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Spotlight on Photoactivatable Liposomes beyond Drug Delivery: An Enabler of Multitargeting of Molecular Pathways

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Abstract

The potential of photoactivating certain molecules, photosensitizers (PS), resulting in photochemical processes, has long been realized in the form of photodynamic therapy (PDT) for the management of several cancerous and noncancerous pathologies. With an improved understanding of the photoactivation process and its broader implications, efforts are being made to exploit the various facets of photoactivation, PDT, and the associated phenomenon of photodynamic priming in enhancing treatment outcomes, specifically in cancer therapeutics. The parallel emergence of nanomedicine, specifically liposome-based nanoformulations, and the convergence of the two fields of liposome-based drug delivery and PDT have led to the development of unique hybrid systems, which combine the exciting features of liposomes with adequate complementation through the photoactivation process. While initially liposomes carrying photosensitizers (PSs) were developed for enhancing the pharmacokinetics and the general applicability of PSs, more recently, PS-loaded liposomes, apart from their utility in PDT, have found several applications including enhanced targeting of drugs, coloading multiple therapeutic agents to enhance synergistic effects, imaging, priming, triggering drug release, and facilitating the escape of therapeutic agents from the endolysosomal complex. This review discusses the design strategies, potential, and unique attributes of these hybrid systems, with not only photoactivation as an attribute but also the ability to encapsulate multiple agents for imaging, biomodulation, priming, and therapy referred to as photoactivatable multiagent/inhibitor liposomes (PMILS) and their targeted versions—targeted PMILS (TPMILS). While liposomes have formed their own niche in nanotechnology and nanomedicine with several clinically approved formulations, we try to highlight how using PS-loaded liposomes could address some of the limitations and concerns

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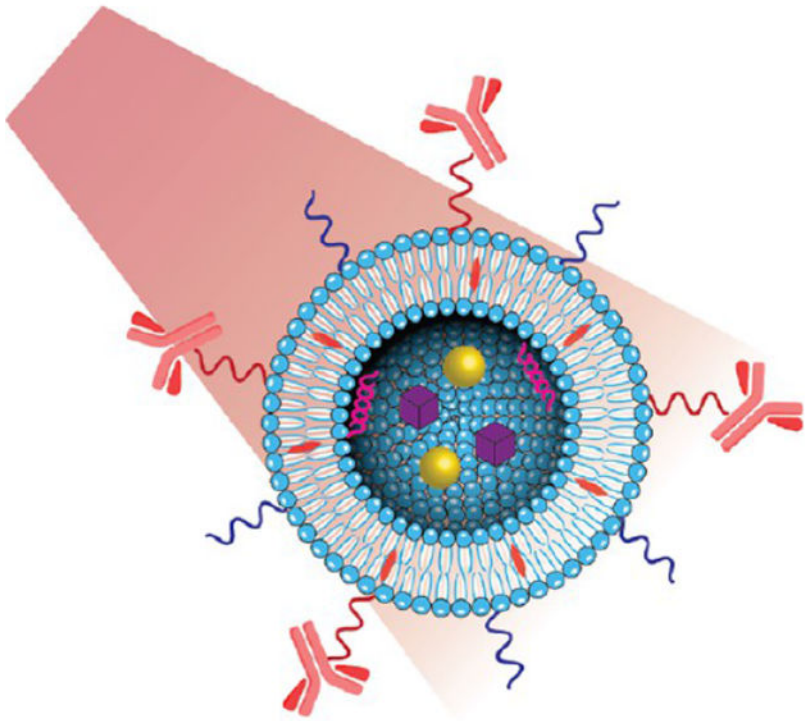
usually associated with liposomes to overcome them and enhance their preclinical and clinical utility in the future.

Graphical Abstract



INTRODUCTION

Photodynamic activation is a cascade of chemical reactions triggered by photons through their interaction with light-sensitive chemical species referred to as photosensitizers (PSs). The process bears similarities with photochemical reactions associated with photosynthesis and vision and involves photodynamic activation of PSs resulting in the generation of toxic molecular species. The therapeutic component of this activation process is commonly known as photodynamic therapy (PDT). PDT is approved for clinical treatment of various cancerous and noncancerous diseases[1–4] by administering PSs followed by their activation using light at a specific wavelength. As most clinically used PSs are hydrophobic molecules, PS formulations were initially designed to improve their solubility and biological activity. While for some PSs such as Photofrin, dissolution in aqueous buffer (5% dextrose) is possible, other PSs such as Temoporfin require the use of organic solvents (ethanol and propylene glycol);[5,6] HPPH (2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a) requires the use of 2% ethanol and 1% polyoxyethylene sorbitan monooleate (Tween 80), Verteporfin is formulated as liposomes,[7,8] and Purpurins are formulated with Cremophor EL emulsion, making them viable for clinical use.[9–11] Although these methods for solubilizing PSs are straightforward, the use of organic solvents and other chemicals for clinical application remained an issue.

Loading PSs in lipid-based particles, including micelles and liposomes, was thus a natural way forward for solubility and formulation purposes, while the exciting field of biomedical nanotechnology was evolving in parallel over the years.[12–14] In the past decade or so, there has been a convergence of these two fields because the hybrid (PS-loaded liposomes) offers some unique features of external activation allowing for controlled cytotoxicity, drug delivery, and microenvironmental modulation, simultaneously. While encapsulation of chemotherapeutic agents in liposomes was important to reduce their systemic toxicity, liposomal encapsulation of PSs serves to enhance not only their pharmacokinetics but also their stability. Lipid-based formulations of some clinically relevant PSs tested in the clinic for various malignancies are shown in Table 1.[4,13,15,16]

Although PS-loaded liposomes were initially conceived and formulated as PS carriers to enhance PS solubility, maintain phototoxicity, and achieve efficient localization and destruction of target tissues, the use of PS-loaded liposomes has since then evolved to an extent where PSs, rather than being just the therapeutic agents, also impart liposomes with exciting photoactivatable features. These include (i) fluorescence imaging; exploiting the limited quantum fluorescence yield of PSs, (ii) modulation of vasculature and tumor stroma to enhance drug/nanomedicine uptake and distribution, (iii) providing phototriggerable features for tuning drug release profiles, (iv) assisting in lysosomal escape of drug/biologics through the process of photochemical internalization (PCI), and (v) imparting cytotoxicity and overcoming treatment resistance (Figure 1). Moreover, drug compartmentalization in liposomes and the possibility of simultaneously coloading drugs assists in synchronizing pharmacokinetics thus deriving maximum benefits from the synergistic potential of the coloaded agents, including the PS, by delivering them in the "*right place*" at the "*right time*". Many of these features can also be achieved by encapsulating PSs in solid-lipid nanoparticles and micelles;[14,28–30] however, drug compartmentalization as achieved in liposomes cannot be achieved in these systems. In micelles, the coloaded small molecules may be close to the PS molecules and hence prone to destruction, even before their activity is realized, by the RMS generated during irradiation of the PS. Liposomes protect the coloaded agents from immediate destruction by the RMS generated during PS irradiation, by spatially segregating the two agents in separate compartments (the hydrophobic liposomal membrane and the hydrophilic lumen), thereby maintaining efficacy of these agents. This review discusses various aspects related to the design and application of PS-loaded liposomes and how photochemical processes can be exploited to overcome certain limitations associated with current liposome applications, as summarized in Figure 1.

LIPOSOMES IN PHOTODYNAMIC THERAPY

The strategy of encapsulating PSs in liposomes, although complex as compared to traditional dissolution techniques, offers several advantages due to their optimal size, enhanced localization at target sites, coencapsulation of multiple pharmacological agents, and ability to conjugate targeting ligands.[8,13,31–34] Since its initial conceptualization as vehicles for drug delivery,[35,36] liposomes have evolved and are still relevant as drug delivery systems in both preclinical and clinical practice.[37,38] For further reading into the evolution of this exciting technology readers are referred to excellent recent and dated reviews.[37–40]

The focus of the current review, however, is to highlight the advantages conferred by the hybrid system what we refer to as PS-loaded liposomes.

Despite advances, current liposomal technology has certain limitations. First, liposomes cannot be tuned to achieve desired drug release profiles. Tunability and achieving a sustained drug release are important factors in nanomedicine where maintaining a desired dose of therapeutic agents over prolonged periods is necessary.[42] Liposomes are generally taken up by cells either through endocytosis or through fusion with the cell membrane to release their payload (Figure 2).[41,43,44] Tuning drug release profiles of liposome-based delivery formulations has not been achievable thus far,[45,46] although several strategies such as prolonging circulation time through PEGylation or encapsulating liposomes in gels/polymers have yielded encouraging results.[47–49] Second, the delivery of biologics through liposomes is still challenging and requires modifications in the lipid composition to achieve delivery of these agents in an active form. As mentioned earlier, liposomes are usually internalized through endocytosis,[41,50] resulting in the exposure of the payload to the lysosomal components where the low pH and high enzymatic activity (proteases and nucleases) may result in the degradation of therapeutic agents delivered by liposomes (Figure 2). In this regard, attempts have been made to incorporate cationic lipids (such as DOTAP; 1,2-dioleoyl-3-trimethylammonium-propane) to assist in lysosomal escape through the proton-sponge effect and the development of fusogenic liposomes that transfer the payload via fusion with target cell membranes thus avoiding contact with the lysosomes.[43] While these strategies have been successful to an extent, modification of liposome content specifically with cationic lipids can alter the protein corona formation in circulation and induce rapid opsonization and clearance from circulation.[51–53] Third, liposomes are inherently nontargeted and achieve desired accumulation at their respective target sites through enhanced permeability and retention (EPR)-based passive accumulation.[54] Although incorporation of targeting moieties has been reported to improve tumor specificity in preclinical studies, the efficacy of actively targeted liposomal formulations is being explored in several clinical trials.[55,56]

Despite these limitations, liposomes have remained useful, and their clinical acceptance is increasing, suggesting a clear opportunity to use liposomes synthesized from the library of available lipids.[57] However, there are continuous efforts to overcome the limitations associated with liposomes and improve their performance. In this context, photochemical activation provides a unique niche which employs the incorporation of PSs in liposomes, combining the benefits and delivery potential of liposomes with the therapeutic and imaging potential of these light activatable chemicals.

PHOTOCHEMICAL AND PHOTOBIOLOGICAL OVERVIEW OF PHOTODYNAMIC THERAPY (PDT)

PDT, as discussed earlier, involves the irradiation of light-activatable molecules leading to a cascade of events resulting in the production of reactive molecular species (RMS) including reactive oxygen species (ROS).[1,2] The time duration between PS administration and irradiation is referred to as the drug light interval (DLI) and plays an important

role in the subsequent action and consequences of the photodynamic activation process (Figure 3A). While short DLIs favor vascular effects due to the presence of PS in the vasculature,[58] longer DLIs result in the accumulation and distribution of the PS in the target sites with more diverse effects including microenvironmental (of both cellular and acellular components) modulation and cytotoxicity.[58–60] For cancer management, PDT with longer DLIs has been found to be more efficient in restricting tumor growth and inducing tumor cell death.[58] However, specific applications such as treatment of age-related macular degeneration (AMD), where vasculature closure is required, PDT with short DLIs is the approved FDA treatment regimen.[61–63] Irrespective of the DLI, the photochemistry of the process is the same where PS irradiation leads to its excitation to a singlet excited state (PS^1*) from the ground state (PS1). PS from the singlet excited state (PS^1*) can either emit radiation in the form of fluorescence or alternatively undergo intersystem crossing to form a long-lived triplet excited state (PS^3*). Subsequent electron (type 1 reactions) or energy (type 2 reactions) transfer from PS^3* to biomolecules leads to the generation of RMS resulting in cytotoxicity or biomodulation, as has been emphasized in recent studies (Figure 3B).[1,59,60,64]

There have been several modes of cell death associated with PDT, ranging from apoptosis and necrosis to the somewhat rare but important mechanisms such as paraptosis, ferroptosis, pyroptosis, and necroptosis.[1,65,66] The mechanism of cell death induced by PDT is mainly determined by the type of PS and its subcellular localization at the time of irradiation. Importantly, cell death induced by most PSs and PDT regimens has been demonstrated to be immunogenic resulting in robust long-term systemic antitumor immune responses which further assist in enhancing the therapeutic efficacy of this treatment modality.[67–70] While adaptive immune responses induced by PDT are now well established and form the basis of several combination therapies of PDT with immune checkpoint inhibitors,[71,72] the systemic responses of PDT and its antimetastatic effects are also observed in immunocompromised preclinical models. These responses are attributed to PDT-mediated (i) reduction of cancer stem (-like) cells, characterized by CD44 and CXCR4 expression, involved in treatment resistance and metastasis,[59] (ii) reduction in the expression of CXCL12 and its receptor CXCR7, a paracrinesignaling axis facilitating metastatic colonization,[60] and (iii) possible modulation of cytokines involved in metastatic disease progression.[73,74]

Photochemistry and Liposomal Composition—Design Considerations for Synthesis of Photosensitizer-Loaded Liposomes.

