identified using an explore Locus CT system (Okamura et al., 2009; Okamura et al., 2010a). These results indicate that the H12-(ADP)-liposomes circulate in the bloodstream in a stable form until reaching the site of a vascular injury, and successfully augments hematostatic effects.

Retention in the blood is also an important factor in the evaluation of the hematostatic effects of H12-(ADP)-liposomes, because if the systemic half-life of the H12-(ADP)-liposome is too short, it cannot effectively function as a platelet substitute. From the viewpoint of future clinical applications, an allometric prediction of human pharmacokinetics based on data obtained from animal studies—so called, "animal scale-up"—is important for the determination of optimal doses and intervals (Izumi et al., 1996). In fact, we successfully predicted the blood retention properties of hemoglobin-vesicles (HbV), the liposomal characteristics of which have similar characteristics in terms of liposomal structure to H12-(ADP)-liposomes. This was accomplished using an allometric equation that is generally applied in animal scale-up studies to extrapolate the half-life of pharmaceuticals in humans. In the present study, we showed that the predicted half-life of H12-(ADP)-liposomes in humans would be approximately 18 hrs (Figure 5) using the above approach. The results obtained for a single-dose pharmacokinetic study of recombinant factor VIIa (rFVIIa), which is widely used as a hemostatic agent in clinical settings, showed that its half-life was 2-3 hrs in patients with hemophilia (Lindley et al., 1994). These results indicate that H12-(ADP)-liposomes would be expected to adequately function as a hemostatic agent in the treatment of massive bleeding in humans.

Since H12-(ADP)-liposomes were developed as synthetic platelet substitute, it is necessary to characterize the biodegradable properties of these particles, such as the

determination of their metabolism and excretion pathways. Liposomes are generally captured and degraded by mononuclear phagocyte system (MPS) in the liver and spleen, such as by Kupffer cells and splenic macrophages (Kiwada et al., 1998). As expected, more than 10% of initial dose of the H12-(ADP)-liposomes were distributed to the liver and spleen (Figure 2), which is in good agreement with a previous *in vivo* study using HbV (Sakai et al., 2001; Sakai et al., 2004). In addition, an *in vitro* finding also reported that the specific uptake and degradation of HbV were observed only in macrophage cells but not in parenchymal and endothelial cells in the liver (Taguchi et al., 2009). Furthermore, linear pharmacokinetics were found for the H12-(ADP)-liposomes within the dose of 40 mg lipids/kg (Figure 4). These results strongly suggest that the majority of the H12-(ADP)-liposomes are also scavenged and degraded by the MPS, such as by Kupffer cells or splenic macrophages, and that this process was not saturated at a doses of 40 mg lipids/kg.

The findings herein also showed that most of the ADP in H12-(ADP)-liposomes was mainly metabolized to allantoin and excreted into the urine within 7 days after the injection of the ^{14}C, ^{3}H labeled H12-(ADP)-liposomes (Figure 3). It is well known that uric acid is the final metabolite of purines, such as adenosine 3', 5'-phosphate, in mammals. On the other hand, the principal metabolite of exogenous cyclic nucleotides in the rat is allantoin, and not uric acid (Coulson, 1976). Furthermore, another study showed that, in rats, hepatic uricase converts most of uric acid into allantoin, a form that allows it to be excreted in the urine more readily (Friedman and Byers, 1947). Taken together these findings indicate that the ADP encapsulated by H12-(ADP)-liposome was completely metabolized and excreted into the urine even though ADP was encapsulated within liposome.

