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Polymeric Lipid Assemblies as Novel Theranostic Tools

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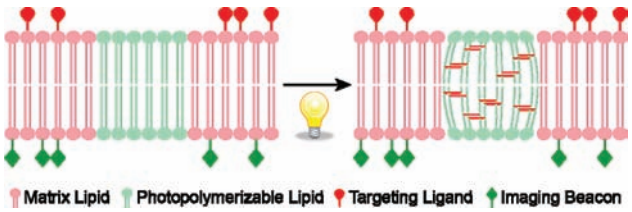

Polymerizable lipids have been used in research and medical applications such as membrane models, imaging platforms, drug delivery systems, vaccine carriers, biosensors, and coating materials. The polymerization of these lipid molecules forms a covalent bond between lipid moieties, which improves the noncovalent interactions that maintain the lipid lamellar phase architecture and increases the stability of the polymerized system. Because such lipid molecules form nanoassemblies with modifiable structures that acquire the stability of polymers following covalent bond formation, these lipids are of considerable interest in the emerging field of theranostics.

In this Account, we summarize the biomedical applications of polymerizable lipids (primarily phospholipids) in the context of various nanoplatforms. We discuss stable nanoplatforms, which have been used in a variety of theranostics applications. In addition, we describe methods for assembling triggerable theranostics by combining appropriate nonpolymerizable lipids with polymerizable lipids.

Polymeric lipids hold promise as nanotools in the field of medical imaging, targeting, and on-demand drug delivery. Because of their similarity to biological lipids, long-term toxicity issues from polymerizable lipid nanoplatforms are predicted to be minimal. Although the field of polymeric nanocapsules is still in development, intensive efforts are underway to produce systems which could be applied to disease diagnosis and treatment. We envision that nanoimaging platforms coupled with localized drug delivery technology will have a significant impact on cancer therapy and other related diseases. The existing wealth of clinical knowledge both in the photochemistry of imaging agents and/or drugs and modifications of these agents using light will prove valuable in the further development of polymeric theranostic lipid-based nanoparticles.

Introduction

The structural basis of the architecture for the cell membrane is a lipid bilayer of about 4 nm thick, made up of two monolayers of lipids.[1,2] According to the classical Singer–Nicholson model, membrane-embedded proteins perform their functions while floating unencumbered in a sea of lipids.[3] According to the model, the lipids play a passive role as a solvent for membrane proteins and no special consideration is given to the particular environment in which membrane proteins function. However, it has been recognized that many membrane functions (e.g., fusion, signaling, and permeability) are strictly dependent on the particular nanoenvironment in which these processes take place.[4,5] Development of emerging techniques to study membrane phenomena at the nanoscale has been instrumental in furthering our understanding of these membrane functions.[6−8] The current view is that membranes are patchy with nanoscale segregated regions of structure and function (nanodomains) and that lipid regions vary in thickness and composition.[9,10] Monolayers, multilayers, and liposomes have frequently been used as simple model membranes in attempts to gain insight into more complex natural structures and nanodomain formation.[9,11] In order to probe the domain structure and motional dynamics of biological

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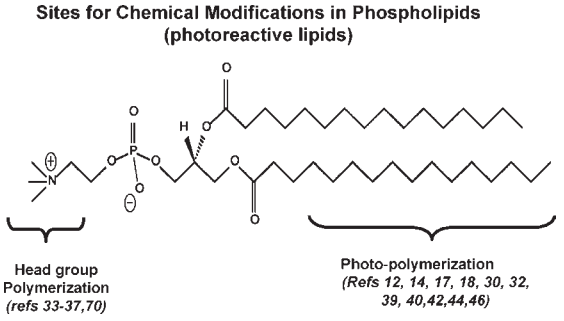

FIGURE 1. Sites for chemical modifications in phospholipids (photoreactive lipids). Two major parts of phospholipids that can be chemically modified to generate photosensitive molecules. The lipid parts, headgroup and fatty acyl chains, are described with their proposed modifications. The references correspond to the currently available designer lipids. The modifications in the glycerol backbone are typically introduced to modulate responses to enzymes such as phospholipases.