While encapsulation in lipid bilayers, in general, improves solubility and pharmacokinetics, lipid composition can have an influence on the photochemical and photophysical properties of the encapsulated PS. Importantly for hydrophobic PSs with a tendency to aggregate, formulation in liposomes have been shown to promote monomerization and enhance PDT efficacy.[75–78] PS encapsulation in liposomes requires specific considerations to maintain the photochemistry of the encapsulated PS. In general, higher PS loading ratios (>15 mol percentage of the total PS and lipid content) tend to destabilize the liposomes and result in aggregation.[8] Most liposomal formulations comprise phospholipids (Figure 4) with varying degrees of unsaturation and acyl chain lengths. Further incorporation of cholesterol and

polyethylene glycol (PEG) imparts stability and improves pharmacokinetics for in vivo applications.[79]

Lipid Composition and Photochemistry.—Photosensitization is a complex process and is influenced by the microenvironment of the PS and the relative oxygen levels. In general oxygen solubility is higher in lipids with high fluidity (higher degree of unsaturation).[80] Moreover, the lifetime of singlet oxygen, generated after PDT, is also relatively higher (13–35 μs) in lipid bilayers as compared to aqueous solutions (3–4.4 μs).[81–84] Therefore, singlet oxygen generated from PSs that are intercalated deep in the lipid layer get more time to exert damage on lipid structures surrounding the PS, while diffusing to the surface where they can interact with other biomolecules.[83,84] While for PSs encapsulated in liposomes this may influence liposome permeability and drug leakage, for PSs partitioned into cellular membranes, after delivery through liposomes, this can result in damage of membrane lipids and associated proteins leading to cytotoxicity. For this reason, PSs with higher lipid intercalation have been suggested to have higher PDT efficacy.[83] Saturated phospholipids, on the other hand, are more rigid, impermeable, and less susceptible to singlet oxygen-based peroxidation. In the context of PS loading, the lower fluidity of liposomes comprising saturated lipids hinders PS intercalation.[81,85] In a study by Massiot et al.[81] the leakage of calcein from model liposomes synthesized with DOPC was found to be higher as compared to the ones synthesized with SOPC and SLPC. While all three lipids SOPC, SLPC, and DOPC contain double bonds, the presence of two double bonds on each acyl chain of DOPC resulted in a more pronounced effect on lipid peroxidation and membrane permeability of hydrophobic PSs, thus leading to enhanced calcein release. Similar observations were made by Luo et al. where incorporation of DOPC in porphyrin phospholipid (PoP) liposomes accelerated doxorubicin release and enhanced treatment efficacy in in vivo pancreatic tumor xenograft models.[86]

Although incorporation of free PSs in lipid membranes, as discussed previously, is straightforward, it however results in premature leakage of the PS. Moreover, it also influences the adsorption of multiple agents in the liposomes as reported by Mir et al., where incorporation of Verteporfin in liposomes influenced the subsequent adsorption of Cetuximab (anti-EGFR).[32] Despite several interesting concepts for PS-loaded liposomes, PSs are prone to rapid exchange with serum components, thereby defeating the purpose of advanced liposome design.[18,87] To circumvent this issue, strategies involving conjugation of PSs to lipids have also been attempted which can then form PS-lipid nanostructures (micelles, lipid vesicles, and liposomes) through self-assembly and limit premature leakage of the loaded PS.[70,88–93] As covalent conjugation of PSs to lipids is expected to influence their photochemical and photophysical properties, Obaid et al. developed liposomes with Verteporfin conjugated to various lipids (16:0 Lyso PC, 20:0 Lyso PC and DSPE-PEG_{2000}) and compared them with the clinical formulation of Verteporfin–Visudyne, containing the lipids EPG and DMPC. In this study, Visudyne showed the highest rate of photobleaching (4.97 nM/s) which was attributed to the quenched state of Verteporfin leading to photobleaching by self-oxidation. While the photobleaching rate was similar for liposomes synthesized with 16:0 Lyso PC and 20:0 Lyso PC, photobleaching for DSPE-PEG_{2000} liposomes was relatively lower and comparable to that of the lipidated PS in DMSO. Singlet

oxygen formation, as detected by the fluorescent probe SOSG, negatively correlated with the photobleaching results and Visudyne showed the lowest rate of SOSG fluorescence increase. Importantly, the photophysical and photochemical properties of the different liposomes did not correlate with the phototoxicity observed in in vitro OVCAR-5 cells, suggesting that parameters such as interaction of PSs within the cells, their subcellular localization and interaction with serum proteins (protein corona), possibly plays a deciding role in phototoxicity and should be taken into consideration while designing such formulations (Figure 5).[51,53,90,94–96]

Photosensitizer Hydrophobicity, Membrane Insertion, and Photochemical Parameters.—As discussed earlier, hydrophobic PSs tend to get inserted into the hydrophobic lipid tails in the liposomal membranes, whereas amphiphilic PSs with hydrophilic moieties insert partially.[81] As membrane inserted PSs tend to have a higher contact surface area with membrane lipids, their ability to oxidize membrane lipids tend to be higher, more so because of the higher solubility and the longer lifetime of O_2 and ROS, respectively, in the lipid layer.[81,82] Lajunen et al. studied the effect of Indocyanine Green (ICG) incorporation either in the lipid bilayer or in the PEG layer on liposomal surface. It was found that ICG adsorption in the PEG layer was also capable of inducing a release of the encapsulated payload (calcein and FITC-dextran) through phototriggered effects (although mostly thermal) leading to membrane instability.[97,98] In another study, Guirguis et al. demonstrated that liposomes prepared with membrane-protruding DSPE-PEG-IRDye700DX PS-lipid conjugates (Figure 6A) provide rapid phototriggered release of the encapsulated agent in comparison to liposomes synthesized with a membrane-inserting BPD-PC PS-lipid conjugate where the encapsulated agent (Calcein) was released in a sustained manner (Figure 6D). This correlated with the higher singlet oxygen (Figure 6B), hydroxyl radical, and peroxynitrite anion (Figure 6C) formed by Lipo IRDye700DX as compared to the membrane inserted PS-Verteporfin liposomes.[99] However, the cytotoxicity was significantly higher for the liposomes with membrane inserted BPD as compared to the Lipo IRDye700DX (Figure 6E). The contrasting findings reported with respect to PS properties and subsequent liposomal membrane damage, post irradiation, suggest that parameters beyond solubility and membrane insertion may be important in governing the properties of PS-loaded liposomes. These include the photochemical properties of the PS, quantum yield of ROS generation, and lipid modifications (such as PEGylation status) which should also been taken into consideration while designing these PS-loaded liposomes.

FUNCTIONAL SIGNIFICANCE OF PHOTOSENSITIZER-LOADED LIPOSOMES

The incorporation of PSs in liposomes enhances the pharmacokinetic properties of the PS. For PS-loaded liposomes, prepared by either passively incorporating PSs during the synthesis process or generating PS-lipid conjugates and self-assembling them into bilayers,[88,90] PSs usually partition into the lipid bilayers due to their amphiphilic/hydrophobic nature, with a few exceptions.[97,98,100] This leaves the lumen of the liposomes vacant and provides an opportunity for coloading of other imaging or therapeutic agents. The ability to coload PSs with other agents in liposomes yet keeping these agents spatially

segregated forms the basis of several unique features of these hybrid PS-loaded liposomes which are discussed in this section.

Image-Guided Therapies.

PSs have a finite fluorescence quantum yield and therefore can be utilized for their fluorescent properties. Some of the clinically approved applications of PSs exploit their fluorescence properties for diagnosis and treatment guidance such as fluorescent-guided surgeries (FGS).[101–104] While the use of PSs for guiding fluorescence-based surgical resection is well accepted and clinically approved, the limited fluorescence quantum yield of PSs and the general lack of depth profiling conferred by fluorescence imaging are major limitations which can be overcome through other complementary imaging methodologies. Liposomes provide a platform to incorporate multiple contrast agents and enable multimodal imaging where fluorescence imaging can be complemented by other imaging methodologies such as photoacoustic imaging (PAI), Positron Emission Tomography (PET), Computed Tomography (CT), and Magnetic Resonance Imaging (MRI) (Figure 7).[105–109]

While incorporating additional contrast agents in PS-loaded liposomes provides appropriate complementation to improve detection, these imaging techniques can also assist in guiding PDT by assisting in detection of tumor volumes for deep tissue PDT. Another possible application of these multiagent liposomes is in guiding surgical resections where current protocols of identifying tumor margins through fluorescence imaging have limitations. Complementary imaging enabled by multiagent liposomes can provide enhanced contrast and a volumetric profile of the tumor tissues for guiding surgeries. As the presence of microscopic tumor tissue is a major cause of recurrence, post-tumor resection surgeries, the inherent photosensitization property of PSs can be exploited to treat the residual tumor tissue by irradiation of the tumor bed, as has been suggested in several preclinical studies.[110–112] While such complementary imaging methodologies are interesting, the sequence of imaging and PDT is important as has been suggested in a study by Xavierselvan et al., where ROS generated by Verteporfin oxidizes ICG and limits its photoacoustic imaging capabilities,[113] highlighting the importance of carefully designing and utilizing these multiagent liposomal constructs.

Imparting Tumor Tissue/Cell Specificity.

Liposomes and other nanomaterials, in general, passively accumulate in tumor tissues, due to the Enhanced Permeability and Retention (EPR) effect.[54] Incorporation of PSs can provide a second level of selectivity through irradiation of confined volumes focused on target sites. This inherent dual selectivity of PSs has been exploited to minimize off-target effects usually associated with other cancer therapies. However, regions where confinement of irradiation volumes is not possible, as in the case of micrometastatic ovarian disease where irradiating the entire peritoneal space is required, tumor cell specificity is preferred to minimize off-target toxicities associated with PDT. This was indeed the case, as initial clinical attempts with PDT of micrometastatic ovarian cancers resulted in dose-limited off-target toxicities including bowel perforation.[114–116] Similarly, PDT of pancreatic tumors involving the gastroduodenal artery, requiring illumination of larger volumes, resulted in cases of gastrointestinal bleeds.[117] Therefore, in an attempt to enhance tumor specificity,

molecular targeted PSs have been developed through conjugation of PSs to antibodies targeting receptors overexpressed on tumor cell surfaces—EGFR, HER2, Transferrin Receptors, Folate, etc. (Figure 8B).[118–125] These antibody-PS conjugates find application in the detection, treatment, and treatment guidance of tumor tissues,[111,124,126,127] as highlighted by the recent clinical approval of photoimmunotherapy in head and neck cancers in Japan and active phase III clinical trials in USA (NCT03769506) for patients with head and neck cancers. Several clinical trials with antibody fluorophore conjugates are also exploring the utility of these probes for guiding tumor resection surgeries.[128–131] While the generation of photosensitizer-antibody conjugates (Photoimmunoconjugates; PICs) provides the much-needed specificity, the delivery of PSs to target cells through PICs is relatively lower as compared to free PSs, thus requiring much higher light doses for their activation and phototoxic activity. To enhance PS uptake while maintaining specificity, Huang et al. reported the development of PIC-NC developed by the conjugation of PICs to nanoconstructs (NCs). These PIC-NCs take advantage of the “carrier effect” and enhance the amount of PS taken up by the target cells (Figure 8D).[132,133] Conjugating PICs on nanoconstructs provides the much-needed specificity and enhanced PS uptake in target cells; however, the conjugation of PSs away from the lipid bilayer limits their ability to provide a phototriggered pay-load release.

Alternatively, molecular-targeted PS-loaded liposomes, using antibodies, antibody fragments, peptides, and other ligands, have been developed.[134–136] Initial studies demonstrating the development of antibody-based targeted liposomes relied on passive adsorption of antibodies on PS-loaded liposomes (Figure 8E),[32] which can result in dissociation of the adsorbed agents upon contact with serum proteins and upon prolonged storage.[32] Several studies have since reported the development and utility of antibody conjugated PS-loaded liposomes (Figure 8F).[64,91,137,138] Obaid et al. presented a comparison of stochastic versus site-specific conjugation of Cetuximab in targeting photoimmunonanoconjugates (PINs) to EGFR overexpressing human cancer cell lines (Figure 9). PINs with stochastic Cetuximab conjugation (Cet-PINs) were approximately 2.72-fold more efficient at EGFR binding and cellular internalization as compared to PINs with site-specific Cetuximab conjugation (Cet-Pz-PINs) (Figure 9A, B, and C). Cet-PINs showed tumor specificity as validated by photoacoustic imaging of Cet-PIN-IRDye800 (Figure 9D–G) with a perivascular penetration ranging from 174 to 473 μm. The Cet-PINs induced tumor necrosis in heterotypic PDAC tumors, even at a 10-fold lower clinical photosensitizer concentration, highlighting the potential of specificity tuned photonanomedicine in increasing efficacy and reducing collateral damage associated with the administration of high PS payloads.[64] While single receptor targeted liposomes specifically target tumor subpopulations expressing the corresponding cognate receptor, heterogeneity in receptor expression often encountered in tumors poses challenges.[139,140] Moreover, heterogeneity in receptor expression is often associated with variable treatment responses encountered in cancer treatment.[141] To address this, dual- and triple-targeted nanoconstructs have been designed to further enhance tumor cell specificity and improve treatment outcomes in heterogeneous tumor populations (Figure 8G).[70,92,142,143] Bano et al. demonstrated an enhanced efficacy of triple receptor (EGFR, HER2, and transferrin receptors) targeted PS-loaded liposomes in a heterocellular 3D tumor model developed

with human cancer cell lines with varying expression levels of EGFR, HER2, and transferrin receptors (Figure 9H and I).[70,92] While it is not yet clear if such multitargeting approaches would be as successful in vivo, it nevertheless is an intriguing approach which proposes a strategy to address molecular heterogeneity usually observed in tumors. Further preclinical validation would shed more light into the actual efficacy of these multitargeted nanoconstructs.

Photodynamic Priming - Photosensitization of the Microenvironment.

Although initially conceived as a local treatment modality, PDT and the associated phenomenon of photodynamic priming (PDP) have been shown to have pleiotropic effects mainly dictated by the PS microenvironment at the time of irradiation (Figure 10).[1] These effects can be exploited to (i) enhance the efficacy of other drugs through sensitization of otherwise nonresponsive target cells,[144–146] (ii) enhance drug accumulation at the target site by increasing vascular permeability and extracellular matrix (ECM) modulation,[64,91,147–149] (iii) relieve immune suppression and activating immune responses against target cells,[67,71,150] and (iv) prevent the selection and enrichment of treatment resistant cellular populations.[59] The process of PDP is generally associated with PDT due to the heterogeneity in PS distribution and nonuniformity of target site irradiation leading to differences in the effective PDT dose (product of PS concentration and light dose). While cells receiving a toxic dose tend to undergo cell death, other cells receiving a subtherapeutic PDT dose have been demonstrated to undergo transcriptomic, proteomic and metabolomic changes.[151] Apart from changes at the cellular level, PDP can also bring about changes in the acellular structures of the tumor microenvironment depending upon the PS localization.[64,91,147,149]

It has been previously established that for nanoparticles in the size range of 15–100 nm, active targeting to tumor cells achieves 0.1% to 11.8% targeting efficiency, whereas the rest of the administered dose entering the tumor microenvironment either gets trapped in the acellular regions or is taken up by macrophages.[152] As most FDA approved chemotherapeutic drug loaded liposomal formulations are in the size range of 60–100 nm,[153,154] the ability of these formulations to interact and internalize in the desired tumor cells is expected to be low. Moreover, as the targets of chemotherapeutic agents are usually intracellular, it is imperative for these clinical liposomal formulations to be internalized and make the released cargo available for action. As most liposomal formulations rely on target cell fusion and internalization to release the loaded cargo, the process of photoactivation can offer advantage in this context by triggering drug release, through external irradiation, even after deposition of the liposomes in the tumor interstitium. PDT has been established to modulate ECM[64,91,147] and results in an increased accumulation and homogeneous distribution of nanomedicine in tumors resulting in enhanced treatment efficacy.[59,60,64,147] Studies from our group and others suggest that PDT reduces collagen density, and more importantly it is the increase in collagen fiber nonalignment that correlates with overall survival and reduced tumor burden in mice (Figure 11A and B).[91] Loosening of ECM, after PDT, is suggested to be a major reason for enhanced accumulation of several chemotherapeutics including Doxil and Onivyde in the tumor interstitium.[59,60,149,155] Huang et al. reported an approximately 10-fold increase in the accumulation of nanoliposomal

irinotecan (nal-IRI), after PDT (Figure 11C) which resulted in a long-term response in MIA PaCa-2 and AsPC-1 PDAC tumors as compared to nanoliposomal irinotecan (Figure 11D and E).

