and 8 mol% in Lactosome. The total concentrations of ICG and amphiphilic polydepsipeptide are 0.48 μ M (a). The mouse image of ICG in H₂O at 5 min after administration (c). The Fluorescence ranges were set to be from max count of 1500 to min count 380. The dotted line indicates liver.

***In vivo* evaluation of anti-tumor activity**

As a pilot study, a radionuclide therapy using ¹³¹I-Lactosome in combination with PEIT was initiated here with relatively a small group of tumor-bearing mice (Figure 9).

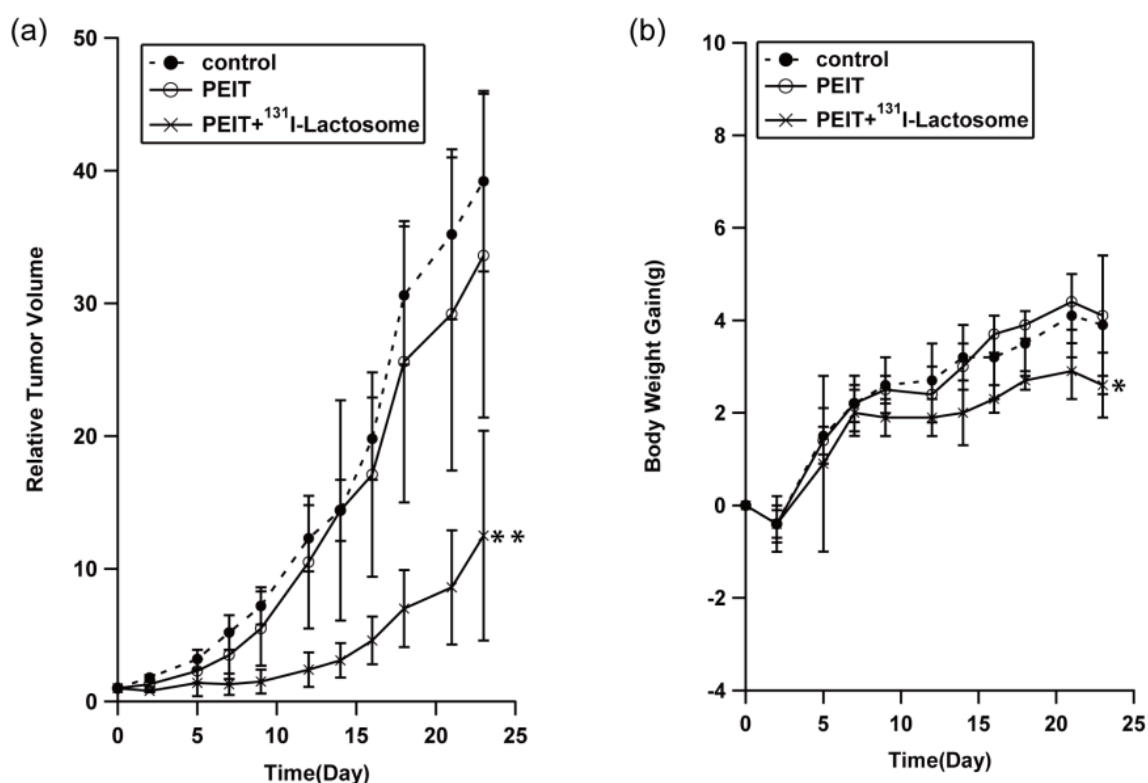


Figure 9 Time courses of (a) relative tumor volumes in mice, and (b) body weight gain (n=5 per group). Radiation dose of ¹³¹I-Lactosome was set to be 2.0×10^2 MBq/kg, and the effect was examined with the 4T1 transplanted tumor model mouse. ●: control (saline), ○: PEIT only, and ×: PEIT in combination with ¹³¹I-Lactosome. * $p < 0.05$, ** $p < 0.01$

¹³¹I-Lactosome (2.0×10^2 MBq/kg) was injected to mice in combination with or without PEIT, and time courses of tumor size and body weight were traced for 16 days (Figure 10). At 16 days after the dosage, the relative tumor volume (RTV) was suppressed to 5.32 ± 1.06 (Figure 10c), which was significantly lower than those of PEIT alone (15.9 ± 5.0) (Figure 10c) and control (no treatment) (21.3 ± 5.9) (Figure 10a and Figure 10c). The ¹³¹I-Lactosome in combination with PEIT (Figure 10c) therefore showed a strong anti-tumor activity, which was not attained without PEIT (Figure 10a). On the other hand, neither PEIT alone nor PEIT in combination with [¹³¹I]NaI showed any anti-tumor activity. Figure 10b and Figure 10d show that any significant loss of

body weight was not observed under these experimental conditions.