The [^{3}H]cholesterol in H12-(ADP)-liposomes was mainly excreted into feces within 7 days after the injection of ^{3}H labeled H12-(ADP)-liposomes. This result is in good agreement with the disposition of HbV, using HbV labeled with ^{3}H after an injection of HbV (1400 mg Hb/kg), which revealed that the majority of outer lipids component (cholesterol) was excreted via feces within 7 days (Taguchi et al., 2009). Kuipers et al. previously reported that cholesterol in vesicles reappear in the blood mainly as lipoprotein-cholesterol complexes after entrapment in Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol complex by hepatocytes (Kuipers et al., 1986). Therefore, a knowledge of whether the behavior of cholesterol as the lipids components of H12-(ADP)-liposome is the same as that of endogenous cholesterol after the metabolization of H12-(ADP)-liposome in the MPS would be highly desirable. On the other hand, we did not directly examine the disposition of the DPPC, DHSG, PEG-DSPE, and H12-PEG-Glu2C18 in H12-(ADP)-liposomes. Previous reports have shown that the phospholipids in liposome are metabolized in the MPS and reused as cell membranes or are excreted into the bile (Dijkstra et al., 1985; Verkade et al., 1991). Therefore, it is also possible that phospholipids in H12-(ADP)-liposome are also metabolized and excreted in the same manner as the other liposome components, as mentioned above.

From the standpoint of biodegradable properties, it is also important to realize the possibility that H12-(ADP)-liposomes and components might accumulate in tissues, because it is well known that cholesterol is a risk factor for several diseases, including arterial sclerosis and hyperlipidemia. The findings reported herein indicate that both H12-(ADP)-liposomes and components derived from them disappeared from the bloodstream and organs within the 7 days after the injection of the

H12-(ADP)-liposomes, indicating that H12-(ADP)-liposomes and components derived from them possess low accumulative properties. Therefore, H12-(ADP)-liposomes contain the appropriate components and have the potential for use as a synthesis platelet substitute, because they possess acceptable biodegradable properties.

Based on the present findings, we provide the first demonstration to show that the disposition of H12-(ADP)-liposomes and components derived from them, occurs as follows. After being systemically administrated, the H12-(ADP)-liposomes are stable and circulate in an intact form in the circulation. As a result, some of the H12-(ADP)-liposomes would be specifically recruited at an injury site and would exert a pharmacological action, while the rest mainly are distributed to the liver and spleen, where they are degraded by the MPS. Finally, the encapsulated ADP and membrane components are eliminated mainly to the urine and feces, respectively, as final metabolites. In addition, our pharmacokinetic study, using different animal species, enabled us to predict that the half-life of H12-(ADP)-liposomes in humans is 18 hours, which is sufficient for them to function as hemostatic agents. The above findings provide further support for the effectiveness and safety of the H12-(ADP)-liposomes in clinical situations for use as a platelet substitute.

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Authorship Contributions

Participated in research design: Taguchi, Otagiri and Maruyama

Conducted experiments: Taguchi, Ujihira, Ogaki, Fujiyama, Doi

Contributed new reagents or analytic tools: Ikeda and Handa

Performed data analysis: Taguchi, Ujihira,Watanabe

Wrote or contributed to the writing of the manuscript: Taguchi, Takeoka, Handa, Otagiri, Maruyama

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Footnotes

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Figure legends

Figure 1

(A) Structure and regiospecifically-^{3}H, ^{14}C radiolabled of H12-(ADP)-liposome. (B) Time course for the plasma concentration of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome after intravenous injection at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. (*n*=4).

Figure 2

The tissue distribution of (A) ^{3}H and (B) ^{14}C radioactivity at 2, 6, 24 hours after an intravenous injection of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. (*n*=4).

Figure 3

Time course for radioactivity in urine (A) and feces (B) after the administration of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome to rats. Each point represents the mean ± S.D. (*n*=4). **(C) Scheme of metabolism pathway from ADP to allantoin in rodents. (D) Chromatogram of standard mixture and urine sample analyzed by HPLC.** The standard peaks are (a) allantoin, (b) ADP, (c) uric acid, (d) hypoxanthine and (e) xanthine. The urine sample was collected 6 hour after intravenous injection of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats.

Figure 4

Dose-dependent plasma concentration curve of (A) ^{3}H and (B) ^{14}C radiolabled H12-(ADP)-liposome after intravenous injection at a dose of 10, 20 and 40 mg

lipids/kg to rats. Each point represents the mean ± S.D. (*n*=4). **(C) Relationship between the dose of H12-(ADP)-liposome and the area under the blood concentration-time curve.** The linear regression of logarithmic values was calculated using the least-squares method (y=119.48x+178.92, r^2=1)

Figure 5

Allometric relationships between body weight and distribution volume (V_{dss}) (A) and body weight and clearance (CL) (B). The linear regression of the logarithmic values was calculated using the least-squares method (A, $y=279.19x^{1.2908}$, r^2=0.982; B, $y=10.058x^{0.8937}$, r^2=0.97). The extrapolated human values based on a body weight of 60 kg (open circle) are also shown.