While the concept of photodynamic priming and microenvironmental modulation through photoactivation of PSs is now a well-accepted phenomenon in cancer biology, its application and potential in the field of antimicrobial PDT is gaining popularity and the readers are referred to some recent reviews for further reading.[1,156]

Enabling Mechanism-Based Combination Therapies.

In cancer therapeutics, it is increasingly evident that combination therapeutic regimens targeting nonoverlapping cell death pathways have maximum impact on therapeutic outcome. Single agent therapies, although induce therapeutic responses, may also lead to treatment resistant phenotypes over the course of treatment.[157] PDT, apart from permeabilizing the TME to maximize drug uptake and distribution, has also been established to enhance the therapeutic efficacy of other drugs by directly destroying several anti-apoptotic proteins (Bcl-2 and Bcl-XL) and drug transporters (P-glycoprotein ATPase) to overcome treatment resistance often observed in cancer cells.[144,146,158,159] PDT has been shown to enhance therapeutic efficacy of several chemotherapeutic agents and resensitize treatment-resistant tumor tissues/tumor cell lines to subsequent chemotherapies.[118,121,144,145] As suggested previously, the ability of PDT to act against chemo- or radiotherapy resistant tumors is primarily due to the pleiotropic nature of the therapy and the ability of reactive molecular species to directly destroy biomolecules in their vicinity. In addition, the mechanistically distinct and non-overlapping modes of cytotoxicity induced by PDT and chemotherapy further assists in enhancing the synergistic potential of these combination therapies. While these studies highlight the importance of combining photodynamic priming with chemotherapy, the therapeutic potential of these combinations is expected to enhance further through coformulation in nanoconstructs. In this context, liposomes hold an advantage as they provide an opportunity to load these agents into different compartments—the lipid layer and the hydrophilic lumen, thereby segregating them physically yet delivering them in the “same place” at the “same time” to maximize their synergistic potential.[34]

To maximize the therapeutic potential of PDT-based combination therapies several nanoformulations with PSs and chemotherapeutics have been reported.[34,59,91,160,161] Liang et al. reported a nanoliposomal formulation combining Verteporfin and irinotecan where Verteporfin conjugated anti-EGFR antibodies (Photoimmunoconjugates; PIC) were further conjugated to irinotecan loaded liposomes generating the formulation PIC-Nanoliposomes (PIC-NL) (Figure 12A).[133] The efficacy of the construct was found to be EGFR specific and showed 20% more cytotoxicity as compared to the combination of PIC + Nal – IRI.[133] The enhancement in cytotoxicity of the construct was attributed to the enhanced uptake of the nanoconstruct, due to the “carrier effect” (Figure 12B)[132] and the synergy between the 3-way combination therapy of Cetuximab, Verteporfin-PDT, and irinotecan enabled via a liposomal nanoconstruct operating by EGFR downregulation, mitochondrial depolarization, and DNA damage (Figure 12C and D).

As toxicity of chemotherapeutic agents is a major hurdle in continuing treatment in the clinic, combination therapy with PDT has been shown to allow the use of several-fold lower dose of chemotherapeutic agents while maintaining their efficacy. Anbil et al. demonstrated a 75% dose reduction in nal-IRI, while maintaining treatment efficacy in preclinical heterotypic models of stroma-rich pancreatic tumors, in combination with Verteporfin-PDT and a stromal modulation therapy by Vitamin D receptor activation using Calcipotriol.[60] In a separate study, an XL184 (Cabozantinib) concentration which was ~1/6700 of the daily oral XL184 monotherapy dose was found to be effective, in preclinical pancreatic tumor models, when administered through a photoactivatable multiinhibitor liposome (PMIL).[162] The PMILs were synthesized with Verteporfin in the lipid layer of the liposomes, imparting them photoactivatable features, while the chemotherapeutic agent XL184 was encapsulated in PLGA nanoparticles and loaded into the lumen of the liposomes. The proposed PMILs targeted distinct molecular pathways all at the same time. While PDT results in the induction of tumor cell apoptotic pathways and microvessel damage, XL184 inhibits VEGF and MET signaling to further suppress tumor angiogenesis, vascular regrowth, cancer cell motility, invasion, and metastatic escape in response to tumor hypoxia.[162] To further increase the therapeutic efficacy of such combination therapies, targeted constructs loaded with multiple therapeutic agents; TPMILs (targeted photoactivable multi-inhibitor liposomes) have been developed and demonstrated to provide tumor cell specificity and enhanced therapeutic efficacy even at 8.1% patient equivalent dose of irinotecan, as evaluated on a preclinical desmoplastic PDAC model.[91]

With the recent emergence of immunotherapy in cancer management and realization of the immunogenic potential of PDT-mediated therapeutic regimens, several combination approaches involving PDT and immune modulatory agents have been attempted. Although the adaptive immune responses mediated by PDT are well studied now; PDT, however, as mentioned earlier, also leads to therapeutic responses at remote sites in immunocompromised animals, thus highlighting the potential and unique features associated with this treatment modality.[59,73] Most popular immunomodulatory agents (immune checkpoint inhibitors) target specific receptors on immune cells (PD1 and CTLA4) which are not usually confined in the TME and may be present in secondary lymphoid organs (lymph nodes and spleen). Therefore, the maximum therapeutic potential of these inhibitors is usually realized when administered as separate entities and not through coloading with PSs into liposomes or other nanoconstructs. However, several small molecule immune modulatory agents including metabolic checkpoint inhibitors such as 1-methyl tryptophan and immune adjuvants such as CpG oligodeoxynucleotides (for DC activation),[72,163] have taken advantage of coloading with a PS in nanoconstructs, as their targets are present in the TME, while the effects are systemic. With promising preclinical results, several ongoing clinical trials (NCT04305795, NCT04836429, NCT04400S39) are evaluating the potential of PDT in combination with immunomodulatory agents for the treatment of various cancers.

Phototriggered Drug Release—Tuning Drug Release Profiles.

A major limitation of liposomal delivery vehicles is the lack of control over release of the encapsulated payload, thereby limiting their use as sustained release vehicles. Most liposomes release the encapsulated drug upon cellular internalization and subsequent

disruption of the liposomal membrane leading to drug leakage.[41,43] Sustained release formulations of liposomes rely on strategies to enhance circulation time or embedding liposomes in sustained release gels, both of which limit their use and function.[47–49]

In contrast, PS-loaded liposomes where the PS is loaded in the liposome membrane can be triggered externally to initiate drug release. This is achieved mostly due to PS-mediated generation of ROS which results in oxidation and subsequent disruption of the lipid membrane of the liposomes. Other phenomenon such as photo-cross-linking, photoisomerization, photothermal, and photocleavage of lipids in the liposomes has also been reported and utilized to trigger drug release via an external trigger.[165] Phototrigger using suboptimal light doses has been shown to attain sustained release of the encapsulated therapeutics by partial disruption of the liposomal membrane.[81,97,164,166–170] As mentioned earlier, the phototriggered release of drugs from liposomes is influenced by lipid composition of the liposomes, and photosensitizer properties,[81] both of which can influence the release of the encapsulated cargo in a size-dependent manner.[171] Several studies have now demonstrated the preparation of optimized PS-loaded liposomes where phototriggered drug release is possible. Importantly, in these studies the drug release was found to be strictly associated with irradiation and alternating light and dark cycles was shown to release the encapsulated drugs in a stepwise manner suggesting resealing of the liposomal membrane after the irradiation.[93,164] Enzian et al. demonstrated the release of Calcein from two model liposomes synthesized with 5,10-DiOH, but with different lipid compositions. While the liposomes synthesized with low cholesterol content (molar ratio of 2.3) showed an almost complete (100%) Calcein release with 10 irradiation cycles (420 nm and 20 mW/cm^2 for 20 s) (Figure 13A), liposomes synthesized with a higher cholesterol content (molar ratio of 9.6) showed a maximum of 20.46% release of Calcein during the same time duration with 10 irradiation cycles. This was attributed to the stability conferred by cholesterol (Figure 13B).[172] The potential utility and selectivity of light triggered chemotherapeutic agent releasing liposomes was demonstrated by Carter et al. where irradiation with a 658 nm laser light (200 mW/cm^2 for 12.5 min (150 J/cm^2)) after injection of Dox–PoP–liposomes in nude mice bearing subcutaneous KB tumors. Biodistribution analysis revealed the presence of 3-fold higher Dox in the tumors of irradiated mice as compared to the non-irradiated tumors. The regions surrounding the tumor (muscle and skin) also showed significantly higher dox accumulation as compared to other organs where dox accumulation was not significantly different as compared to the non-irradiated mice. Importantly, a single dose of Dox—PoP–liposomes (10 mg/kg Dox dose) was enough to cure tumors with 80% mice showing permanent tumor cure and no recurrence after 90 days of therapy.[93] Yet another interesting strategy to attain sustained release of encapsulated payloads was developed by Spring et al. They demonstrated the deposition of XL184-loaded PLGA-NPs in xenograft models of PDAC, through phototriggered liposomes. In this study, irradiation was shown to result in PLGA-NP release which then sustained the release of RTKi (XL184) for a few days, allowing the use of relatively lower concentrations of the drug (1/6700-fold lower XL184) which otherwise causes high off-target toxicity (Figure 13C—E).[162]

While phototriggered release is an interesting feature with high clinical potential, the ability to irradiate desired sites multiple times over prolonged periods to attain sustained release of therapeutics is still challenging. Advances in the field of photonics have led to the

development of sophisticated light delivery methods which can be used for this application. Moreover, the use of bioluminescent molecules which can be codelivered with PSs and assist in photoactivating the latter through bioluminescence resonance energy transfer (BRET) is also a forward-looking approach for irradiating PSs in deep-seated tumors, thus bringing about the desired photochemical reactions.[173]

Photochemical Internalization—Implications in the Delivery of Biologics.

Chemotherapeutic agents and biologics are usually prone to degradation through lysosomal action resulting in a decrease in their therapeutic efficacy. They are therefore delivered by formulating in positively charged polymers such as poly(L-lysine), polyethylenimine (PEI), etc., which facilitate lysosomal escape through lysosomal membrane rupture by the proton sponge effect.[174–176] In the case of liposomes, apart from cationic lipids such as DOTAP and DOTMA, which mediate lysosomal release,[177,178] incorporation of lipids such as dioleoylphosphatidyl ethanolamine (DOPE) and dipalmitoyl succinyl glycerol (DSPG) has been shown to promote lysosomal membrane incorporation and destabilization thus facilitating the release of the loaded content.[179] However, the efficiency of these methods remains modest at this point.

In this context, photochemical internalization (PCI) has been demonstrated to be effective in the cytosolic delivery of therapeutic agents in an active form.[180–182] PCI involves the localization of a PS in the endolysosomal complex followed by its activation through irradiation to facilitate lysosomal membrane reorganization and rupture resulting in the leakage of lysosome-entrapped drugs into the cytosol (Figure 14A). Since the first reports of PCI-mediated cytosolic delivery of type I ribosome inactivating proteins, horseradish peroxidase (p21ras-derived peptide), and a plasmid encoding green fluorescent protein by Berg et al. in 1999, PCI has since been utilized for increasing the cytosolic delivery of antibodies, proteins, nucleic acids, and chemotherapeutic agents.[183] As the phenomenon of PCI utilizes photochemical processes similar to PDT, there is some phototoxicity usually associated with the process. While optimizing the light dose and PS concentrations can limit the toxicity and increase the efficiency of the process, the associated phototoxicity can however be an advantage in cases such as cancer therapy where effects mediated by PDT can synergize with chemo/biological therapeutic agents delivered through PCI. In general, amphiphilic PSs such as disulfonated tetraphenyl chlorin ($TPPS_{2a}$) and aluminum phthalocyanine disulfonate ($AlPcS_{2a}$) are more efficient in mediating PCI due to their ability to associate and intercalate with lysosomal membranes where the generated ROS can have a more pronounced effect on lipid peroxidation leading to its rupture.[184] Hydrophilic PSs, on the other hand, tend to penetrate membranes and localize in the cytosol away from the lysosomal membranes resulting in a low efficacy during the PCI process.[184,185]

Since the initial demonstration of PCI, disulfonated tetraphenyl chlorin (TPCS2a) (Fimaporfin, Amphinex) has been used in Phase-I clinical trials for enhancing the efficacy of bleomycin in patients with solid cutaneous or subcutaneous malignancies (NCT00993512). Bleomycin is a chemotherapeutic agent, and its hydrophilic character and large molecular weight limit its membrane penetration leading to its accumulation in endolysosomal vesicles.[187,188] PCI has also been tried in a Phase-I dose-escalation study of gemcitabine

in patients with extrahepatic bile duct cancer (cholangiocarcinoma) (NCT01900158).[189,190] Apart from the delivery of chemotherapeutic agents, a major utilization of the PCI process has been in the delivery of biologics including antibodies and nucleic acids, which are susceptible to proteases and nucleases in the lysosomes.[191,192] An interesting study in this context was reported by Wang et al. in 2015 where the delivery of anti-Ki67 antibody was reported in HeLa cells. The strategy involved PCI-based internalization, using Verteporfin as the PS, of TuBB-9-FITC (a FITC conjugated antibody against the active form of Ki67). In this study, an initial irradiation at 690 nm triggered the activation of the PS–Verteporfin resulting in the endolysosomal escape of TuBB-9-FITC and its relocalization in the nucleus (Figure 14B). The next irradiation at 490 nm activated the FITC bound to TuBB-9 resulting in the targeted inactivation of Ki67 in tumor cells, through a process referred to as chromophore-assisted light inactivation (CALI).[193,194] This process decreased the cell viability up to approximately 13%, highlighting the potential of this strategy in efficiently delivering antibodies to cytosolic targets (Figure 14C).[186] Tangutoori et al. demonstrated the coencapsulation of Verteporfin and bevacizumab in liposomes for simultaneous delivery of the two agents.[195] This strategy exploits the potential of Verteporfin-PDT to induce PCI and maximize the therapeutic potential of the released bevacizumab to neutralize the rapid but transient burst of VEGF following Verteporfin-PDT. In a similar study, the delivery of siRNA against pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor 1 (PAC1R) was demonstrated in PC12 cells utilizing a liposome coencapsulating Verteporfin and an siRNA. The process achieved a gene knock-down efficiency of approximately 75%.[196] Due to its potential to translocate foreign macromolecules from the lysosome into the cytosol, PCI has been shown to enhance the presentation of externally delivered antigens through MHC class I molecules resulting in a robust CD8 T cell mediated antigen response.[197] Encouraging results lrom preclinical studies led to phase I clinical trials (NCT02947854) assessing the safety and tolerance of fimaporfin in combination with HPV16 E7 peptide antigens, Keyhole Limpet Hemocyanin (KLH) protein, and an adjuvant Hiltonol. The results from these trials suggest more consistent and enhanced CD8+ T-cell and humoral responses, respectively,[198] highlighting the potential of PCI in the delivery of lysosome sensitive therapeutics.