Risk of relapse will become high with PEIT when the safety margin is not taken wide enough [8, 9]. With pretreatment of PEIT, however, ^{131}I -Lactosome could infiltrate effectively into the PEIT region even around the peripheral of the tumor, where the survived tumor cells after PEIT should be mainly remained. That should be the reason for the observed tumor suppression effect of ^{131}I -Lactosome with PEIT.

Combining PEIT and ^{131}I -Lactosome could take advantage of their virtues with each other to bring the synergetic effects. The author adopted here PEIT because the author has used mice for these studies which did not allow to use RFA treatment due to lack of RFA instrument for mice. Since RFA is also known to induce inflammation as well as PEIT, a combination use of ^{131}I -Lactosome and RFA should be effective for tumor proliferation suppression as well.

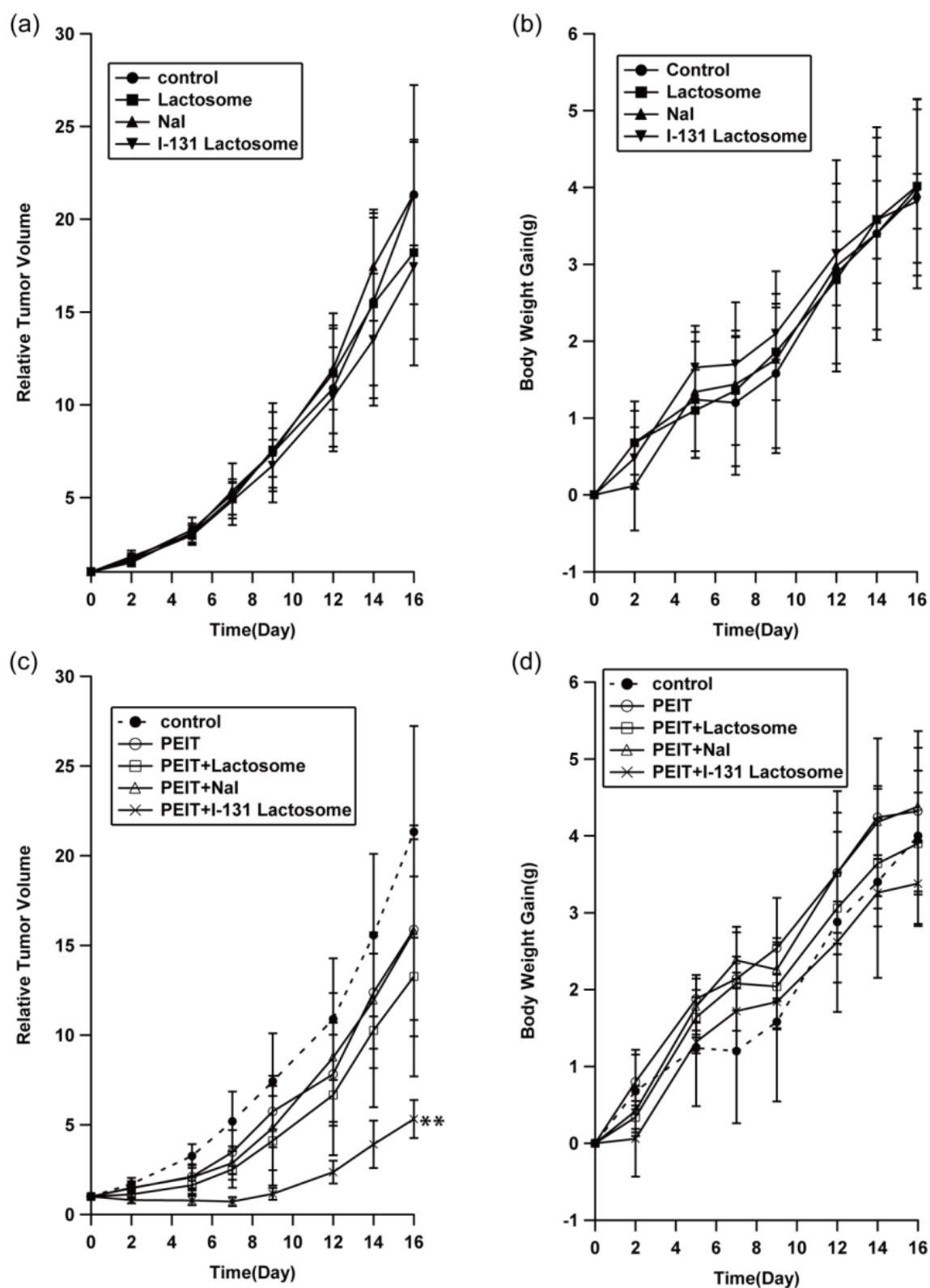


Figure 10 Time courses of (a) relative tumor volumes and (b) body weight gain without PEIT (n=5 per group). ●: control (saline), ■: Lactosome, ▲: [^{131}I]NaI, and ▼: ^{131}I -Lactosome (100 μL) was respectively intravenously administrated. Time courses of

