Liposome-based Biosensors and Diagnosis Imaging Agents

Nobuhito Hamano[*] and Yoichi Negishi

Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy,
Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

The development of biosensors and contrast imaging agents is urgently required to detect and diagnose diseases. Liposomes have been used in various fields as biosensors and imaging agents owing to their biocompatibility, ability to modify the liposomal surface, and encapsulation efficacy for various biologically active agents. In this review, we summarize the current developments and applications of liposome-based biosensors and diagnostic agents, including computed tomography, ultrasound imaging, magnetic resonance imaging, and positron emission tomography. We also discuss future perspectives and challenges associated with the development of liposomal biosensors and imaging agents.

1. Introduction

Biosensors are analytical tools composed of biological materials, biologically derived materials, or biomimetics that are used as recognition molecules. Biosensors have been employed in several fields, including the food industry, environment, agriculture, and medicine, owing to their user-friendliness and high sensitivity.[1–3] *In vitro*, biosensors react with samples such as blood and saliva in a test tube, microtiter plate, or test strip, enabling the detection of contaminants such as viruses. Biosensors generally have four characteristics: selectivity, reproducibility, stability, and sensitivity.[4] *In vivo*, biosensors function as bioimaging sensors for image diagnosis, making them valuable as imaging agents to enable precise diagnosis in clinical settings. Therefore, biosensor/imaging agents are increasingly important in clinical settings.

Liposomes are artificial vesicles mainly composed of phospholipids and cholesterol. As phospholipids are one of the main cell membrane components, liposomes exhibit excellent biocompatibility, non-toxicity, and a lipid bilayer structure. Accordingly, liposomes have been used as biosensors. Liposomes were first discovered by Dr. Alec Douglas Bangham, a hematologist at the Cambridge Babraham Institute, in the 1960s.[5,6] Originally, liposomes were used as a biological cell membrane model. Then in the early 1970s, it was demonstrated that liposomes could serve as a carrier system for drug delivery.[7,8] Since the introduction of PEGylated liposomes, whose surfaces are coated with polyethylene glycol, by Klibanov *et al.* in 1990,[9] liposomes have been extensively investigated. Moreover, in 1995 Doxil® (PEGylated liposomes containing doxorubicin) was approved by the FDA as the first liposomal

formulation.[10] To date, several functional liposomes have been developed, such as targeting liposomes (e.g., peptide-modified or antibody-modified liposomes)[11,12] and stimuli-responsive liposomes [e.g., pH-responsive, light-activated, and ultrasound (US)-responsive liposomes],[13–16] with over 10 liposomal formulations approved by the FDA.[17]

In the present review, we focus on liposomes as biosensors or imaging agents and present recent promising examples of liposomal biosensors and diagnostic imaging agents. In addition, we discuss associated challenges and future perspectives.

2. Liposomes as Biosensors

Typically, liposomes are classified into small unilamellar vesicles (SUVs; 20–100 nm), large unilamellar vesicles (LUVs; 100–1000 nm), multilamellar vesicles (MUVs; over 500 nm), and giant unilamellar vesicles (GUVs; >1 mm) and have a lipid bilayer structure with a thickness of 4–5 nm.[18,19] Notably, the liposomal surface can be conjugated with diverse biorecognition molecules [e.g., antibodies, peptides, saccharides, and DNA segments[20–23]]. In addition, liposomes possess a large cavity and lipid bilayer structure and can encapsulate hydrophilic molecules in the cavity or hydrophobic molecules in the bilayer, including dyes, enzymes, DNA, and organic nanoparticles.[24–27] In addition, liposomes have a high surface area and can amplify signal intensities,[28,29] making them excellent materials for developing biosensors. In this section, we introduce liposomal biosensors developed since the 1990s.