CONCLUSIONS: CHALLENGES AND CONSIDERATIONS FOR CLINICAL TRANSLATION OF PHOTOACTIVATABLE LIPOSOMES

Clinical applications of nanomedicines have increased significantly in the past few decades with liposomes being at the forefront of this exciting revolution. The advantages conferred by nanoformulations are primarily attributed to their nanodimensions (enabling a high surface area to volume ratio), improved pharmacokinetics, reduced off-target effects, and coloading multiple agents to facilitate synergy and enhance therapeutic efficacy. In the late 20th century and beginning of the 21st century, there has been interest in externally activated nanoconstructs and light has entered as a reagent for this activation process. The phenomenon ofphotoactivation gained tremendous attention with several light-activatable PSs developed and used in preclinical and clinical applications of PDT. The most notable of these was the approval of Visudyne (a liposomal formulation of Verteporfin) for the treatment of wet age-related macular degeneration (AMD). Several million treatments have

been performed using Visudyne which received FDA approval within 6 years of early preclinical studies.[61,62] In addition, Visudyne has been used for cancer treatments in the clinic.[25,26,199] In general, most of the PSs that are in clinical use are lipophilic and are therefore formulated in lipid-based structures. With advances in synthetic chemistry, availability of plethora of lipids and an improved understanding of both lipid and PS chemistry, multiagent photoactivatable liposomes have been engineered with a capability of disabling multiple pathological pathways simultaneously. This has resulted in the use of light-activated liposomes with effects beyond local tumor shrinkage, including the reduction of metastatic disease. In addition, photochemistry-based priming of biological targets and the ability to switch on and off the release of liposomal-encapsulated entities makes these nanoconstructs valuable and versatile. Combination treatments enshrined in a single construct with light-controlled release simultaneously or sequentially for maximal impact are in principle possible. These treatment strategies utilize the capability of the first treatment to prime and reinforce the subsequent therapies thus maximizing treatment outcomes. Features such as phototriggered drug release, photochemical internalization (PCI), multitargeting, facilitating delivery of lysosome-sensitive drugs, modulating the TME along with codelivery of synergistically acting therapeutic agents makes photoactivatable liposomes particularly attractive for nanomedicine.

While these exciting and unique features associated with PS-loaded liposomes have been established in preclinical models, their translation into the clinic is still challenging. As with most PDT-based applications irradiation of deep tissues and the general hypoxic nature of tumor tissues may limit the efficacy of PDT. However, technological advances in endoscopic methods and use of optic fibers for irradiation have to a large extent overcome the issue of irradiating deep-seated tumors. Other approaches such as the use of up-conversion nanoparticles, X-ray based PDT, or the incorporation of bioluminescent agents (molecular light sources) for facilitating PDT (BL-PDT) have been reported in preclinical models which can potentially be an alternative for irradiating solid tumors.[173,200–202] Moreover, several studies have also reported the potential of nanotechnology-based approaches for overcoming hypoxia to improve PDT efficacy.[107,203 205] The potential of these hybrid systems (PS-loaded liposomes), as demonstrated in several preclinical studies, offers hopes for clinical translation in the future with some exciting research on the horizon.

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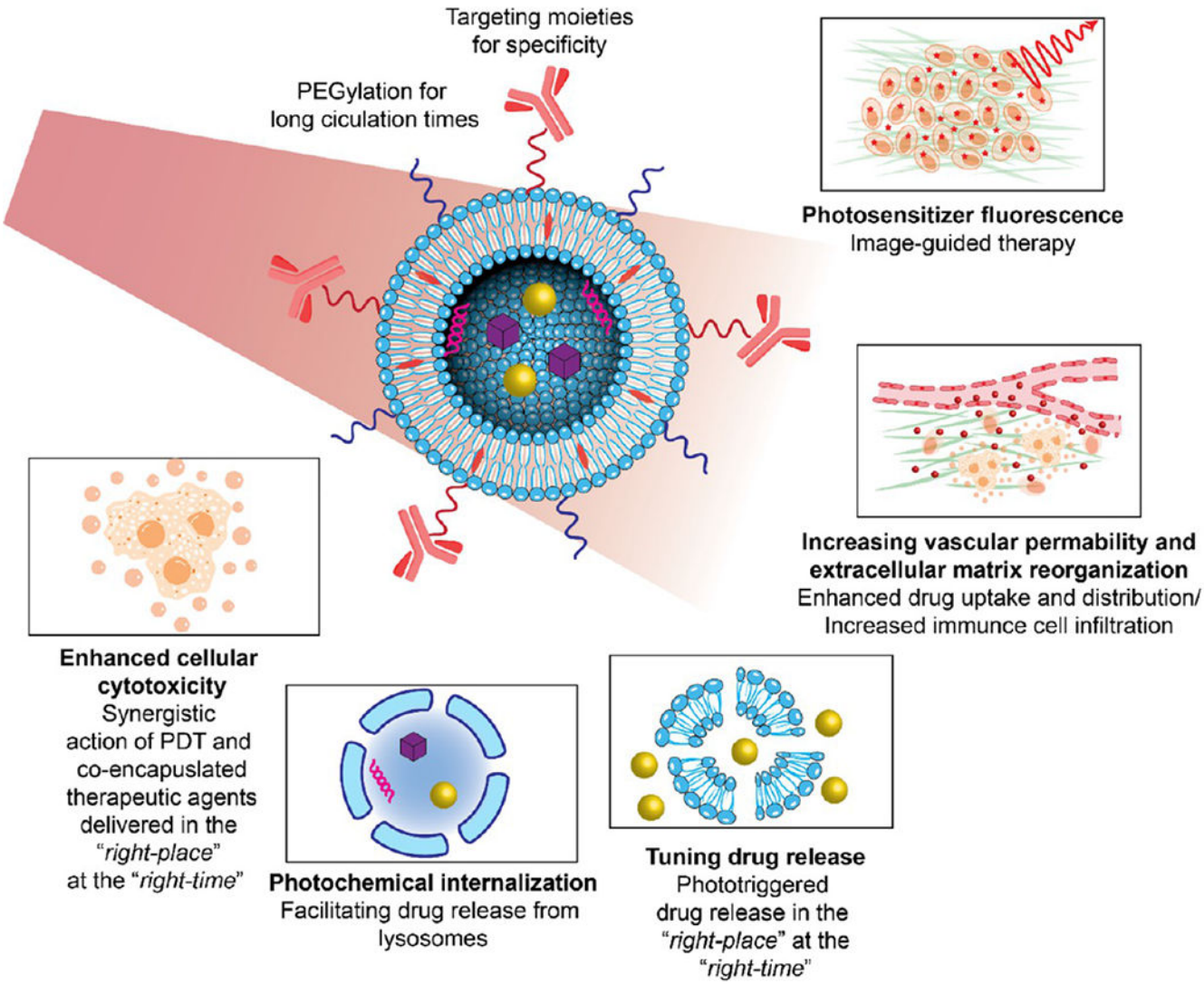

Figure 1.

Photoactivation of liposomes—a brief overview: Photosensitizer-loaded liposomes can be activated by an external irradiation resulting in (i) fluorescence imaging of target tissues, (ii) increasing vascular and stromal permeability leading to increased drug accumulation and distribution, and enhanced immune cell infiltration, (iii) photoactivatable triggered drug release in the "*right place*" at the "*right time*", (iv) photochemical internalization for lysosomal escape of drugs/biologics, and (v) enhanced cellular toxicity through synergistic action of PDT and the coencapsulated drug in the *"right place"* at the *"right time"*.

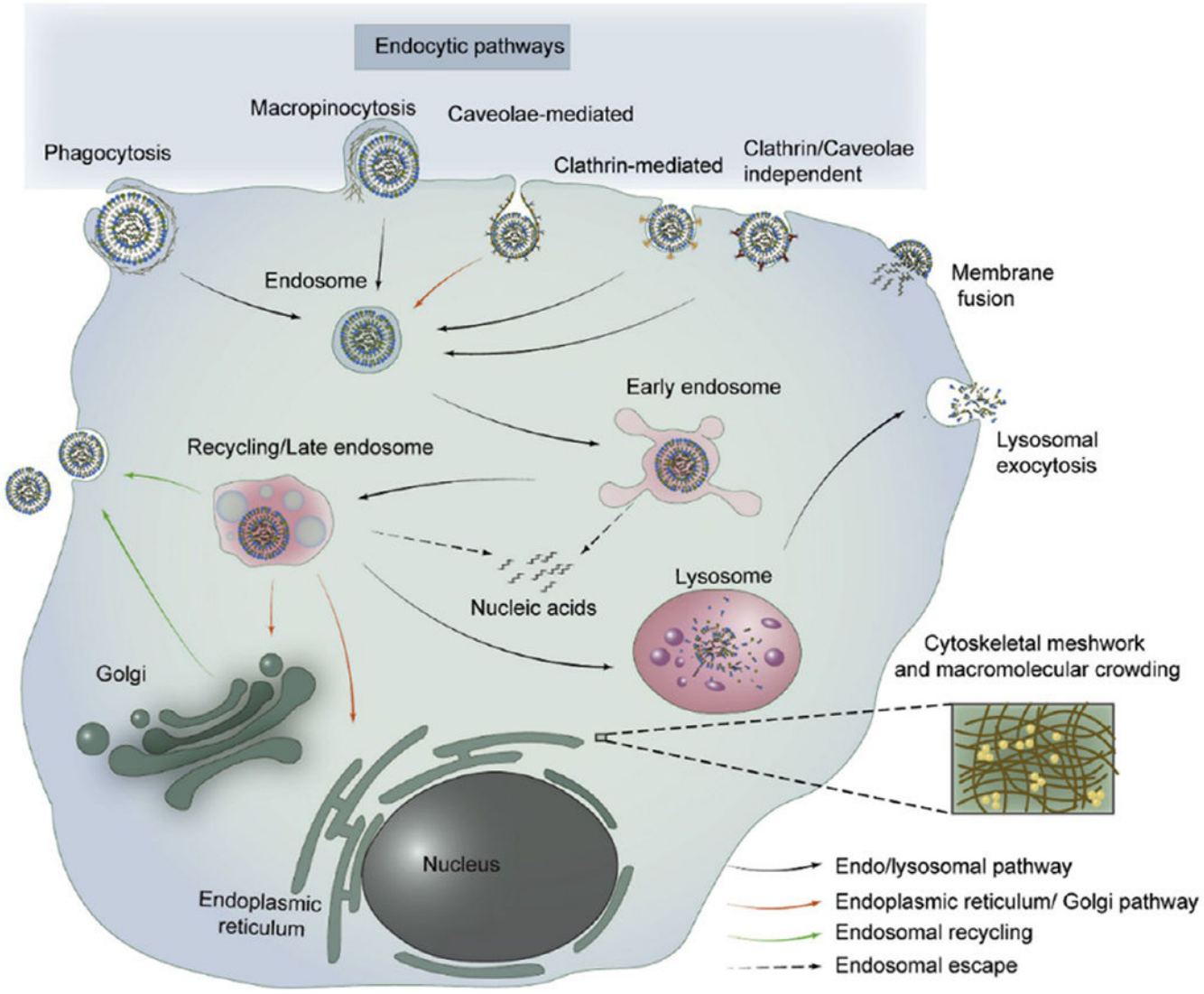

Figure 2.

Cellular uptake and fate of lipid complexes: Lipid complexes including liposomes can be taken up through various endocytic pathways ending up in the endosomes. Endosomes containing the lipoplexes are either recycled or mature into lysosomes where the loaded content is released and may be subjected to nucleases, proteases, and low pH, conditions that may inactivate and degrade the payload rendering it ineffective. Reprinted with permission from ref 41. Copyright 2020 Elsevier.