(c) relative tumor volumes and (d) body weight gain with PEIT (n=5 per group). ●: control (saline), ○: PEIT only, □: PEIT in combination with Lactosome, Δ: PEIT in combination with [¹³¹I]NaI, × PEIT in combination with ¹³¹I-Lactosome. ** $p < 0.01$

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Concluding Remarks

This thesis describes the ABC phenomenon induced by nanoparticles covered with poly(sarcosine) chains and their utilization for tumor targeting and imaging. The results are briefly summarized as follows.

In Chapter 1, *in vivo* disposition of Lactosome was shown to change upon multiple dosages. This ABC phenomenon was explained by the productions of anti-Lactosome IgM and IgG₃ through the immune response related with B-lymphocyte cells, and the memory effect of B-lymphocyte cells lasted for nearly six months in mouse. The epitope moiety of Lactosome was concluded to be poly(sarcosine) based on the competitive inhibition assay. The ABC phenomenon induced by Lactosome was, however, different from that by PEGylated liposome about the IgG₃ production by Lactosome.

In Chapter 2, the Lactosome ABC phenomenon, which was caused by production of anti-Lactosome IgM, could be suppressed by high-dose Lactosome at either first or second administration as far as the transplanted tumor could be imaged. In terms of the anti-Lactosome IgM level, the high dose at the first administration developed a partial immune tolerance, but at the second administration did not. Importantly, any acute toxicity with Lactosome of high doses was not observed. Therefore, high-dose Lactosome is a considerable approach to evade from the Lactosome ABC phenomenon, which makes it possible to use Lactosome for multiple imaging.

In Chapter 3, the peptide-nanosheet despite of coverage with the same poly(sarcosine) chains as Lactosome was shown not to induce the ABC phenomenon. Poly(sarcosine) chains on the surface of the molecular assemblies can become antigens easily with the help of an adjuvant effect of the nanoparticle, Lactosome. However, poly(sarcosine) chains completely lost any epitope activity if they were in a high-density polymer brush state as realized by the peptide-nanosheet. The formation of the high-density polymer brush on the surface of nanoparticles is one of the clues for nanoparticles to evade from the immune system.

In Chapter 4, in order to reduce the production of anti-Lactosome IgM, a nanoparticle composed of (poly(sarcosine)₂₃)₃-*block*-poly(L-lactic acid)₃₀ (A₃B-type) was prepared. The A₃B-type Lactosome at the second administration showed the *in vivo* disposition similarly to that at the first administration due to suppression of the antibody production. The study with using the AB- and A₃B-type Lactosomes and varying the polymeric micelle sizes revealed that the high local density of poly(sarcosine) chains of the A₃B-type Lactosome should be related with prevention of a polymeric micelle from interaction with B-cell receptor.

In Chapter 5, the core-shell type polymeric micelles were controlled about the size in a nm precision with mixing the AB-type and the branched A₃B-type amphiphilic polymers having the same hydrophilic and hydrophobic components, and the B block. The precise size control relies on the regular packing of the hydrophobic helical blocks. In the field of nanomedicine, nanoparticles have been attracting much attention due to their biological responses depending on the nanoparticle sizes, and the present

nanoparticles should be useful for that.

In Chapter 6, the *in vivo* blood clearance times of Lactosomes decreased with incorporation of poly(lactic acid)s in the order of PDLA>PLLA>PDLLA. Stereochemistry of poly(lactic acid)s at the hydrophobic core of polymeric micelles is therefore an important factor for determining the physical stability in the blood stream. The amount of the accumulated Lactosome at the tumor region was the highest with using ICG-Lactosome(D). On the other hand, the signal contrast at the tumor region became the highest with using ICG-Lactosome(DL). For application of nanocarriers to drug and/or signal agent delivery, the *in vivo* blood clearance of the nanocarriers should be precisely controlled according to the objectives. Utilization of stereochemistry at the hydrophobic core of nanocarriers is therefore simple and easy approach to control this point.