Different types of assays have employed liposomes for biosensing by simply mixing the liposomes with test samples, such as immunoassays [liposome agglutination, liposome immunoaggregation, liposome immunoblotting, immunochromatographic liposome, and liposome-linked immunosorbent assays[30,31]]. Liposomes have also been employed as biosensors to detect various analytes (e.g., toxins, pesticides, enzyme, and viruses) (Table 1). Since the mid-1990s, liposomes have been used as biosensors to detect toxins [*botulinum toxin*, *cholera toxin*, and *Salmonella*[24,32,33]] as well as pesticides [alachlor, carbofuran, and herbicide[34–36]]. The common features in these liposomal systems were low-molecular-weight encapsulated liposomes (SRB or Tb) and strip assays. In the 2010s, horseradish-peroxidase (HRP)- and polypeptide-encapsulated liposomes have been used to detect enzymes [matrix metalloproteinase (MMP)-9 and phospholipase A2 receptor (PLA2)[25,37]]; these systems have been used for enzyme-linked immunosorbent assay (ELISA) and colorimetric analysis. As another enzyme detection system, Chowdhury and Park revealed that sphingomyelinase could be detected at a markedly low detection limit (>0.01 mU/mL) using methylene blue (MB) and electrochemical differential pulse voltammetry (DPV).[38]

An excessive production of reactive oxygen species (ROS) has been implicated in the development of various chronic and degenerative diseases, including cancer and respiratory, neurodegenerative, and digestive diseases.[39] Recent evidence has shown that ROS play a key role as messengers in normal cell signal transduction and cell cycling. ROS-detectable liposomal systems have been reported,[27,40] such as a Förster resonance energy transfer (FRET) system for ROS detection. FRET is an effective technique for probing minimal changes occurring at a distance, such as the separation between donor and acceptor fluorophores of less than 10 nm. It

Table 1
Summary of liposome-based biosensors.[*]

Analytes	Type of liposome	Liposome composition	Marker	Detection signals (method)	Detection limit	Ref.
Toxin						
Botulinum toxin	Ganglioside-modified liposomes	DPPC, DPPG, chol, DPPE-ATA	SRB	Fluorescence	15 pg/mL	24
Cholera toxin	Ganglioside-modified liposomes	DPPC, DPPGchol, GM1	SRB	Fluorescence	0.01 pg/mL	32
Salmonella	Antibody-modified liposomes	DPPC, DPPG, chol, biotin-X-DHPE, DPPE	SRB	Fluorescence	100 CFU/mL	33
Pesticides						
Alachlor	Dye-encapsulated liposomes	DPPC, chol	SRB	Fluorescence	5–10 ng/mL	34
Herbicide	Antigen-sensitized liposomes	Lecithin, chol, decetyl Atrazine-DMPE	Calcein or SR 101	Fluorescence	0.13 ng/mL	36
Carbofuranv	Tb-encapsulated liposomes	LPC, PE	Tb	Fluorescence	0.01–10 ng/mL	35
Enzymes						
MMP-9	Lipopeptide-modified liposomes	POPC, chol	HRP	Absorbance (ELISA)	N/A	25
PLA2	Polypeptide-loaded liposomes	POPC	Polypeptide	Colorimetric	0.07–7 pmol/mL	37
Sphingomye linase	Bare liposomes	Sphingomyelin, chol	MB	Differential pulse voltammetric (electrochemical DPV process)	0.1–10 mU/mL	38
ROS	Boronate-modified liposomes	DOPE-based lipids DPPC, DOPE, PA	ANTS/DPX	Fluorescence (FRET)	N/A	40
	Chlorophyll a-embedded liposomes	DPPC, chol, DSPE-PEG2000	UCNPs/ AuNPs	Fluorescence (FRET)	25–50 mM (H$_2$O$_2$)	27
Viruses						
Dengue RNA	DNA probe-modified liposomes	DPPC, DPPG, chol, DPPE	DNAprobes	Electrochemi-luminescence	10 pfu/mL	23
Chikungunya virus E1 protein	Antibody-modified liposomes	DOPC, DOPG, DSPE-PEG2000	MB/QDs	Electrochemical and fluorescence	0.03–0.56 pg/ml	44
SARS-CoV-2 RNA	Bare liposomes	DMPC, chol	CRISPR-FDS	Fluorescence	N/A	26

[*]Abbreviations: chol = cholesterol; SRB = sulforhodamine B; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PA = l-α-phosphatidic acid sodium salt from chicken eggs; ANTS = 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX = p-xylene-bis-pyridinium bromide; UCNPs = upconverting nanoparticles; AuNPs = gold nanoparticles; PG = phosphatidylglycerol; MB = methylene blue; QDs = quantum dots.