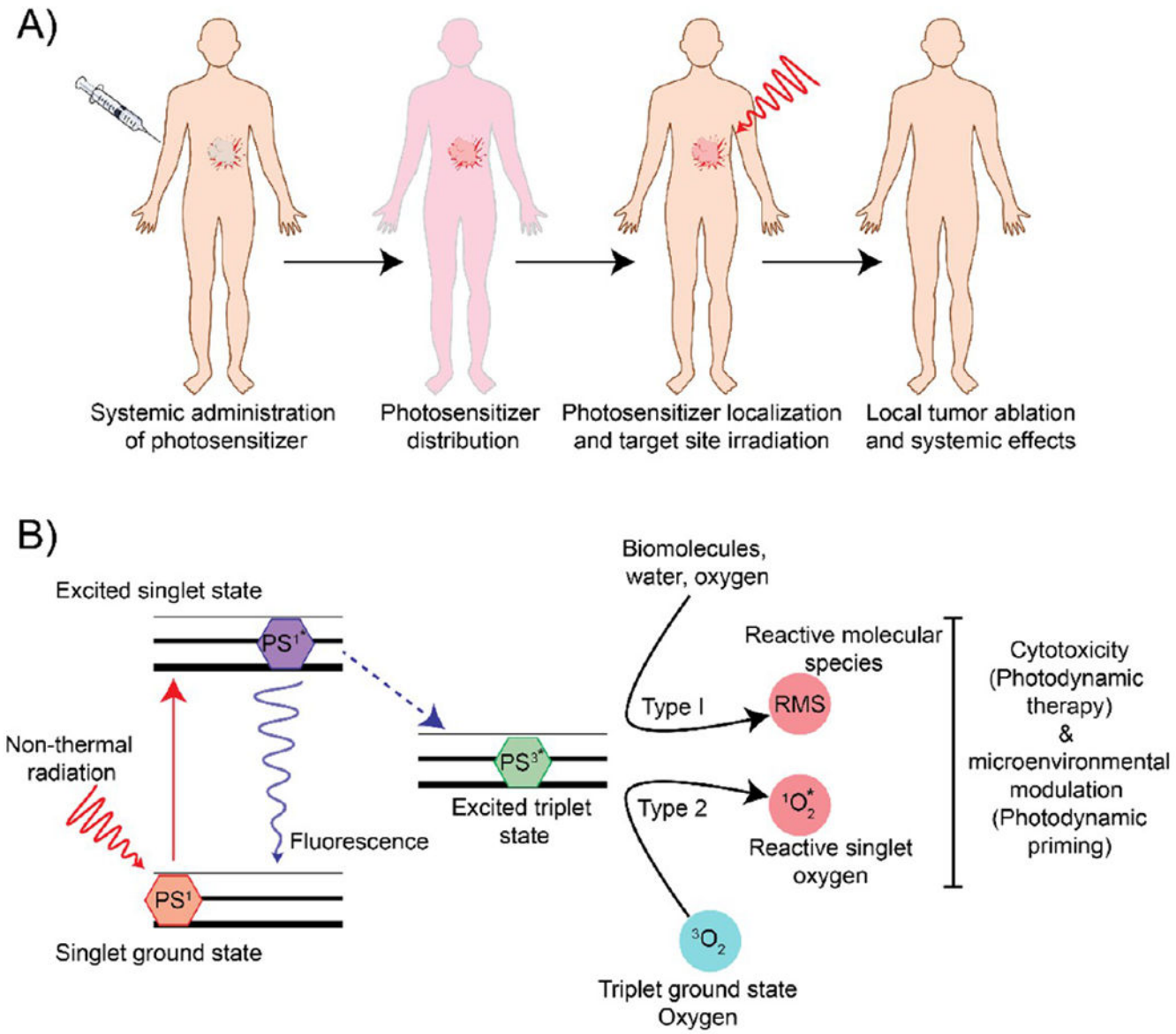

Figure 3.

Biological and chemical overview of photodynamic therapy. (A) Schematic representation of different steps in the PDT process: PS is administered systemically followed by its distribution and preferential localization in the target site after an appropriate interval of time referred to as the drug light interval (DLI). Subsequent irradiation of the target site leads to photochemical activation of the PS. (B) Diagrammatic representation of the Jablonski diagram demonstrating the excitation of a PS from its ground state (PS^1), upon irradiation, to a higher energy excited singlet state (PS^1*). PS from the excited singlet state (PS^1*) can relax back to the ground state by emitting energy in the form of fluorescence radiation. Alternatively, it can undergo intersystem crossing leading to the generation of a long-lived excited triplet state (PS^3*). Subsequently, electron (type 1) or energy (type 2) transfer from the PS^3* to biomolecules, water, or ground state molecular oxygen results in the formation

of cytotoxic reactive molecular species (RMS). The generated RMS can result in either cytotoxicity or microenvironmental modulation.

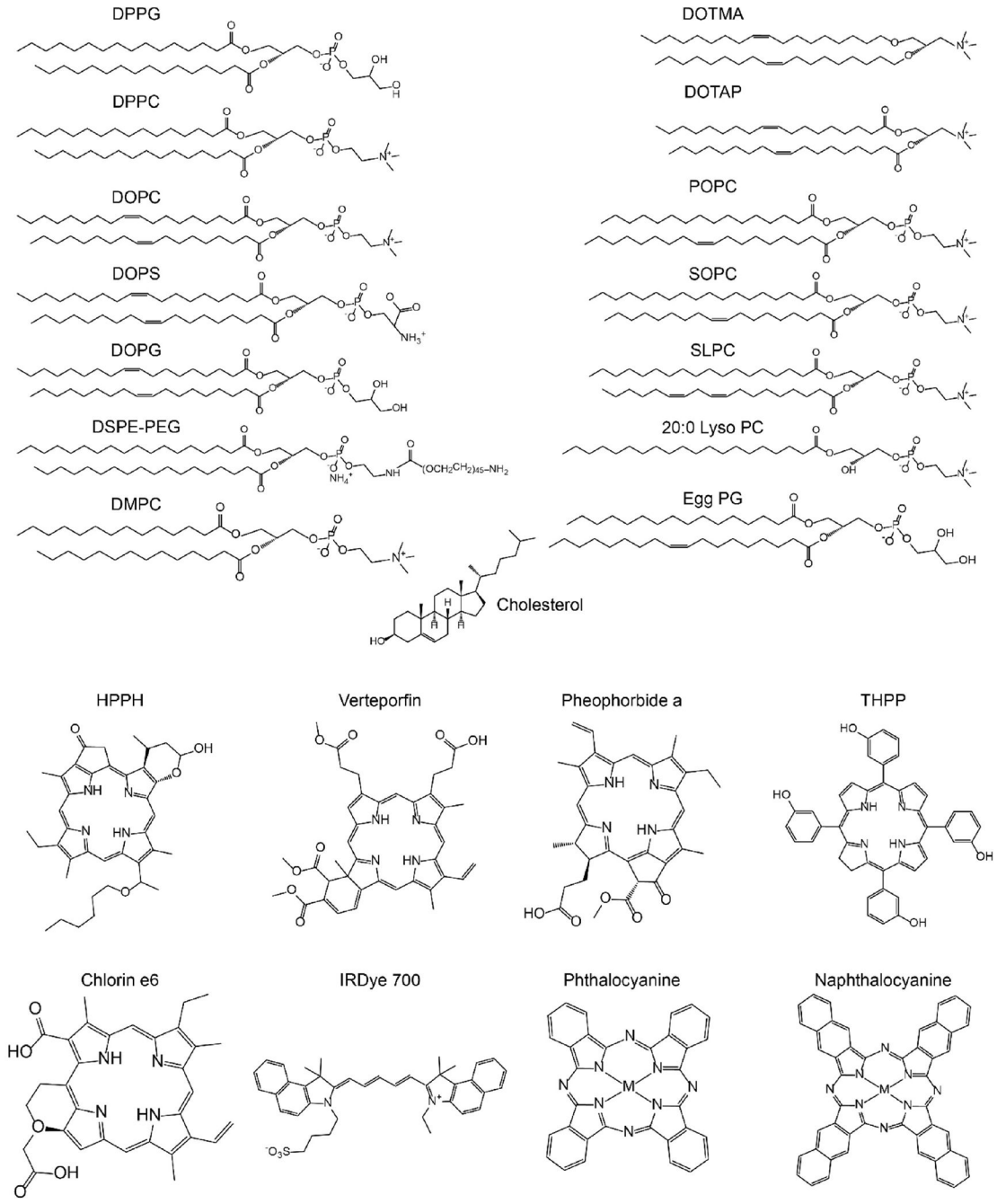

Figure 4.

Chemical structures of lipids, lipid derivatives, and photosensitizers used for synthesizing photosensitizer loaded liposomes: DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000]; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOTMA, 1,2-di-*O*-octadecenyl-3-

trimethylammonium propane; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; POPC, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SLPC, 1-stearoyl-2-linoleoyl-*sn*-glycero-phosphocholine; 20:0 Lyso PC, 1-arachidoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; and EggPG, L-*α*-phosphatidylglycerol. M in the phthalocyanine and naphthalocyanine structures refers to a metal atom.

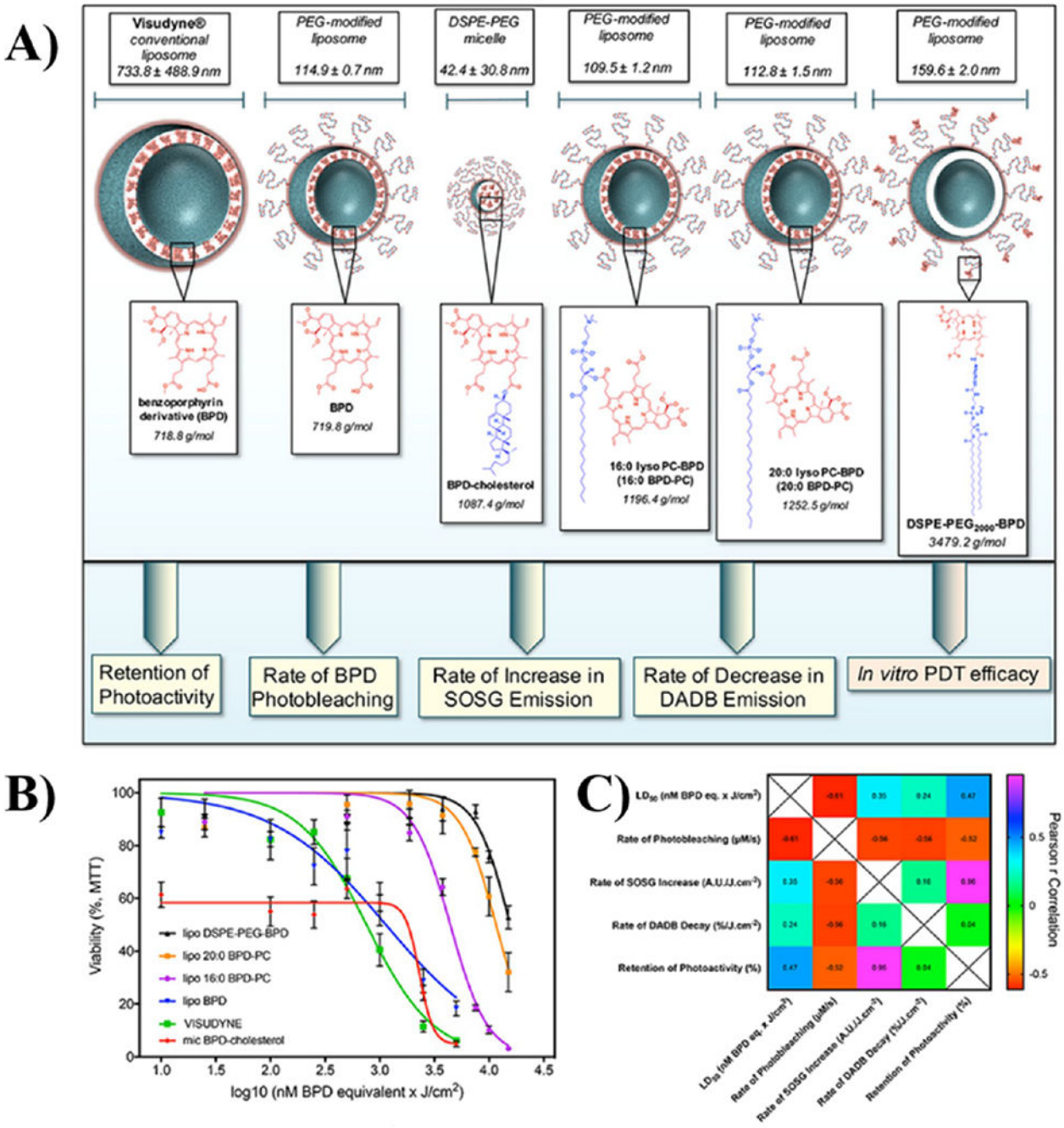

Figure 5.

(A) Diagrammatic representation and size characterization of Verteporfin-based liposomal formulations, where Verteporfin is conjugated to different lipids—Cholesterol, 16:0 Lyso-PC, 20:0 Lyso-PC, and DSPE-PEG. The photoactivity, photobleaching, singlet oxygen production (fluorescence emission of Singlet Oxygen Sensor Green (SOSG), and diethyl-3-30-(9,10-anthracenediyl)bisacrylate (DADB)) of the Verteporfin-based liposomes were evaluated with respect to their in vitro PDT efficacy in OVCAR-5 cells (B). (C) Pearson's *r* correlation matrix of liposomal LD50 values in OVCAR-5 cells with their

respective photochemical and photophysical properties. Reprinted with permission from ref 90. Copyright 2018 The American Society of Photobiology.