membranes and their model systems, photosensitive moieties have been incorporated into lipid structures.[12−15] Photopolymerizable diacetylenic lipids have been extensively studied in lipid model membranes in the context of membrane structure and domain formation.[16−19] Since these photopolymerizable lipids combine the plasticity of lipids with the robustness of polymers, they have received much attention in the biotechnology arena.[20,21] The lipid-based scaffolds, once polymerized, form extremely stable structures which may be used in surface coatings for biocompatible materials, supporting matrices for biosensing molecules, and carrier vehicles for drugs.[21] The aim of this Account is to summarize the biomedical applications of polymerizable lipids (primarily phospholipids) in the context of various nanoplatforms that are currently available and being developed. The first part of this Account will deal with the stable nanoplatforms, which have been used in a variety of theranostics applications. In the second part, we will describe a way to trigger nanoplatforms that contain photopolymerizable lipids in a stable lipid matrix for on demand drug delivery applications.

Principles of Polymerization

The concept of using phospholipid polymers as tools in the medical field originated in the early 1980s.[22] Biomedical applications of the lipid polymers include biosensors,[23,24] micropatterned membrane biomemetics,[25] rechargeable batteries,[26] imaging agents,[27] and drug delivery carriers.[28−31] The basic design of a photopolymerizable lipid relies on two important parameters: (a) self-assembly properties of the lipids (or related molecules) and (b) strategic chemical synthesis schemes for the introduction of photoactivable bonds in these molecules. Phospholipids such as phosphatidylcholine (PC, Figure 1) can be considered as a prototype molecule to direct the design of polymerizable lipid molecules for multifaceted applications. The PC molecule can be divided into three major parts, headgroup, glycerol backbone, and fatty acyl chains; each of these regions has been modified by either the introduction of additional groups or modification of existing chemical bonds such as polymerizable moieties to produce light sensitive nanoassemblies of lipids.

In this Account, we will only focus our discussion on the light-activable lipid molecules (including phospholipids and nonphospholipids) that utilize the principle of photopolymerization (photo-cross-linking) and will later summarize their biological applications. A general overview of the drug delivery applications of light-sensitive lipid-based nanoparticles has recently been published.[20]

The photoreactive chemical bonds in a photopolymerizable molecule are primed to undergo photo-cross-linking (polymerization) upon activation with a light source; the modifications are expected to introduce minimum perturbations in overall self-assembly features of the nanosystem being investigated (such as monolayers, bilayers, and/or lipid vesicles). Typically, light-triggered photo-cross-linking reactions result in irreversible polymerization due to inter- or intramolecular chemical reactions between the photoactive groups; however, a few examples exist where these reactions have been shown as reversible phenomena. Various polymeric lipids that have been designed to date utilizing distinct polymerization principles are described below.

a. Reversible Polymerization. During the early 1980s, Singh, Regen, and colleagues described the synthesis and characterization of a thiol-bearing phospholipid, with the aim to generate vesicles that can undergo reverse polymerization.[32] The structure of a class of one such lipid (1,2-bis(11-mercaptoundecanoyl)-sn-glycero-3-phosphocholine) is shown in Figure 2i. The principle of the reversible polymerization of this lipid entails a "switched on/switched off" mechanism by oxidation/reduction, respectively. Polymerization (via the S−S bond formation) could be achieved by either direct UV (254 nm) treatment or oxidation in the presence of hydrogen peroxide. Although an interesting platform, biological applications of this approach have not been documented yet. Moreover, the light source and the effective concentration of the oxidizing-reducing agents that will be compatible with biological systems may pose limitations for this approach.

b. Free-Radical-Initiated Photopolymerization. The examples of free radiation-initiated polymerization reaction include the headgroup polymerizable phospholipids.