is also a sensitive tool for detecting molecular binding and changes in protein conformation, as well as for chemical and biological analyses.[41–43] Liposomes have a bilayer structure and a thickness of approximately 5 nm; accordingly, FRET is highly suitable for biosensor applications. Moreover, a liposomal nanoplatform reported by Wan *et al.* encapsulated upconversion nanoparticle (UCNP)–gold nanoparticle (AuNP) nanocomplexes, which could measure ROS concentrations via FRET imaging while also scavenging excessive ROS.[27]

Viral diseases are widespread infections caused by viruses, a type of microorganism. If community risk or transmission levels are substantial or high, monitoring or screening tests are especially important, as in the case of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Virus-detectable liposomal systems have been reported.[23,44] In 2021, SARS-CoV-2 RNA was detected in human clinical samples using liposomal systems.[26] In general, quantitative reverse-transcriptase polymerase chain reaction (qRT–PCR) has been the gold standard for analyzing respiratory samples to diagnose coronavirus disease 2019 (COVID-19).[45] However, one critical limitation is that the SARS-CoV-2 RNA level in the upper respiratory tract rapidly decreases after infection.[46] In addition, the sensitivity for SARS-CoV-2 RNA in plasma is insufficient owing to the dilution and degradation of viral RNA released into the circulation.[47] Therefore, Ning *et al*. collected plasma samples from patients with COVID-19 and captured extracellular vesicles (exosomes) that contained viral RNA. They developed reverse transcriptase (RT) and recombinant polymerase amplification, clustered regularly interspaced short palindromic repeat (CRISPR) 12a-encapsulated liposomes, and established SARS-CoV-2 RNA-positive detection systems by mixing liposomes and extracellular vesicles. Interestingly, the extracellular plasma vesicles collected from six patients with negative nasal RT-PCR results strongly reacted in the assay system.[26]

Accordingly, liposomes have been widely adopted as biosensors for various analytes. For biosensor applications, virus detection systems, such as those developed by Ning *et al*., are crucial from the perspective of point-of-care testing (POCT).[26,48] POCT enables rapid testing, thus allowing healthcare professionals to make quick decisions and take appropriate treatment measures; however, some challenges persist. In the systems of Ning *et al*., liposome-encapsulated RNA, Cas12a, and enzymes are associated with liposomal stability. Accordingly, to overcome this issue, the development of novel liposome-based technologies is crucial. For instance, it was recently reported that the combination of microfluidic technology and pH-responsive lipids could generate stable RNA-encapsulated lipid nanoparticles.[49–51] Thus, this pharmaceutical technology could be valuable for developing novel biosensors.

3.   Liposomes as Imaging Agents for Diagnosis

Medical imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI), US, and positron emission tomography (PET) play an important role in medicine by facilitating disease diagnosis and treatment. Contrast imaging agents, such as gadolinium (III) (Gd) and iodine-based contrast media, are often employed to enhance images or the disease detection ratio. As previously mentioned, liposomes can encapsulate drugs, and the liposomal size is <200 nm. Typically, the width of the tight junction regions between blood endothelial cells ranges from 12 to 20 nm.[52] However, in some vasculature abnormalities (e.g., tumor vascular or inflammation area), the vascular endothelium is disjointed, irregular, and allows the penetration of nanocarriers such as liposomes; this phenomenon is called the enhanced permeability and retention effect.[53] Therefore, liposomes can be valuable as carriers for diagnostic imaging agents. In this section, we introduce liposomal diagnostic imaging agents used in CT, MRI, US, and PET (Table 2).

Table 2
Summary of liposome-based imaging agents.[*]