In Chapter 7, the radioactivity of ^{18}F -labeled Lactosome was found to be stable in the blood circulation and maintained a high level between 10 min to 6 h after the tail vein injection. The uptake of the radioactivity gradually increased specifically at the model tumor region, which was obtained by graft of HeLa cells in the femoral region. The mean uptake ratios of tumor/muscle, tumor/digestive tract, and tumor/brain were 2.7, 2.8, and 4.9, respectively, at 6 h after the injection. In addition, tumor PET imaging of ^{18}F -labeled Lactosome was achievable, and the imaging was similar to tumor NIRF imaging by NIRF-labeled Lactosome. These nanocarriers might be useful for tumor PET imaging due to the EPR effect. Tumor imaging using ^{18}F -labeled Lactosome as a nanocarrier may be therefore a potential candidate for a facile and general tumor

imaging technique.

In Chapter 8, ^{131}I -Lactosome radionuclide therapy became effective for tumor treatment with PEIT. Lactosome is considered to be accumulated easier with inflammation induced by PEIT due to the enriched leaky neovascularity. The survived tumor cells after PEIT were effectively damaged by the beta-ray therapy of ^{131}I -Lactosome. As a result, therapeutic performance was clearly improved. In future, the importance of therapy without large-scale surgeries will accrete. The radionuclide therapy with low dose, for example, of ^{131}I -Lactosome for safety can be combined with the local treatments of PEIT and RFA, which possibly be useful for tumor treatment with less stress to body.

In this thesis, the author has studied the rational and precise desine of polymer nanoparticles. Especially, the study for evasion from the ABC phenomenon has increasesd the possibility of the nanoparticle utilization for tumor imaging and targeting. Based on this study, in the future, clarification of the mechanisms of these phenomenon found with the nanoparticles will lead not only development of nanoparticles which have no immunogenicity but also control of immune system like a suppression of autoimmune disease and a development of vaccine.

List of Publications

Chapter 1 Pharmacokinetic Change of Nanoparticulate Formulation “Lactosome” on Multiple Administrations

Hara, E.; Makino, A.; Kurihara, K.; Yamamoto, F.; Ozeki, E.; Kimura, S.

International Immunopharmacology **2012**, 14, 261–266

Chapter 2 Evasion from Accelerated Blood Clearance of Nanocarrier Named as “Lactosome” Induced by Excessive Administration of Lactosome

Hara, E.; Makino, A.; Kurihara, K.; Sugai, M.; Shimizu, A.; Hara, I.; Ozeki, E.; Kimura, S.

Biochim Biophys Acta general subjects **2013**, 1830, 4046–4052

Chapter 3 Suppressive Immune Response of Poly(sarcosine) Chains in Peptide-Nanosheets in Contrast to Polymeric Micelles

Hara, E.; Ueda, M.; Kim, C. J.; Makino, A.; Hara, I.; Ozeki, E.; Kimura, S.

Journal of Peptide Science **2014**, 20, 570–577

Chapter 4 Factors Influencing *in Vivo* Disposition of Polymeric Micelles on Multiple Administrations

Hara, E.; Ueda, M.; Makino, A.; Hara, I.; Ozeki, E.; Kimura, S.

ACS Medicinal Chemistry Letters **2014**, 18, 873–877

Chapter 5 Size Control of Core-Shell-type Polymeric Micelle with a Nanometer Precision

Makino, A.; Hara, E.; Hara, I.; Ozeki, E.; Kimura, S.

Langmuir **2014**, 30, 669–674

Chapter 6 Control of *in Vivo* Blood Clearance Time of Polymeric Micelle by Stereochemistry of Amphiphilic Polydepsipeptides

Makino, A.; Hara, E.; Hara, I.; Yamahara, R.; Kurihara, K.; Ozeki, E.; Yamamoto, F.; Kimura, S.

Journal of Controlled Release **2012**, 10, 821–825

Chapter 7 Radiosynthesis and Initial Evaluation of ^{18}F Labeled Nanocarrier Composed of Poly(L-lactic acid)-*block*-Poly(sarcosine) Amphiphilic Polydepsipeptide

Yamamoto, F.; Yamahara, R.; Makino, A.; Kurihara, K.; Tsukada, H.; Hara, E.; Hara, I.; Kizaka-Kondoh, S.; Ohkubo, Y.; Ozeki, E.; Kimura, S.

Nuclear Medicine and Biology **2013**, 40, 387–394

Chapter 8 Radionuclide Therapy Using Nanoparticle of ^{131}I -Lactosome in Combination with Percutaneous Ethanol Injection Therapy

Hara, E.; Makino, A.; Kurihara, K.; Ueda, M.; Hara, I.; Kawabe, T.; Yamamoto, F.; Ozeki, E.; Togashi, K.; Kimura, S.

Journal of Nanoparticle Research **2013**, 15, 2131

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Eri Hara