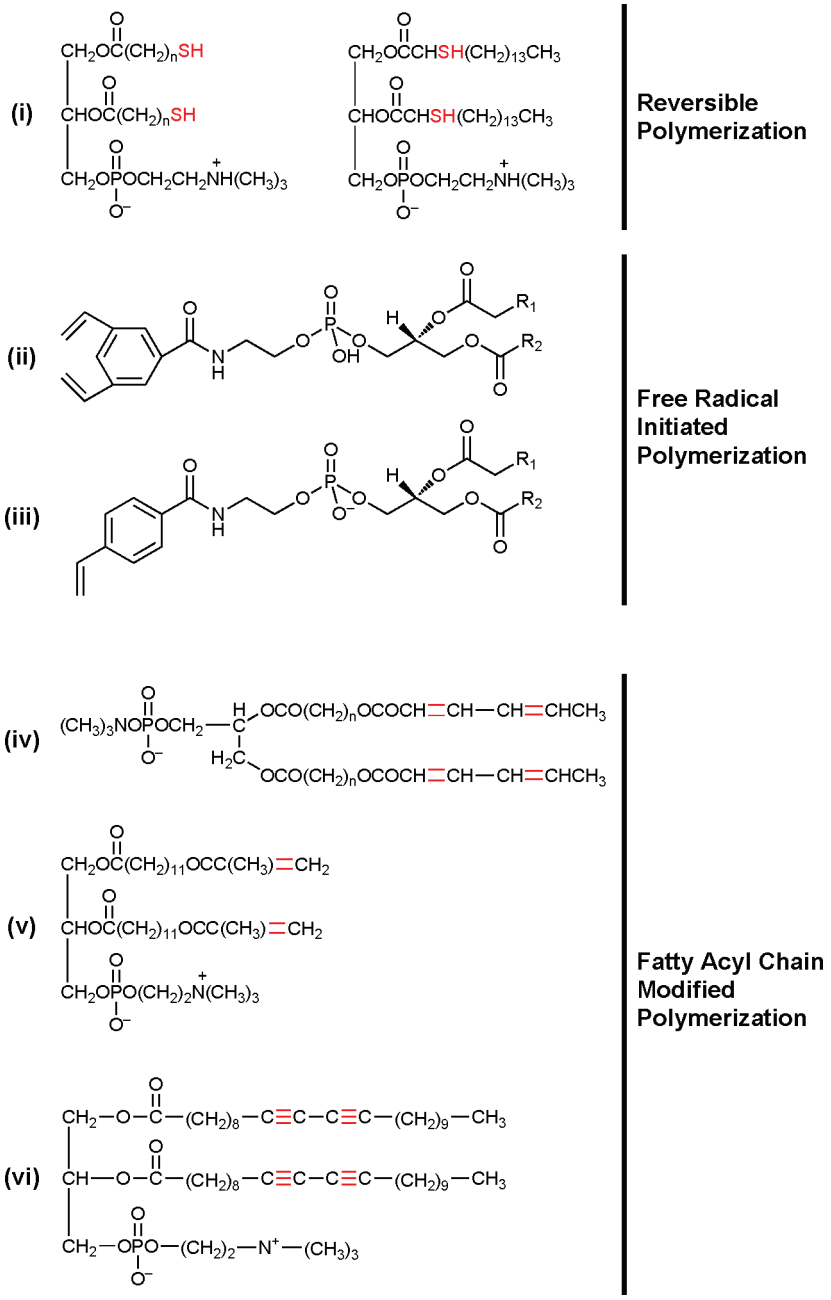

FIGURE 2. Polymeric lipids. Chemical structures of various photoactivable phsopholipids are shown: (i) lipids beating SH groups for reversible polymerization; (ii, iii) headgroup polymerizable lipids to generate stable nanocapsules (ii, DVBA; ii, styryl modifications); (iv–vi) fatty acyl modified lipids (iv, bis-Sorb PC; v, dipolymerizable lipid(DPL); vi, diacetylenic lipid ($DC_{8,9}PC$)).