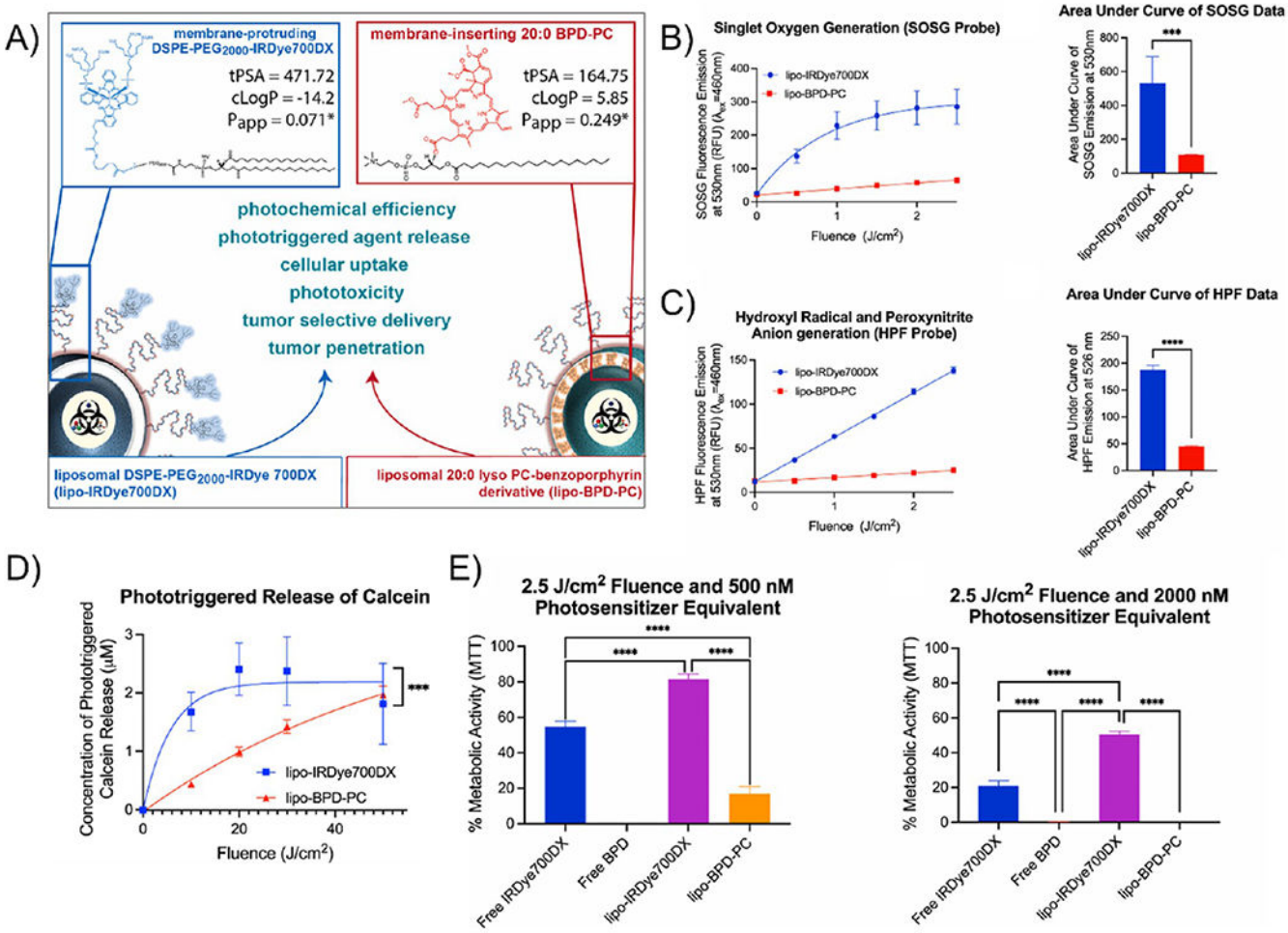

Figure 6.

(A) Diagrammatic representation of liposomal photonanomedicines developed with membrane-protruding DSPE-PEG IRDye700DX or membrane-inserting 20:0 lyso-PC-benzoporphyrin derivative (BPD-PC). Topological polar surface area (tPSA), octanol/water partitions (cLogP), and apparent biomembrane permeability coefficients (P_{app}) of the PSs are mentioned. (B) Singlet oxygen generation by the two formulations measured by fluorescence emission of singlet oxygen sensor green (SOSG). (C) Hydroxyl radical and peroxynitrite anion generation by the two formulations measured by fluorescence emission of hydroxyphenyl fluorescein (HPF). (D) Phototriggered calcein release from lipo-IRDye700DX and lipo-BPD-PC. Phototriggered calcein release was performed using 690 nm light at an irradiance of 17.86 mW cm^{-2}. (E) Percent metabolic activity at a fluence of 2.5 J cm^{-2} with a PS equivalent of 500 nM and 2000 nM. Reprinted from ref 99. Copyright 2021.

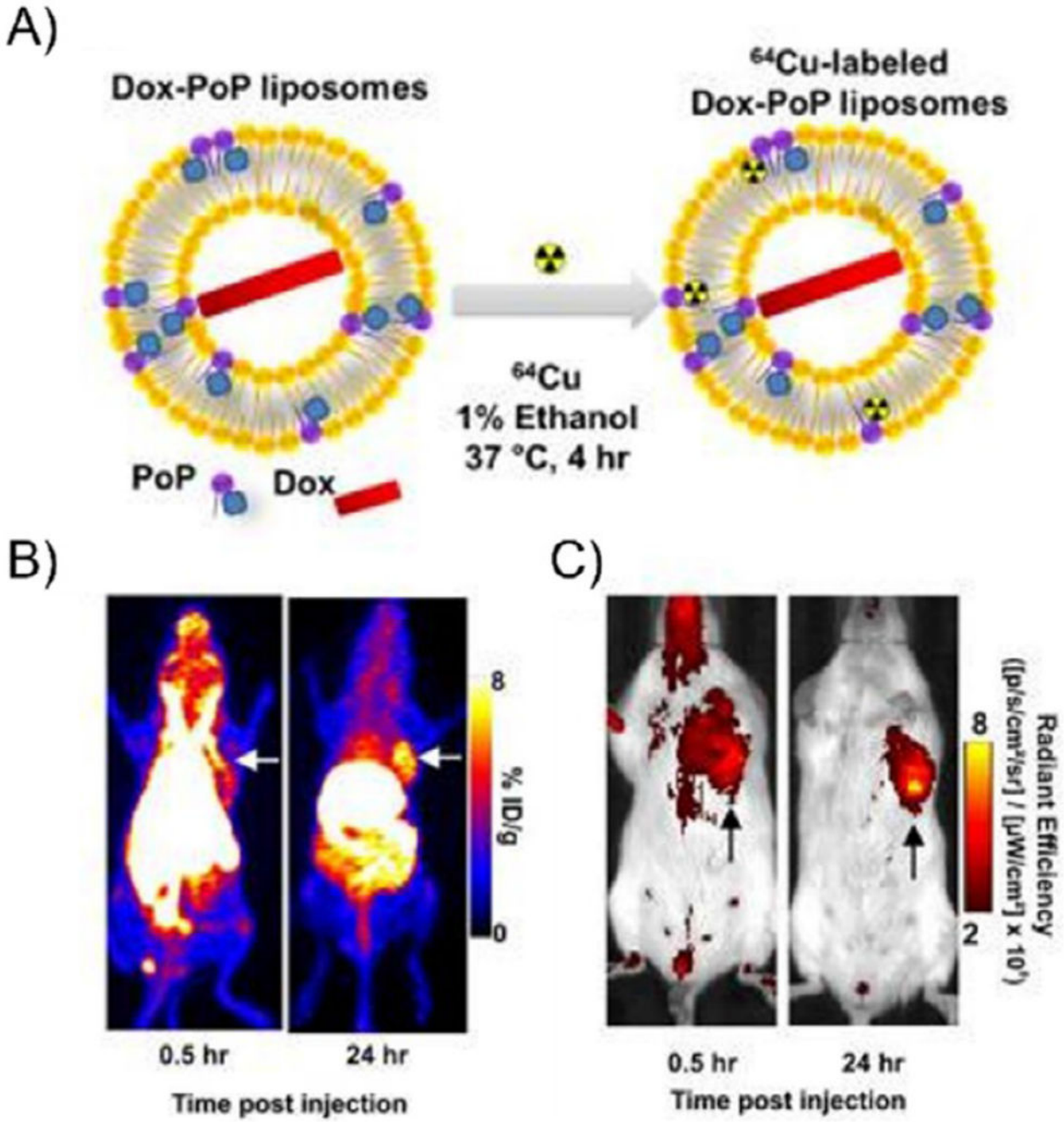

Figure 7.

Multimodal imaging enabled by PS-loaded liposomes. (A) Diagrammatic representation of ^{64}Cu labeled Dox-PoP liposome synthesis. PET (B) and (C) NIR fluorescence imaging of mice following Dox-CuPoP administration (white arrows indicate tumor location). Reprinted with permission from ref 105. Copyright (2017) American Chemical Society.

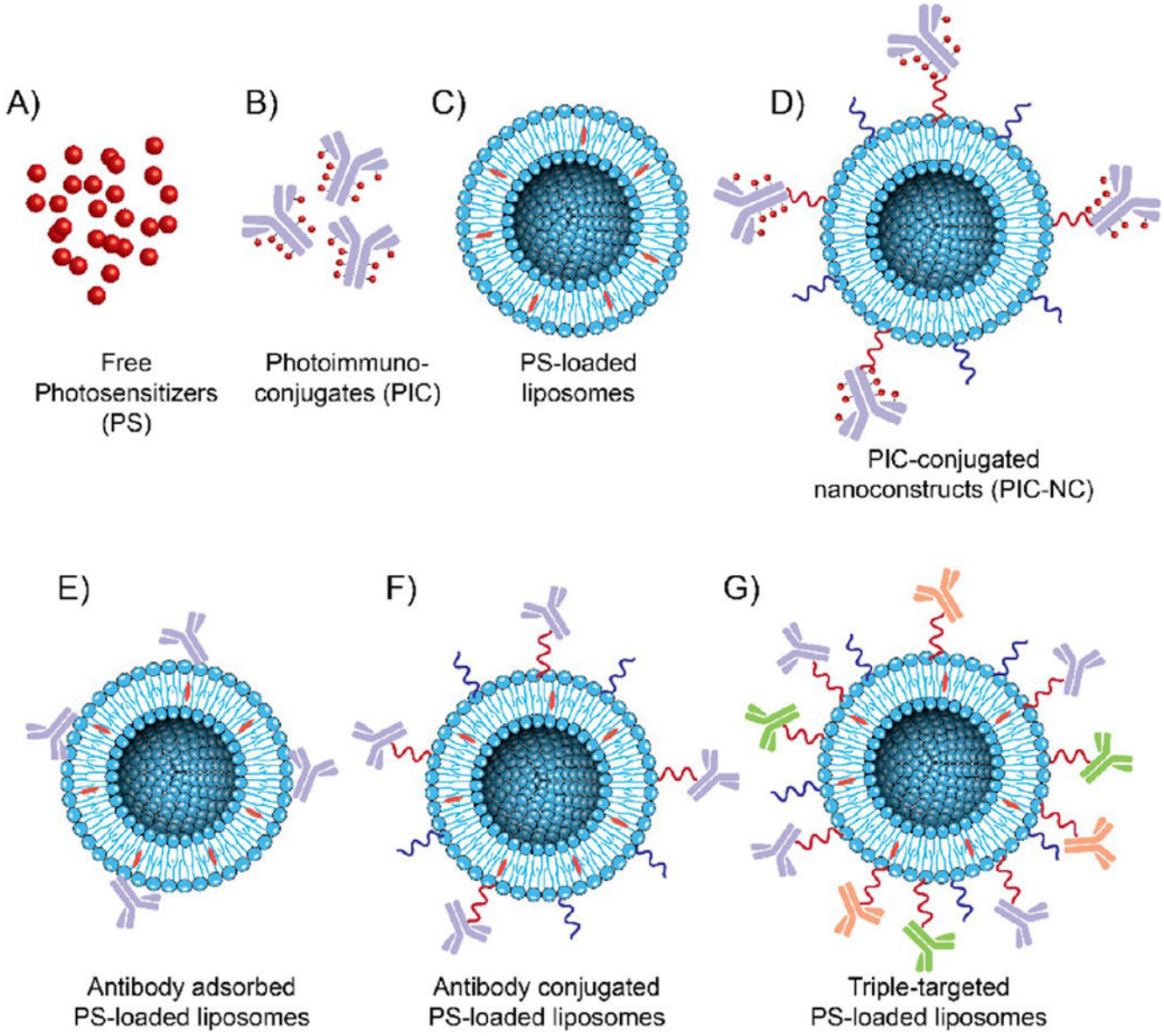

Figure 8.

Evolution of targeted PS-loaded liposomes: (A) Free photosensitizers (PSs) preferentially accumulate in tumor tissues with a second level of selectivity conferred by confining the irradiation volume. (B) Photosensitizer conjugation to antibodies (photoimmunoconjugates (PICs)), provides PS with tumor specificity through molecular targeting of markers overexpressed on tumor cells. (C) PS-loaded liposomes preferentially accumulate in the tumor tissues through the leaky vasculature and dysfunctional lymphatic drainage (EPR effect) and provide an opportunity to coload multiple drugs. (D) PIC-conjugated nanoconstructs (PIC-NCs) provide selectivity and molecular septicity and an opportunity to coload multiple drugs. However, with the PS conjugated to the antibody and not on the lipid bilayer, their ability to provide a phototriggered drug release is limited. (E) Antibody adsorbed PS-loaded liposomes provide tumor specificity and an opportunity to coload

multiple agents; however, there are concerns regarding stability and antibody displacement in vivo upon contact with serum proteins. (F and G) Single- and triple-targeted PS-loaded liposomes provide tumor specificity even in tumor models with heterogeneity in expression of tumor markers.

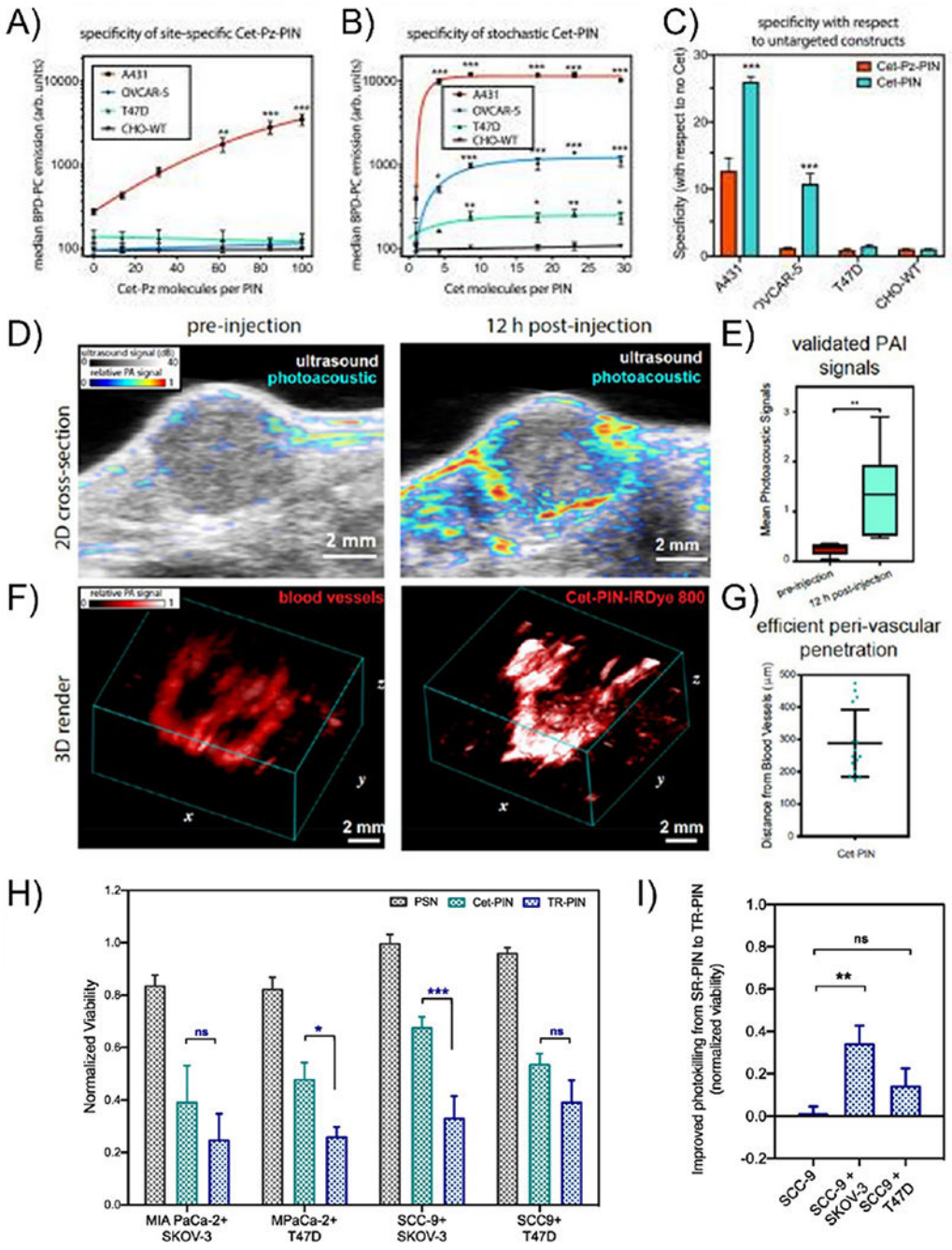

Figure 9.