Imaging modality	Type of probe	Type of liposome	Composition	Size	Experimental model	Ref.
CT	Iomeprol	Tf-modified liposomes	DSPC, chol, DSPE-PEG2000	<150 nm	Rat glioma model	85
	Iodixanol	PEG liposomes	DPPC, chol, DSPE-PEG2000	<140 nm	Primary or metastatic tumor model	57
	Gold nanostars	FA-modified liposomes	DSPC, DPPC, DSPE-PEG2000 silica, Dox	200 nm	HeLa xenograft model	58
MRI	Gd	a4b1-targeted liposomes	TIT0567, DPPC, chol, BSA-DTPA-Gd	120–250 nm	Atherosclerotic plaque model	63
	Gd	EGFR-targeted liposomes	CFL, porphyrin, DSPE-DOTA, DSPE-PEG-cetuximab	90 nm	Mouse colorectal tumor model	65
	Magnevist	PEG liposomes	DPPC, MPPC DSPE-PEG2000	170 nm	Pancreatic cancer PDX model	64
US	Perfluoropropane	Antibody-modified nanobubbles	DSPC, DSPE-PEG2000	500–700 nm	SKOV3 xenograft model	20
	Perfluoropropane	Annexin V-modified nanobubbles	DPPC, chol, DSPE-PEG2000	635 nm	MDA-MB-231 xenograft model	72
	Perfluoropropane	Peptide-modified nanobubbles	DSPC, DSPE-PEG2000	420 nm	Mouse colon26 tumor model	73
	Perfluoropropane	FA-modified nanobubbles	DSPC, DSPE-PEG2000-ICG, DSPE-PEG2000-FA, RV	1290 nm	HepG2 xenograft model	74
PET	^{52}Mn/^{64}Cu	PEG liposomes	HSPC, chol, DSPE-PEG2000	around 100 nm	Mouse colon26 tumor model	77
	^{64}Cu	MM-DX-929 liposomes	DSPC, chol, DSPE-PEG2000, TEA-SOS, ^{64}Cu:4-DEAP-ATSC	<100 nm	HT29 xenograft model	78

[*]Abbreviations: Tf = transferrin; FA = folic acid; Dox = doxorubicin; Gd = gadolinium; TIT0567 = α4β1 integrin antagonist; ICG = indocyanine green; RV = resveratrol; TEA-SOS = triethylammonium sucrose octasulfate.

CT is a non-invasive radiological imaging technique that uses X-rays to produce 3D tomographic (cross-sectional) tissue images. CT can image the whole body, including bone and lungs, although it is difficult to obtain high-quality images that can identify the interface between two different tissues, such as a tumor in an organ or a soft tissue in body fluids.[54,55] Contrast CT, which employs a contrast agent injection, affords better results and enhances the contrast between target and normal tissues. The contrast agent is often iodine, which can increase the CT contrast of blood vessels; however, iodinated contrast media can potentially induce acute kidney injury.[56] Iodine can be easily encapsulated in liposomes. Therefore, liposomal iodine is an attractive CT contrast agent as its persistence in the blood pool eliminates the need for precisely timing the contrast injection during scanning. Furthermore, non-renal clearance via the reticuloendothelial system can decrease nephrotoxicity. Ghaghada *et al*. reported that long-circulating liposomal iodine contrast agents enabled the prolonged visualization of small and large tumors in companion dogs with naturally occurring cancer.[57] In addition, metal-based agents, such as gold nanoparticles, can be employed as contrast imaging

agents. For example, An *et al.* developed gold-nanostar-encapsulated liposomes and presented CT tumor images.[58] Moreover, this platform showed controlled drug release and cancer chemotherapeutic effects by using a combination of doxorubicin and a near-infrared laser.

MRI is a form of nuclear magnetic resonance, which images hydrogen atoms in biological tissues, mainly water and fat in the body. Accordingly, it does not require contrast agents to image anatomical structures or blood flow. However, MRI has limitations such as cost, long imaging times, motion artifacts, and potential foreign body/implant artifacts. MRI contrast agents significantly help detect and differentiate healthy tissues.[59,60] Gadolinium (Gd) is currently the most common MRI contrast agent. Given the toxicity of free Gd ions, clinical contrast agents are available in the form of Gd chelates (Gd III complexes). Nevertheless, some concerns regarding nephrogenic systemic fibrosis and brain deposition of Gd persist.[61] In addition, MRI has a high resolution but low sensitivity;[62] therefore, it often requires numerous imaging agents. On the basis of these findings, liposomal Gd, which can be employed to image atherosclerotic plaques or tumors with high resolution, has been developed.[63–65] In particular, MRI signals could be detected 48 h post-injection of targeted liposomal Gd [epidermal growth factor receptor (EGFR)-targeted liposomes], as developed by Li *et al.*; this effect was not observed with control liposomes.[65] Thus, liposomal Gd formulations can increase the blood circulation time and tissue accumulation by targeting liposomes, which partially resolves the problem of low sensitivity. Also, Gd has been incorporated or encapsulated in almost all liposomal Gd formulations.[63–66] Thus, although there is still the possibility of Gd leakage, liposomal Gd formulations are well tolerated with no safety issues.[63,66]