Figure 2ii, iii shows the chemical structures of two headgroup modified phospholipids containing divinylbenzoyl[33] (Figure 2ii) and styryl[34] (Figure 2iii) functionalities. These molecules were synthesized using either saturated or unsaturated fatty acyl chains in the hydrophobic part, with the aim to stabilize liposome bilayer membranes to improve their drug delivery potential in vivo. The light-induced photo-cross-linking in these liposomes is typically achieved under relatively mild conditions in the presence of a water-soluble free radical initiator. The choice of monomer functionalities and the flexibility to place these monomers in the liposome membrane prior to cross-linking offers attractive possibilities with potential applications for plasma stable vesicles for theranostic (drug delivery and/or imaging) applications. It is critical that the photoreactive monomers should maintain the integrity of the vesicles and their contents (such as pharmaceutical agents) during the photopolymerization step. Introduction of a photoreactive moiety in the headgroup

of the phospholipid (see Figure 2) appears to be an appropriate choice, since these modifications result in polymerization while sustaining the original lipid assemblies. Light treatment of the liposomes (prepared from these phospholipids) has been demonstrated to photo-cross-link without compromising the activity of entrapped enzymes.[35] Although biophysical studies demonstrate that these headgroup polymerizable lipids are potential candidates for generating stable liposomes,[33,34] further studies are needed to evaluate the merit of these lipids for sustained drug delivery and as theranostic tools.[70] In the phospholipid realm, there are only a few examples of headgroup photopolymerizable molecules (Figure 2). During the past decade, Jung, German, and colleagues have also explored similar design of molecules (nonphospholipids) for in situ polymerization in the vesicle bilayers; the biological applications of these molecules, however, have not been explored yet.[36−38]

c. Fatty Acyl-Chain-Modified Photopolymerizable Lipids and Phototriggering. As discussed above, fatty acyl chains (tail region) of the lipids play an important role in self-assembly of the lipidic nanoparticles; modifications in the tail regions are projected to influence stability, structure, and physical properties of the polymerizable nanoassemblies. About three decades ago, a number of studies were reported to this end.[29−31,39] In contrast to relatively few reports concerning chemical modifications in the headgroup region of the lipid molecules[33] (see above), introduction of various photoactivable groups in tail regions of the lipids has extensively been studied. These functionalities include the diacetylenes, methacryloyl, and sulfhydryl modifications.[19,40−42] The objective to introduce light-sensitive fatty acyl modification in the phospholipid structures was multifold including generation of stable vesicles, on-demand drug delivery, and also as tools to understand the membrane structure and function. In general, the light-induced changes in these molecules typically involve direct chemical or photon-catalyzed reactions that lead to polymerization reaction in an organized pattern. Figure 2iv−vi shows a partial list of structures of various tail-region modified photopolymerizable lipids (iv, bis-Sorb PC;[42,43] v, methacryloyl PC (a dipolymerizable lipid);[30,44] vi, $DC_{8,9}PC$[45]).

For photoreactive lipids to undergo photo-cross-linking within the liposome bilayer (photopolymerization), appropriate molecular packing of these molecules is an essential component. Apparently, segregation of polymerizable lipids within the lipid bilayer will favor intermolecular cross-linking of the lipids. This phenomenon is shown in cartoon form in Figure 3 (adapted from refs 12 and 20). The next section

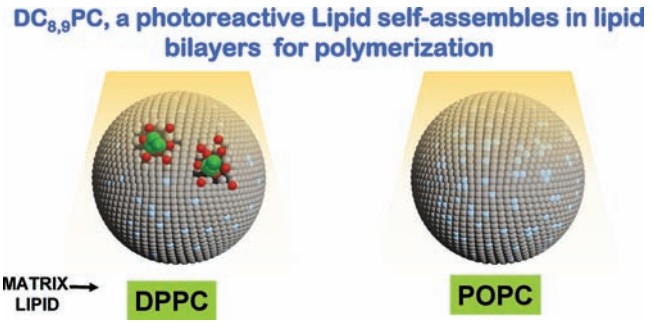

FIGURE 3. Cartoon depicting effect of bulk (matrix) lipids on self-assembly of a polymerizable lipid, $DC_{8,9}PC$ in the lipid bilayers. Gray, matrix lipid (left panel, DPPC (T_m, 41 °C); right panel, POPC (T_m, −2 °C)). Blue, light-activated $DC_{8,9}PC$ (T_m, 44 °C). $DC_{8,9}PC$ clustering in DPPC results in light-induced activation of molecules (shown in blue) that leads to $DC_{8,9}PC$ polymerization. This results in release of drugs (green) or imaging molecules (red). Right panel, $DC_{8,9}PC$ is not clustered in POPC molecules; light treatment results in activation of $DC_{8,9}PC$, but no polymerization and hence no release of contents. Adapted from refs 12 and 20.