Targeted PS-loaded liposomes: Specificity of PINs, as tested on different EGFR expressing cell lines, conjugated at various densities of (A) site-specific Cetuximab (Cet-Pz) and (B) stochastic Cetuximab. (C) Binding specificity of PINs with 100 Cet-Pz molecules and stochastic Cet-PINs with 30 Cetuximab molecules as compared to untargeted nanoconstructs. (D,E) 2D cross sections with quantification and (F,G) 3D renders of heterotypic MIA PaCa-2 + PCAF tumors in vivo before and after intravenous administration of Cet-PIN tagged with the photoacoustic contrast agent IRDye800CW (Cet-

PIN-IRDye800). Quantification of photoacoustic signals suggest significant perivascular tumor penetration of the Cet-PIN-IRDye800. (H,I) Comparison of fractional viability of heterocellular cultures of MIA PaCa-2 and SCC-9 with T47D and SKOV-3 following PDT with the monotargeted Cet-PINs and triple-receptor targeted TR-PINs. The phototoxicity conferred by TR-PINs was significantly higher in heterocellular spheroids as compared to the single receptor targeted Cet-PINs. Reprinted from ref 64, Copyright (2019) American Chemical Society, and from ref 92, Journal of Clinical Medicine.

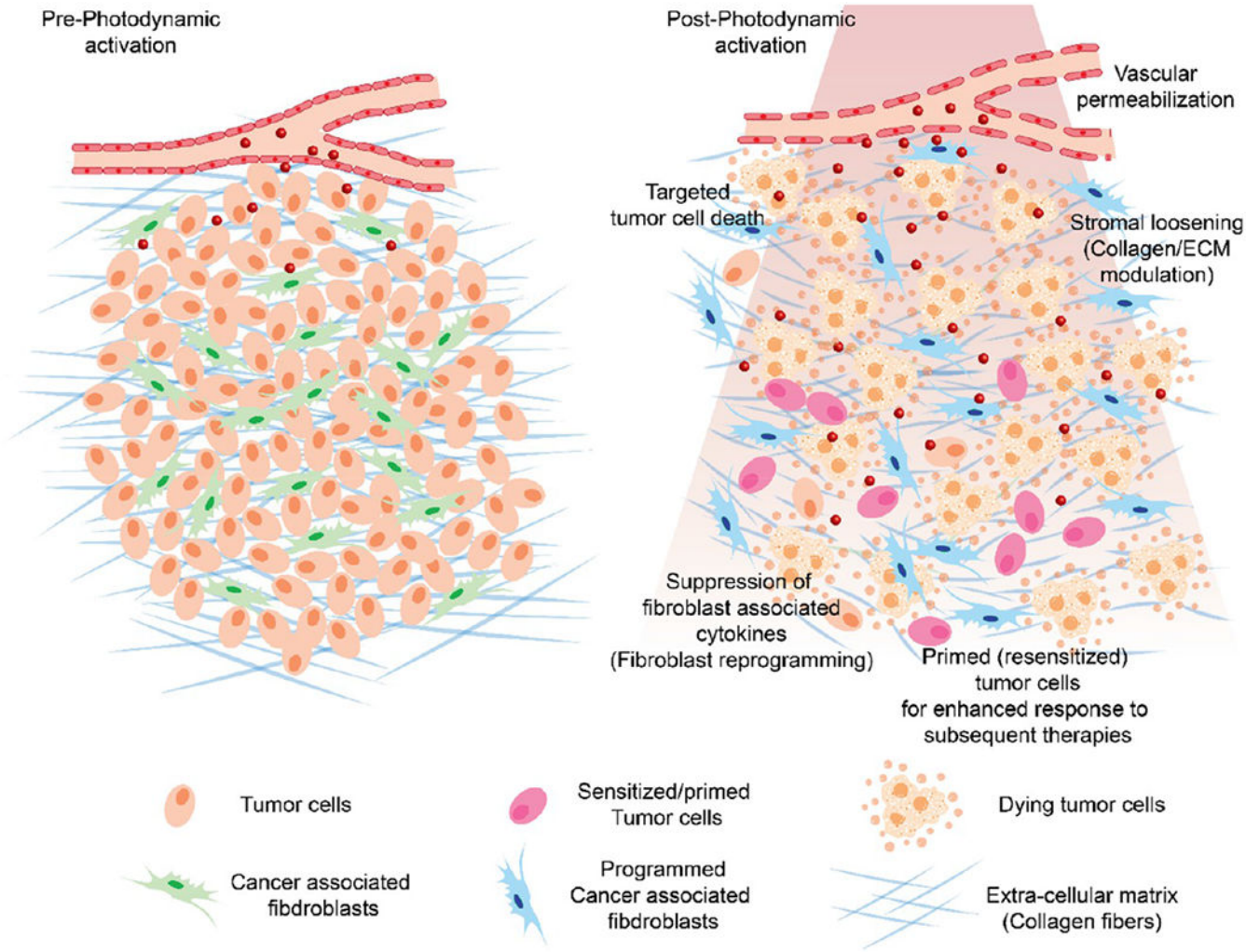

Figure 10.

Diagrammatic representation of the multiple effects of photodynamic priming in the tumor microenvironment: Desmoplastic tumors are generally characterized by high numbers of cancer associated fibroblasts and excessive deposition of extracellular matrix molecules, including collagen which limits the penetration and distribution of drugs. Irradiation of PSs deposited in the tumor microenvironment can result in either cell death or priming depending on the PDT dose (a product of PS concentration and light dose). Photodynamic priming can have different consequences in different TME compartments including, enhanced vascular permeability, ECM modulation, enhanced drug penetration, sensitizing tumor cells to subsequent therapies, and reprogramming of cancer associated fibroblasts.

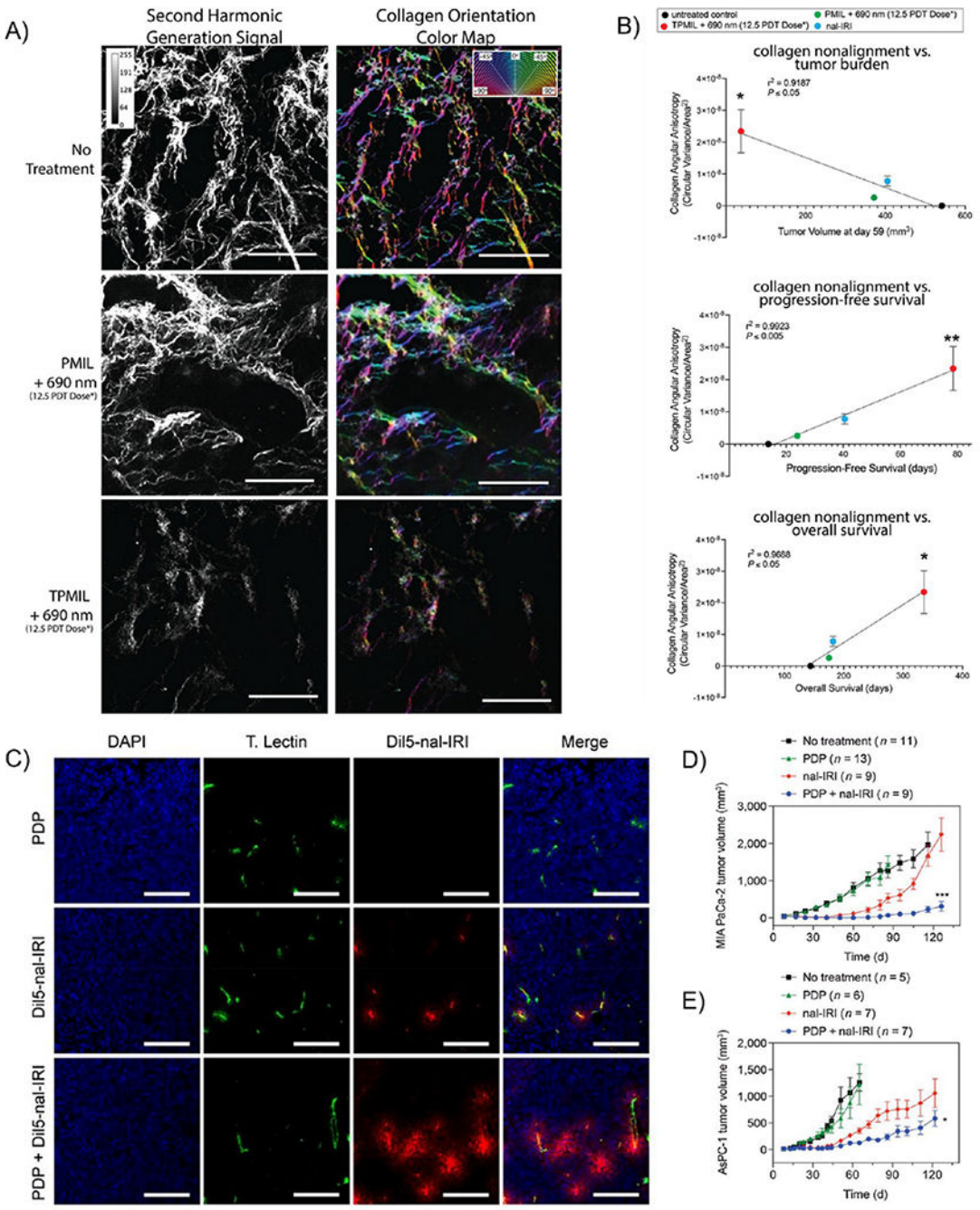

Figure 11.

Photodynamic priming of the tumor microenvironment: (A) Second harmonic generation images (false colored white) and respective collagen orientation color map images (depicting the angles of collagen fibers within the image) of control and treated tumors (either with photoactivable multi-inhibitor liposomes; PMIL, or targeted photoactivable multi-inhibitor liposomes, TPMIL) 72 h following irradiation. (B) Correlations between PDAC tumor burden (at day 59), progression-free survival, and overall survival with collagen nonalignment. (C) Fluorescence microscopy images showing the distribution of Dil5 labeled

nanoliposomal irinotecan (nal-IRI) (red) in combination with PDP (bottom row). The signal of Dil5-nal-IRI in the control (no PDP) group (middle row) was confined to the blood vessels indicated by tomato lectin staining (green). Nuclear staining (blue fluorescence, DAPI); Scale bar, 200 mm. (D and E) Longitudinal monitoring of volumes of orthotopic MIA PaCa-2 (D) and AsPC-1 (E) tumors. A combination of PDP and nal-IRI prolonged and enhanced tumor growth inhibition in both MIA PaCa-2 and AsPC-1 animal models compared with nal-IRI alone. Reprinted with permission from ref 59, Copyright 2022 The Authors, and with permission from ref 91, Copyright 2018, American Association for Cancer Research.