Table 1

The pharmacokinetic parameters of inner ADP ([8-^{14}C]ADP) and outer lipids membranes ([1,2-^{3}H(N)]-cholesterol) derived from ^{3}H and ^{14}C radiolabled H12-(ADP)-liposomes after an intravenous injection at a dose of 10 mg lipids/kg to rats.

$t_{1/2}$: half-life, MRT: mean residence time, AUC: area under the concentration-time curve, CL: clearance, V_{dss}: distribution volume

	^{3}H	^{14}C
$t_{1/2}$ (hr)	12.4 ± 1.75	10.0 ± 2.91
MRT (hr)	14.1 ± 1.41	12.0 ± 2.07
AUC (hr・% of dose/mL)	62.2 ± 7.07	54.4 ± 9.86
CL (mL/hr)	1.63 ± 0.18	1.89 ± 0.32
V_{dss} (mL)	22.9 ± 3.80	22.5 ± 4.99

Each value represents the mean ± S.D. (*n*=4).

Table 2

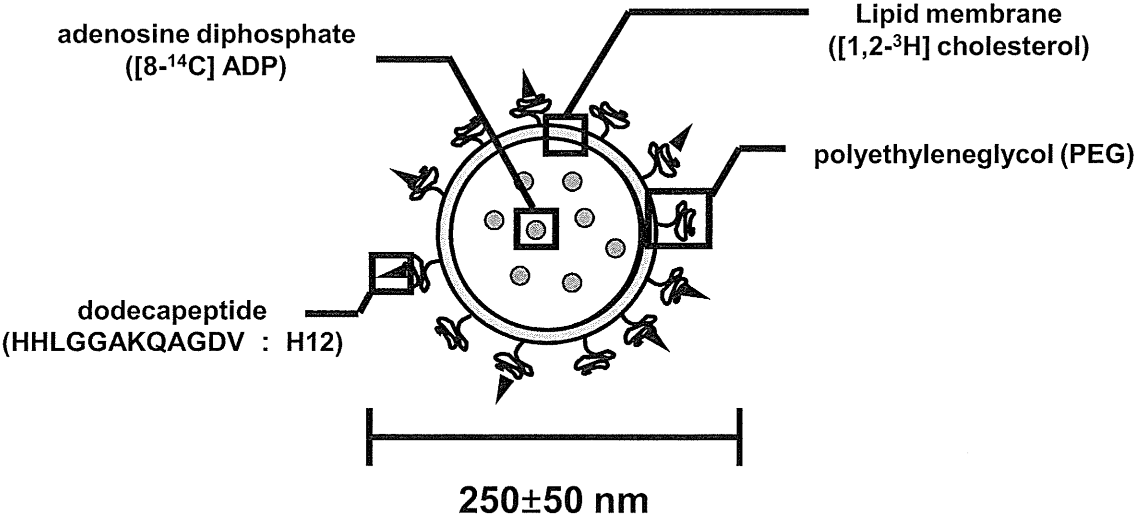
Time course for the % of total detected ^{14}C radioactivity of ADP and metabolites derived therefrom from ^{14}C-ADP in urine after intravenous injection of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats.

	ADP	Hypoxanthine	Xantine	Uric acid	Allantoin
6 hour	1.3±1.2	N.D.	N.D.	N.D.	89.7±12.2
1 day	16.0±15.8	N.D.	N.D.	N.D.	78.7±13.3
3 day	N.D.	N.D.	11.2±10.5	N.D.	71.8±15.9
5 day	N.D.	N.D.	6.7±5.9	3.9±3.4	75.0±20.8

N.D.; not determine, Each value represents the mean ± S.D. (n=4).