describes the properties and theranostic potential of two most-studied photopolymerizable phospholipids containing either bis-Sorbyl or diacetylenic groups.

i. Bis-SorbPC. Initial studies conducted by O'Brien and colleagues examined liposomes containing bis-sorbyl phosphatidylcholine (bis-SorbPC, Figure 2iv) as phototriggerable drug delivery platforms.[43,46,47] Polymerization of bis-Sorb PC occurs via an oxygen radical reaction and is initiated by a photosensitizing lipophilic dye (such as 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)) by visible light (550 nm). Oxygen radicals generated by the photoactivation of DiI trigger polymerization of bis-sorbPC, leading to required defects in the lipid bilayer (see Figure 3).

ii. $DC_{8,9}PC$. The diene-containing lipid molecules have attracted considerable attention in the development of biosensors and as imaging/diagnostics tools. The chemistry of diacetylenes provides opportunities for unique molecular assemblies and light-triggered alterations. A recent review by Cashion and Long[21] describes in detail the fundamental chemical reactions that give rise to defined polymeric lipids and their applications in biomimetics design. Here we will limit our focus on studies that use diene polymers for their biological applications. The photopolymerizable phospholipid 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$, Figure 2vi) can be considered as the best-studied example in this class of polymerizable molecules. Our laboratory is investigating $DC_{8,9}PC$ for on-demand drug delivery application (see below). The biophysical traits necessary for UV-triggered polymerization as well as chemical modifications in monomeric $DC_{8,9}PC$ and/or resulting polymers have been reported earlier.[45,48,49] $DC_{8,9}PC$ is only

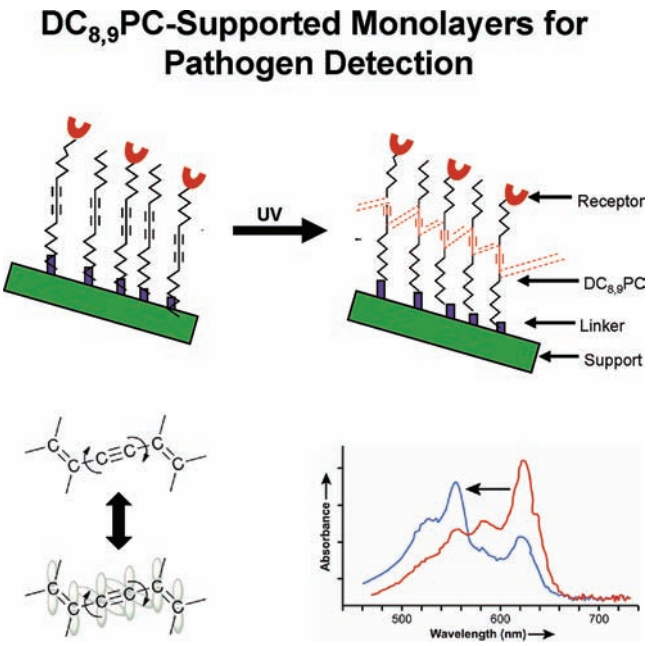

FIGURE 4. $DC_{8,9}PC$-supported monolayers for pathogen detection. This cartoon shows the principle and assembly of cross-linked $DC_{8,9}PC$ on planar surfaces for pathogen detection (top panel). Interaction with pathogens results in changes in chromogenic properties of photo-cross-linked $DC_{8,9}PC$ measured by colorimetric methods (bottom, right). Bottom (left): Schematic presentation of π-conjugated diacetylenes in planar configuration and reorganization of inter- and intramolecular rearrangements of bonds in the polymers. Figure adapted (in part) from ref 59.

found in lower organisms,[50] exhibits unique bilayer packing properties, and undergoes UV (254 nm)-induced photopolymerization in a synchronized fashion.[16,45,51] UV-treatment has profound effects on the plasticity and chromogenic properties of the resulting $DC_{8,9}PC$ polymers. These unique features of $DC_{8,9}PC$ have attracted investigators to explore this lipid for various biological, biomedical, and diagnostic applications. We believe that this molecule may prove to be a viable candidate for future theranostic applications. Currently available reports are discussed below.