US imaging is one of the most widely used medical diagnostic imaging modalities owing to its portability, noninvasiveness, high spatial resolution, low cost, and real-time imaging properties. Similarly to CT and MRI, US imaging can also image tissues without contrast agents; however, to enhance and improve the resolution and sensitivity of imaging, contrast agents called microbubbles are often employed. Currently available commercial US contrast agents (e.g., Sonazoid and SonoVue) are composed of lipids with sizes ranging between 1 and 8 µm.[67–69] However, microbubbles have a relatively short circulation lifetime and low stability owing to their large size.[70,71] Accordingly, nanosized liposome-based US contrast agents have been developed. Moreover, targeting nanobubbles have been formulated for precise imaging. Targeting nanobubbles can be modified by peptides, antibodies, folate, or annexin V to target tumor tissues, including apoptosis areas and tumor vessels.[20,72–74] Moreover, targeting nanobubbles can allow a prolonged imaging time and robust signals. In addition, photoacoustic (PA) imaging, a hybrid optical and US imaging technique, has attracted attention owing to its advantages such as high contrast and excellent spatial resolution because of the optical imaging properties and deeper tissue penetration afforded by US imaging. Zheng's group developed porphyrin and lipid-based microbubbles (MBs),[75,76] which can control the size (small porphyrin MBs, 2 µm; large porphyrin MBs, 6 µm) and obtain both US and PA signals, resulting in US and PA bimodal imaging.[76]

PET, a powerful and widely employed nuclear medicine technology, allows high tissue penetration, high sensitivity, and real-time quantitative imaging analysis after the injection of positron-emitting radionuclides, such as ^{18}F, ^{64}Cu, and ^{89}Zr. However, PET has limitations such

as high cost, rapid attenuation of radionuclides, and radioactive exposure. Therefore, liposome-based PET imaging can overcome some limitations. One potential advantage of liposome-based PET imaging is the increased blood circulation time of radionuclides. Jensen *et al.* reported that the plasma half-life of ^{64}Cu-encapsulated liposomes is longer than that of ^{64}Cu alone (20.3 h vs 12.7 h).[77] Blocker *et al.* assessed the therapeutic efficacy of bevacizumab, an anti-vascular endothelial growth factor drug, using ^{64}Cu-labeled liposomes. They observed that imaging with ^{64}Cu-labeled liposomes might confer a therapeutic effect on monitoring or enable the prediction of the therapeutic effect.[78]

In this manner, using liposomal diagnostic imaging agents, various imaging techniques and combinations are available, such as multimodal imaging and useful tools; however, PEGylated liposomal formulations induce anti-PEG IgG antibodies, resulting in the accelerated blood clearance (ABC) phenomenon.[79–82] The induction of this phenomenon might greatly inhibit the use of liposomal imaging agents.[83] Therefore, to optimize the efficacy of liposomal imaging agents, it is necessary to consider the lipid dose and time interval to minimize the ABC phenomenon.[84]

4. Conclusions and Future Perspectives

Liposomes have been used in various fields as biosensors and imaging agents owing to their biocompatibility, as well as their ability to modify the liposomal surface and encapsulate various biologically active agents. Recently, the concept of "theranostics" has been widely used in nanomedicine.[86] This word is a combination of two words, therapeutic and diagnostic, allowing the combination of diagnosis and treatment. Theranostic systems are currently in the preliminary stages; however, some reports indicate their strong potential for tumor treatment. The carriers of theranostic systems include micelles, lipid nanoparticles, and liposomes.[87–90] One of the advantages of liposomes is that many liposomal formulations have already been approved by the FDA.[17] Moreover, because theranostic liposomal formulations containing imaging agents can be used to monitor the drug or carrier, they may be suitable for dose optimization in preclinical studies. This would allow the results to be used to assess efficacy in preclinical studies or clinical trials, facilitating their clinical use. To launch novel theranostic liposomal formulations, the design of future theranostic systems will need to integrate both formulation properties (e.g., biodistribution) and imaging properties.

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