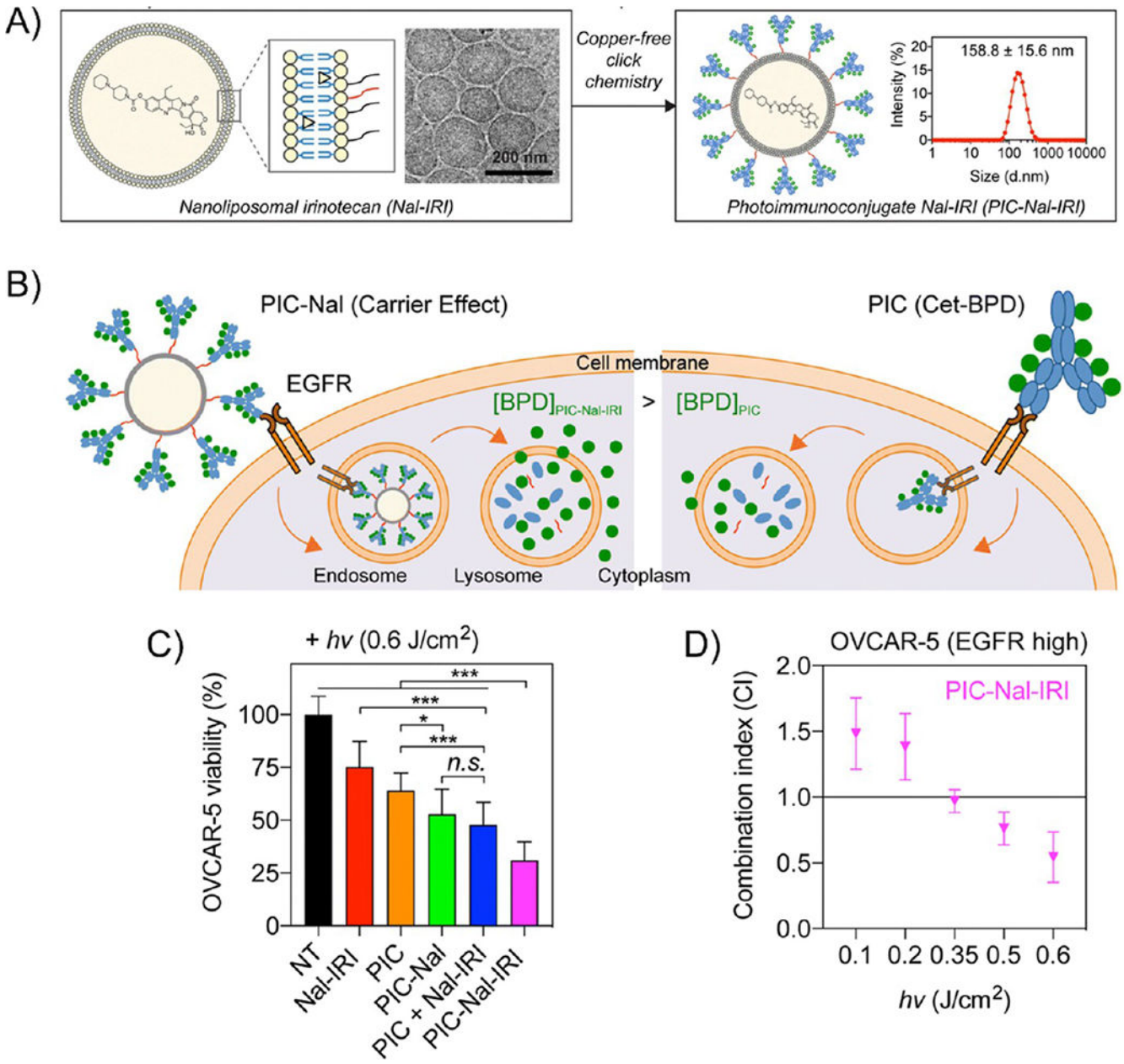

Figure 12.

Synergistic potential of PDT-mediated combination therapies: (A) Diagrammatic representation of the synthesis scheme of photoimmunoconjugate nanoliposomal irinotecan (PIC–Nal–IRI). (B) Schematic illustration of the “carrier effect” of PIC–Nal. The uptake of PS is significantly higher after treatment with PIC conjugated to liposomes as compared to free PICs due to the carrier effect. (C) Viability of Ovcar-5 cells treated with PIC-Nal-IRI was significantly higher as compared to a combination of PIC and Nal-IRI administered separately. (D) Combination index (CI) of PIC-Nal-IRI suggests a light dose dependent synergistic effect of the combination therapy (Synergistic effect, CI < 1; additive effect, CI

= 1; and antagonism, CI > 1. Reprinted with permission from ref 133. Copyright 2020, The Author(s).

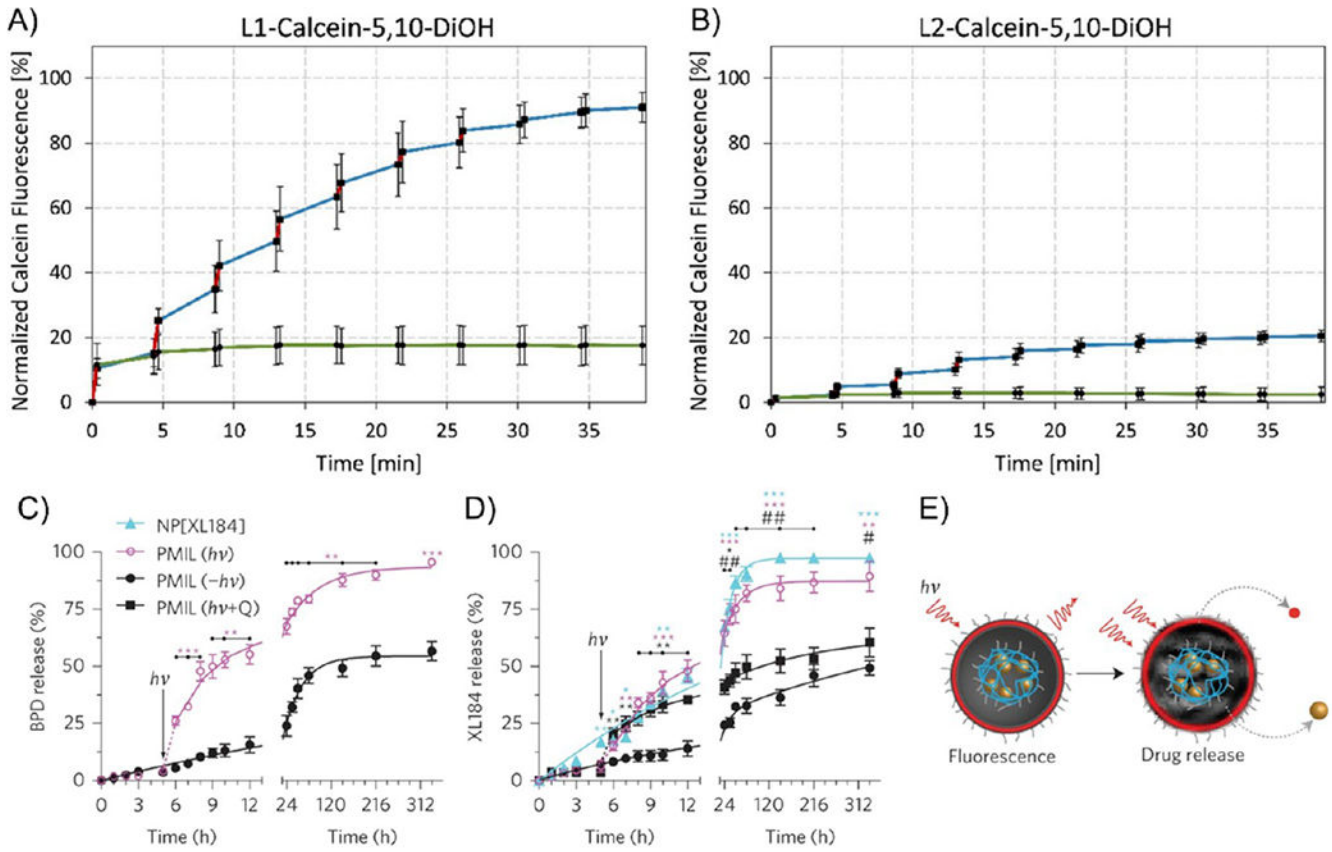

Figure 13.

Phototriggered sustained drug release. Calcein release from 5,10-DiOH loaded L1 (A) and L2 (B) liposomes. Red lines represent the light phase (irradiation at 420 nm, 20 mW/cm^2 for 20 s) while blue lines show the dark phase (no irradiation). As evident, rapid release was observed during irradiation of the liposomes. The release from L2 liposomes was lower due to the high cholesterol content of these liposomes. (C and D) Photoinduced release of drugs (Verteporfin/BPD and XL184) from photoactivatable multi-inhibitor liposomes (PMILs) with and without irradiation (arrows indicate NIR light dose at the 5 h time point (37 °C; 100 mW cm^2; 5 J/cm^2). The photoinduced drug release from PMILs was inhibited in the presence of ROS scavenger (Q) suggesting ROS mediated lipid bilayer being the primary reason for drug release, post-irradiation of the sample. (E) Diagrammatic representation of the photoinduced drug release from PMILs. Reprinted with permission from ref 164. Copyright 2020, American Chemical Society, and reprinted with permission from ref 162, Copyright 2016, Nature Publishing Group.

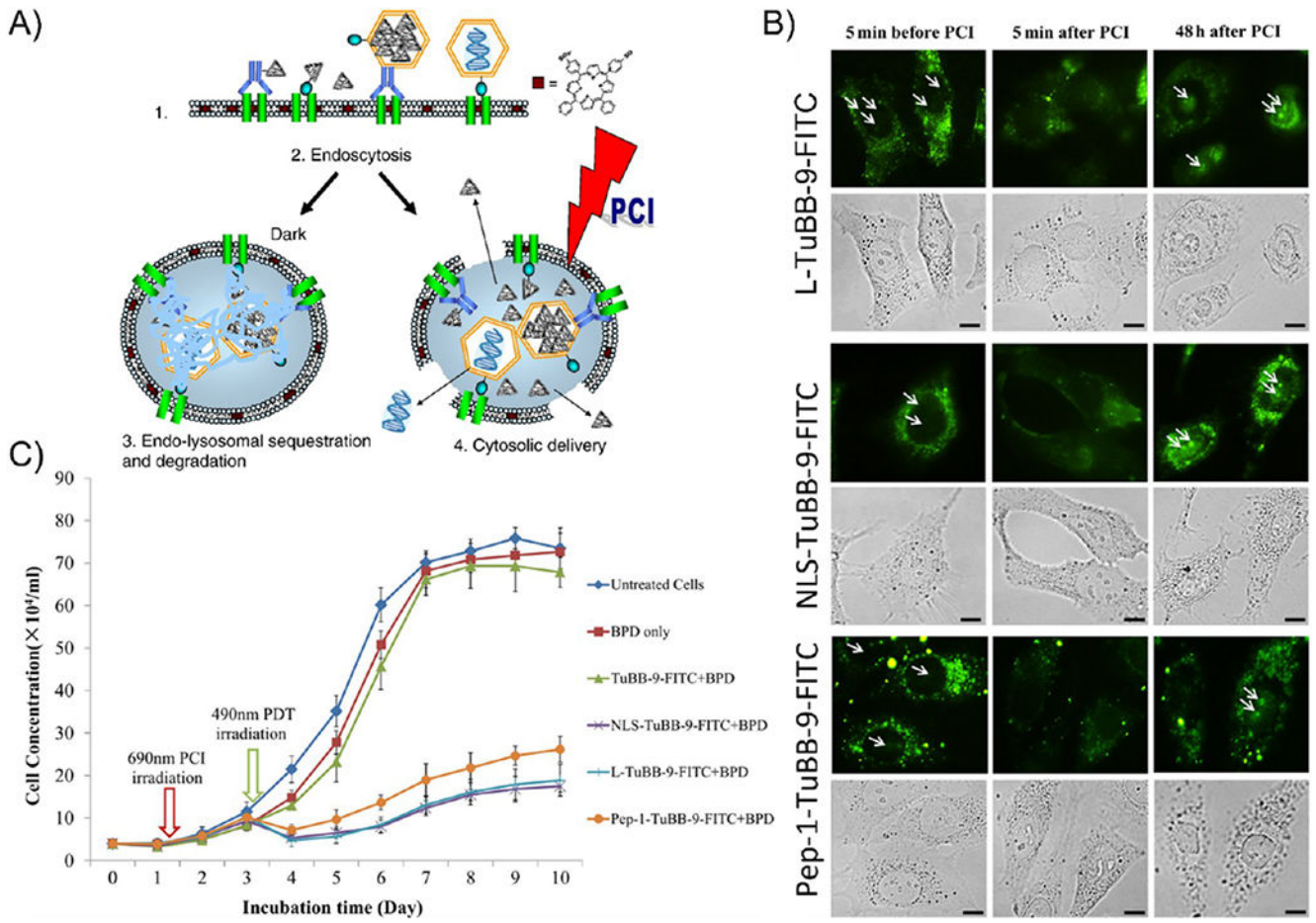

Figure 14.

Photochemical internalization. (A) Schematic illustration of endolysosomal escape of therapeutics by photochemical internalization (PCI). A photosensitizer is administered followed by the therapeutic agent (chemo/biological therapeutic). The PS, due to its hydrophobic/amphiphilic nature, intercalates into the lysosomal membrane along with other membrane rich organelles. An irradiation at this stage induces the generation of RMS resulting in the rupture of the endolysosomal complex leading to the release of the trapped chemo/biological therapeutic agents. In the absence of PS, the chemo/biological therapeutic agents can be degraded by the lysosomal proteases, nucleases, and low pH resulting in their inactivation. (B) Targeting Ki67 in the nucleolar compartment using the TuBB-9-FITC antibody delivered using the PCI phenomenon. FITC signal (green) before PCI for the different constructs targeting Ki67 show cytosolic localization in HeLa cells. Following PCI (using Verteporfin-PDT) fluorescence images suggest nucleolar localization of the different targeting agents. Scale bar: 10 μm. (C) Cell viability assessment of HeLa cells showing a decrease in cell viability after irradiations (first irradiation to induce PCI and second irradiation to photoinactivate Ki67 in the nucleolus). Reprinted with permission from ref 180, Copyright 2010 Elsevier B.V., and reprinted with permission from ref 186, Copyright 2015 American Chemical Society.

Table 1.

Clinically Relevant Photosensitizer-Loaded Lipid Formulations

Photosensitizer	Trade name	Formulation composition	Clinical status	Disease indication	Ref
5,10,15,20-tetrakis(3-hydroxyphenyl)-chlorin or Temoporfin (mTHPC, which is available as Foscan)	Foslip	DPPC, DPPG, and mTHPC (DPPC:DPPG in mass ratio of 9:1) with mTHPC loaded in PS:lipid ratio of 1:12	No clinical studies reported	No clinical study reported for Foslip; Foscan is approved for use in Advanced head and neck squamous cell carcinoma	17–20
5,10,15,20-tetrakis(3-hydroxyphenyl)-chlorin or Temoporfin (mTHPC, which is available as Foscan)	Fospeg	DPPC:DPPG:DSPE-PEG was 9:1:1 and the PS loading ratio was 1:13 PS:lipid	No clinical studies reported	No clinical study reported for Fospeg; Foscan is approved for use in advanced head and neck squamous cell carcinoma	17,18,20,21
Zn-Pc	CGP55847	ZnPc:POPC:DOPS of 1:90:10 (mass ratio)	Abandoned	Squamous cell carcinoma of the upper aerodigestive tract	22–24
BPD-MA	Visudyne	EPG and DMPC (3:5 molar ratio)	Approved	Subfoveal choroidal neovascularization (CNV) due to age related-macular degeneration (AMD), in clinical trials for locally advanced pancreatic cancer	25–27
Purpurin	Purlytin	Cremophor EL (Emulsion)	Approved	Metastatic breast cancer, Kaposi’s sarcoma, in AIDS patients and the treatment of restenosis and psoriasis	9–11

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