Biological Applications of Polymeric Lipids

Although several polymeric lipids are currently available, among these $DC_{8,9}PC$ has been examined for multifaceted applications in the realm of biology including biomimetics, as sensors for pathogen detection,[52,53] drug delivery platforms,[12,30,46,54,55] nanoimaging agents,[27] and as components for DNA delivery[29,56] and vaccine applications.[57] In this Account, we have restricted our discussion to experimental systems where at least cell culture data are available in the literature (see below).

a. Diagnostic Tools for Pathogen Detection. Biosensing devices are considered useful systems for the detection of pathogens such as bacteria and viruses.[58] The principle relies on changes in optical or electrical properties of these sensors upon pathogen-specific interactions on ligand-coated surfaces. Since light-induced chemical modifications in polymerizable lipid molecules typically result in measurable changes in their chromogenicity and overall lipid organization, these molecules have been tested as response-sensitive components of the detection units. Nagy and colleagues were the first to develop a direct colorimetric detection method based on changes in chromogenic properties of diacetylenic lipids[59−61] upon interactions with pathogens. Since influenza virus invades cells via binding to sialic acid residues present on the cellular proteins and/or lipids,[62−64] sialic acid containing lipids were used in this biosensor design.[65,66] Diacetylenic lipids along with other nonpolymerizable lipids and a sialic acid containing lipid was coated on glass slides and then polymerized by exposure to UV (254 nm), yielding a colorimetric reaction. Upon interaction with influenza virus, the polymer linkages presumably undergo a conformational modification resulting in a shift in the chromogenic properties suitable for optical detection. The basic design of this optical sensor and the phenomenon of chromogenic alterations are shown in Figure 4.

Similarly, diacetylenic lipids were also used as a component in the optical biosensor to detect the *Escherichia coli* enterotoxin and botulinum neurotoxin[61] based on selective and sensitive binding to sialic acids. Another application was recently developed by the same group to detect shiga-like toxin producing *E. coli* using the glycodiacetylenic lipids.[53] These colorimetric detection methods may prove to be useful and find practical applications due to the rapid, selective, and sensitive design of these units. However, to our knowledge, these biosensors are not currently available in the market.

b. Nanoimaging Tools. Molecular imaging is a power tool toward diagnosis of cancer and related diseases. Image-contrast agents are widely used for the detection of disease-specific biomarkers (caused by upregulation of specific genes, etc.), and efforts have led to the development and availability of useful probes (PET/SPECT). However, similar to the targeted delivery issues for drugs/pharmaceuticals, it is critical that imaging probes reach desired areas in the body within defined space and time. In this context, the enhanced bioavailability of nanocapsules carrying the image-contrast agents can be achieved by stabilizing these nanocarriers. Choice of polymeric lipids as the components (to provide mechanical stability to nanocapsules) can be considered a

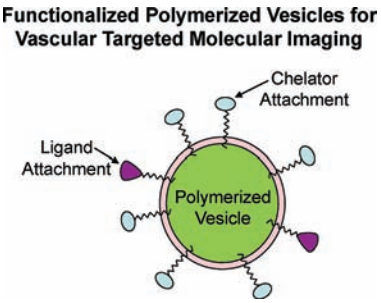

FIGURE 5. Functionalized polymerized vesicles for vascular targeted molecular nanoimaging tools. The core of the vesicle contains a polymerizable lipid and functionalized lipids for chelator attachment for imaging (blue) as well as for ligand attachment for targeting (purple). Cartoon is adapted from ref 27.