Figure 1

(A)



(B)

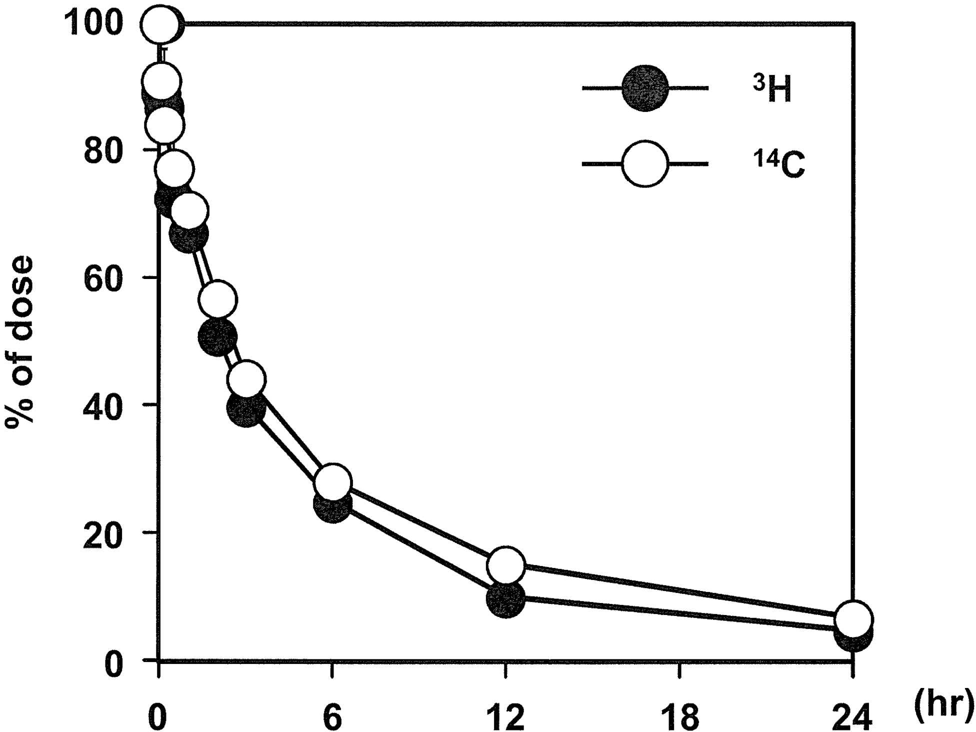
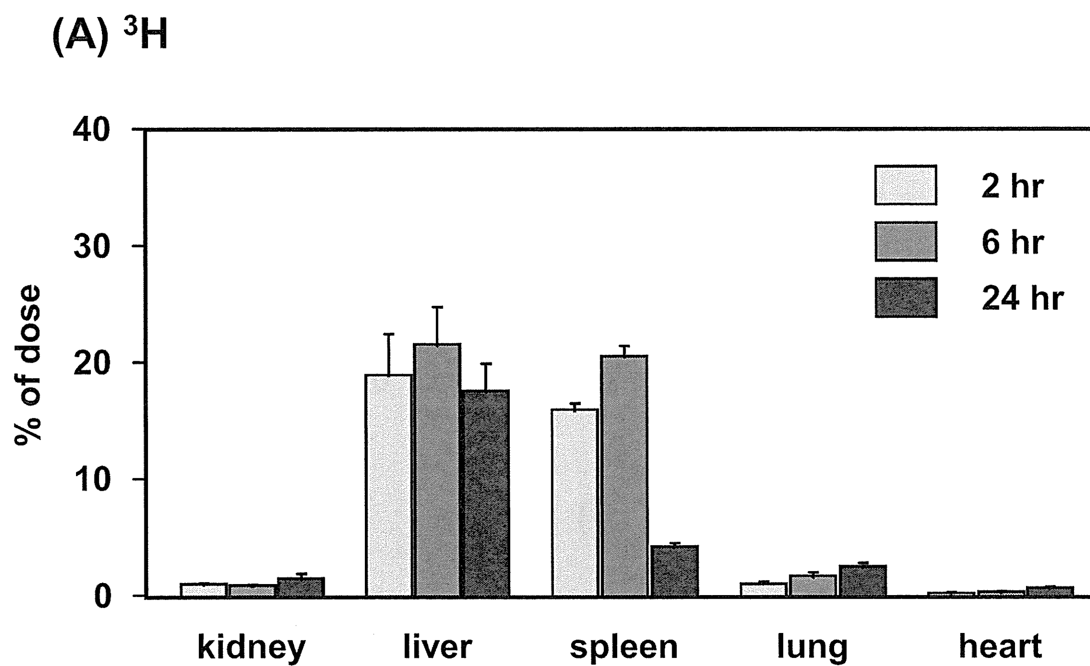