promising strategy, as light-triggered stabilization is expected to cause no or minimum perturbations in the structure and physical properties of the nanocarriers. Principles of such constructs rely on the inclusion of a polymer (such a $DC_{8,9}PC$) in the nanoassembly containing an imaging agent (such as Gd^{3+}) such that light-triggered photo-cross-linking of the polymer promotes stability to the assembled complex. Li and Bednarski were the first to develop functionalized polymerized vesicles for vascular-targeted molecular imaging.[27] The basic assembly of the functionalized polymerized vesicles is shown in Figure 5 (adapted from ref 27). These vesicles serve as a good example of theranostics, as these integrate both an imaging agent (Gd^{3+}) and a ligand for vascular targeting.

Interestingly, animal studies using these polymeric vesicles revealed promising results based on vascular targeting of receptors such as integrins and ICAM. Nevertheless, clinical translation of this promising theranostic platform for humans remains to be seen. Recently, Kumar and colleagues[71] presented an alternate strategy to generate polymerized liposomes bearing adequate functional groups for ligand attachment; this chemistry involves a click reaction (copper-catalyzed azide−alkyne cycloaddition reaction). Nevertheless, biological testing of this system is subject to future developments.

c. In Vivo Studies. Once the potential of polymeric vesicles as tools in biology was realized, it became important to examine the bioavailability and toxicity of the polymerized lipids. In the mid-1980s Regen, Juliano, and colleagues studied the interactions of polymerized liposomes with cultured cells as well their in vivo behavior.[30,31] Later, Li and Bednarski also examined polymeric vesicles as nanoimaging tools and reported biodistribution of Gd^{3+}-loaded polymerized vesicles in animals[27] (described above). Regen's as well as Juliano's group used the synthesized phospholipids (dilipoyl lipids and dipolymerizable lipids, Figure 2v) to prepare polymerized vesicles; it may be noted that these formulations did not include pegylated lipids that confer stealth properties. The biodistribution studies in animals revealed that although polymerized vesicles (with similar size distribution) were cleared from the circulation more rapidly, these exhibited more biostability.[67] The biodistribution analysis in these experiments was based on a radioactive lipid marker C^{14}-cholesteryl oleate. Therefore, the structural integrity of these vesicles in vivo may be questionable; a revisit of these experiments with inclusion of pegylated lipids and entrapped molecules (such as drugs/pharmaceuticals) in polymerized vesicles will shed light on future biological applications.

d. Drug Delivery Applications . i. Stable Nanocapsules. Polymerization of the preformed vesicles loaded with the drugs and/or imaging agents is an attractive technological tool to develop theranostic technologies for future medical applications. Improved stability of the nanocapsules is the primary advantage of this approach. Polymeric lipids bearing either the photoreactive headgroup[33] or the diacetylenic groups in the fatty acyl tail regions[68] have been examined for their potential toxic effects in cell culture systems. In both systems, light treatments had minimal effect of the physicochemical properties of the vesicles.[33−35,68] We have reported that delivery of Piroxicam, an anti-inflammatory agent, to cultured cells by headgroup polymerized lipid vesicles was superior to delivery of Piroxicam by nonpolymerized vesicles or free drug alone;[70] future in vivo studies are needed to assess the practical applications of this system. Recently, Temprana and colleagues studied the effect of light treatment on membrane interfacial properties of diacetylenic vesicles.[68,69] These authors showed that polymerization has a substantial effect on the stability of the vesicles, as the vesicles were reported to be stable up to 30 days at 4 °C. Overall membrane properties were changed as assessed by differential interaction with proteins. Although cell culture experiments indicated that polymerized vesicles did not exhibit cytotoxicity, a detailed examination of the physical and biochemical properties of polymerized vesicles is warranted for their future theranostic applications.

ii. Localized Drug Delivery. Recently, we have examined in situ light-triggered drug release properties of $DC_{8,9}PC$ liposomes.[12,54] According to our hypothesis, $DC_{8,9}PC$ forms aggregates (self-assembles) in the bilayer of phospholipids containing saturated acyl chains, and this packing is prone to create phase boundary defects in lipid model membranes (see Figure 3). In support of this hypothesis, we demonstrated that UV (254 nm)-triggered calcein release

occurs from liposomes containing a mixture of saturated phospholipids and $DC_{8,9}PC$. Here, the UV-triggered mechanism of calcein release was due to the $DC_{8,9}PC$ photopolymerization.