Figure 2

(A) ^{3}H



(B) ^{14}C

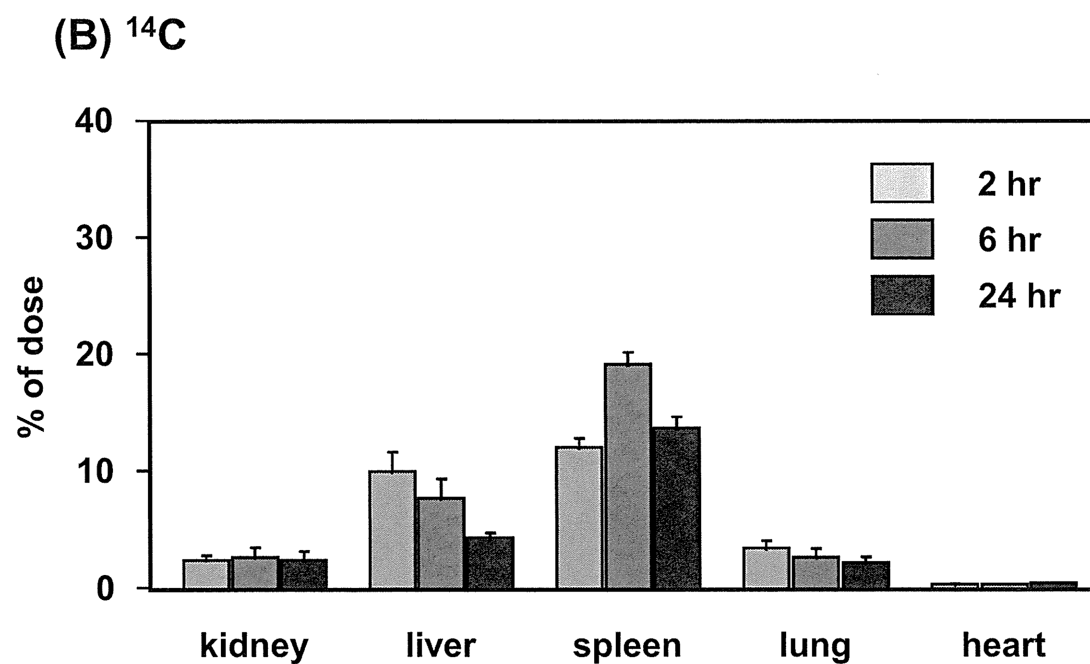
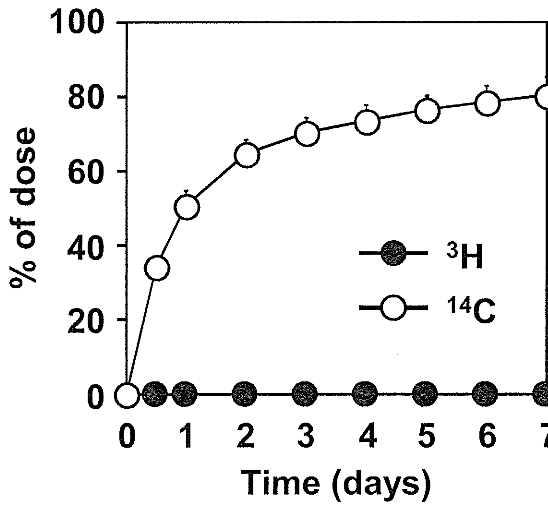
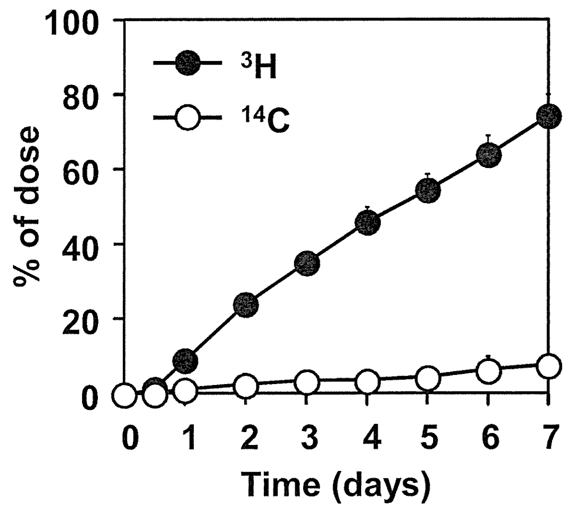
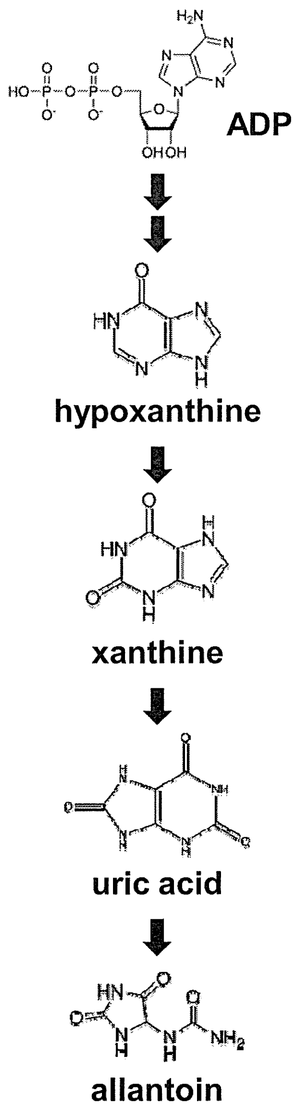


Figure 3







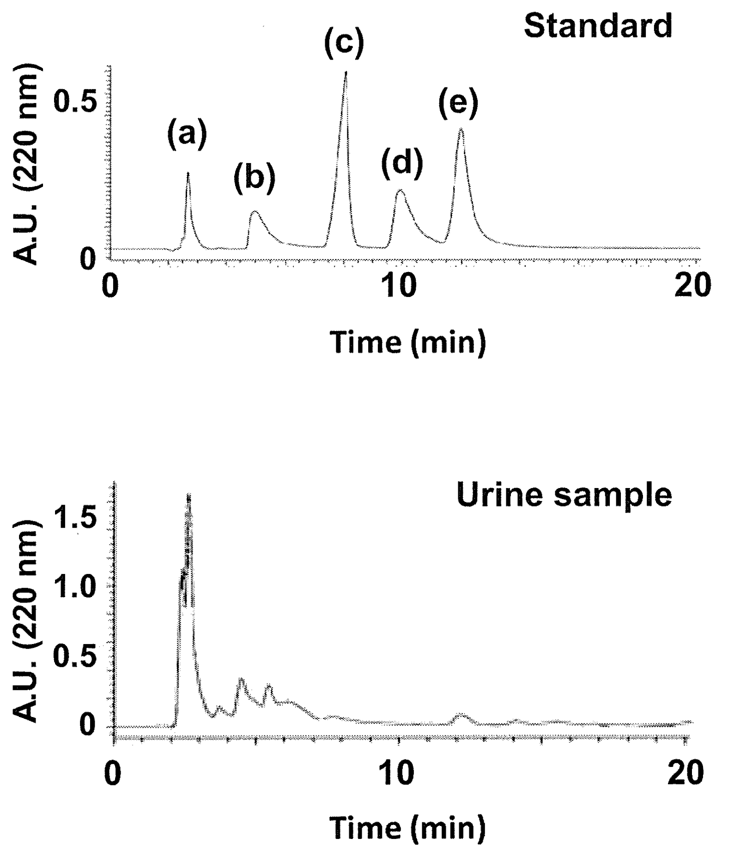


Figure 4