We are currently developing $DC_{8,9}PC$ formulations for their on-demand drug delivery applications. We have demonstrated that visible light (514 nm) treatment of liposomes loaded with a light-sensitive aqueous compound also results in release of contents.[54] Interestingly, in contrast to UV-triggered photopolymerization, visible-light triggered solute release does not occur in concert with the photopolymerization reaction. Visible light triggered release of contents appears to involve reactive oxygen species. The proposed mechanism is based on our observations that the release occurs in a wavelength specific manner and scavengers of oxygen radicals block this release (unpublished data). The exact nature of modifications in the triple bonds by the reactive oxygen radicals is unknown at present and is subject to future investigations. The $DC_{8,9}PC$ formulations appear as promising candidates for future drug delivery because visible-light-triggered release of doxorubicin (an anticancer drug) from these liposomes improved cytotoxicity in our cell culture experiments.[44] We are hopeful that our formulations can be considered as the next-generation of light-sensitive liposomes for on-demand drug delivery applications.

Although a number of light-triggerable formulations have been examined to date, none of the formulations developed so far have been successful for in vivo applications. Lack of success of light-triggered drug delivery is primarily due to two main limitations: first, lack of adequate photon energy produced by the radiation source(s); second, limitations of the available light sources suitable for deep tissue penetration. These topics were covered in detail in our recent review article.[20] We believe that the theranostic approach that will combine development of innovative strategies based on suitable photoreactive lipids combined with an appropriate imaging agent (such as metal ions) as "the helper" components will enable in vivo success in this area. One should keep in mind that the light source(s) used should have minimal effects on the biology of normal cells and tissues. The knowledge about the visible and/or infrared light sources currently in use for photodynamic therapy in patients will certainly be valuable to further develop polymeric vesicles as theranostic tools.

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BIOGRAPHICAL INFORMATION

Anu Puri received her Ph.D. degree in chemistry from the Central Drug Research Institute, Lucknow, India studying the chemical synthesis of modified phospholipids and possible use of their liposomes in drug delivery. She came to the United States in 1985 as postdoctoral fellow at the Hormel Institute, University of Minnesota. In 1986, she joined Dr. Blumenthal as a visiting fellow in the Laboratory of Mathematical Biology, NCI, NIH. Currently she holds the Research Biologist position at the CCR Nanobiology Program, NCI-Frederick, NIH. Her research revolves around several themes including (a) cell biology of viral entry, (b) development of lipid-based nanoparticles for targeted delivery of cancer therapeutics, (c) development of nanoscale diagnostic tools for detection of pathogens and cancer biomarkers, and (d) mechanisms of opportunistic infections in AIDS and related diseases.

Robert Blumenthal obtained his M.Sc. at the University of Leiden, The Netherlands, and his Ph.D. in physical chemistry at the Weizmann Institute, Israel studying mechanisms of active transport across membranes. Following postdoctoral work at the Institute Pasteur and at Columbia University studying molecular mechanisms of membrane excitability in neurons, he came to the NIH and was ultimately recruited by the NCI. In 1978, he was tenured, and in 1980 he became chief of the Section on Membrane Structure and Function. In 2005, he was appointed director of the newly established Center for Cancer Research Nanobiology Program. Dr. Blumenthal has worked in a wide range of areas in membrane biophysics, which includes membrane fusion, membrane transport, membrane domains, membrane channels, cell surface receptors, immune cytotoxic mechanisms, and use of liposomes for delivery of drugs and genes into cells. Dr. Blumenthal's current interest is in viral entry, pathogenesis and vaccines; multifunctional nanoparticles for triggered and targeted delivery of therapeutics; and photoinduced chemical reactions in membranes.

FOOTNOTES

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