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REVIEW

Towards overcoming obstacles of type II photodynamic therapy: Endogenous production of light, photosensitizer, and oxygen

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Abstract Conventional photodynamic therapy (PDT) approaches face challenges including limited light penetration, low uptake of photosensitizers by tumors, and lack of oxygen in tumor microenvironments. One promising solution is to internally generate light, photosensitizers, and oxygen. This can be accomplished through endogenous production, such as using bioluminescence as an endogenous light source, synthesizing genetically encodable photosensitizers *in situ*, and modifying cells genetically to express catalase enzymes. Furthermore, these strategies have been reinforced by the recent rapid advancements in synthetic biology. In this review, we summarize and discuss the approaches to overcome PDT obstacles by means of endogenous production of excitation light, photosensitizers, and oxygen. We envision that as synthetic biology advances, genetically engineered cells could act as precise and targeted "living factories" to produce PDT components, leading to enhanced performance of PDT.

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1. Introduction

Antitumor treatment with photodynamic therapy (PDT) has been extensively investigated in the past few decades. PDT utilizes photosensitizers that can be excited by light of specific wavelengths. Light-activated photosensitizers generate cytotoxic reactive oxygen species (ROS), thereby inducing apoptosis, necrosis, and autophagy of cells[1]. PDT has received approval as a non-invasive option for antitumor treatment[2]. Over the past 30 years, PDT has witnessed a progressive surge in its utilization for the management of diverse solid malignancies[3].

ROS are generated *via* two pathways in PDT. Type I PDT operates in an oxygen (O_2)-independent manner, while Type II PDT relies on the presence of molecular oxygen. The Type II pathway is the primary mechanism of most PDT, wherein the energized photosensitizers interact with O_2 to produce highly toxic singlet oxygen ($^1O_2$, Fig. 1). There are only a limited number of photosensitizers that use Type I mechanism, and most clinically approved PDT use Type II mechanism. Excitation light, photosensitizers, and O_2 are the three essential components for Type II PDT. There are several challenges associated with the three components that hinder PDT efficiency in clinical practice, such as the constrained tissue penetration depth of light, the poor water solubility of conventional photosensitizers, and the presence of hypoxia within the tumor microenvironment (TME)[4].

The penetration depth of the excitation light determines the depth of the tumor that can be treated by PDT. In the context of traditional PDT, Ultraviolet−Visible light (UV−Vis) is commonly employed to excite photosensitizers. The light of such wavelength (400−700 nm) has poor tissue penetration, so the PDT efficiency is limited in treating deep-seated tumors[5]. To overcome this "Achilles' heel", different light sources have been explored. For example, upconversion nanoparticles (UCNPs) are applied to PDT to convert near-infrared (NIR) excitation light to UV−Vis light[6]. There have also been attempts to use X-ray in PDT[7]. In addition to the external excitation light sources, photosensitizers can be activated by self-luminescence, including chemiluminescence (CL)[8] and Cerenkov radiation (CR) luminescence. Such internalized light sources produce excitation light locally at the tumor sites, resolving the tissue penetration limitations of external light. Bioluminescence is a type of chemiluminescence involving luciferase enzymes. The introduction of genes encoding luciferases (Fig. 1) into cancer cells allows for the synthesis of luciferase enzymes, enabling the production of excitation light for *in situ* PDT. This approach has been validated as a successful strategy for enhancing the performance of PDT[9].

The photosensitizers used in PDT are primarily exogenous compounds administered by intravenous injection, subcutaneous injection, and topical application. A plethora of photosensitizers have been synthesized, encompassing both organic and inorganic variants[9,10]; however, they have some common limitations, such as low tissue selectivity, low water solubility, and low biocompatibility. Endogenous small molecule photosensitizers, such as flavin compounds and porphyrin compounds, can be induced in cancer cells for PDT. Protein photosensitizers can also be expressed endogenously in cancer cells by delivering genes encoding the proteins to target cells[11].

Hypoxia represents an inherent attribute exhibited by numerous malignant solid tumors, exerting a profound constraint on the therapeutic effectiveness of oxygen-dependent Type II PDT[12]. Many strategies have been attempted to alleviate hypoxia at tumor sites[13], such as direct delivery of O_2 to tumors[14] and generation of O_2 from MnO_2-catalyzed decomposition of hydrogen peroxide (H_2O_2)[15]. Recently, biological approaches to relieve tumor hypoxia have been successfully applied to PDT. For instance, the use of catalase to breakdown H_2O_2 into O_2 or the use of photosynthetic microbes to generate O_2 *in situ* enhanced the PDT efficiency[16,17].

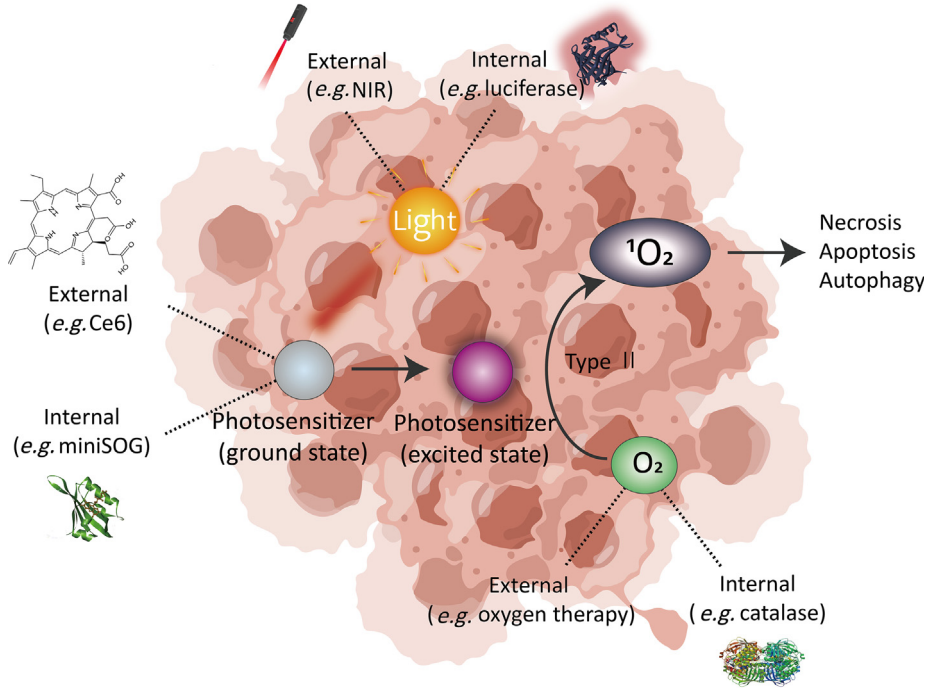

Figure 1 Scheme of the photochemical reaction and three key components involved in type II PDT.

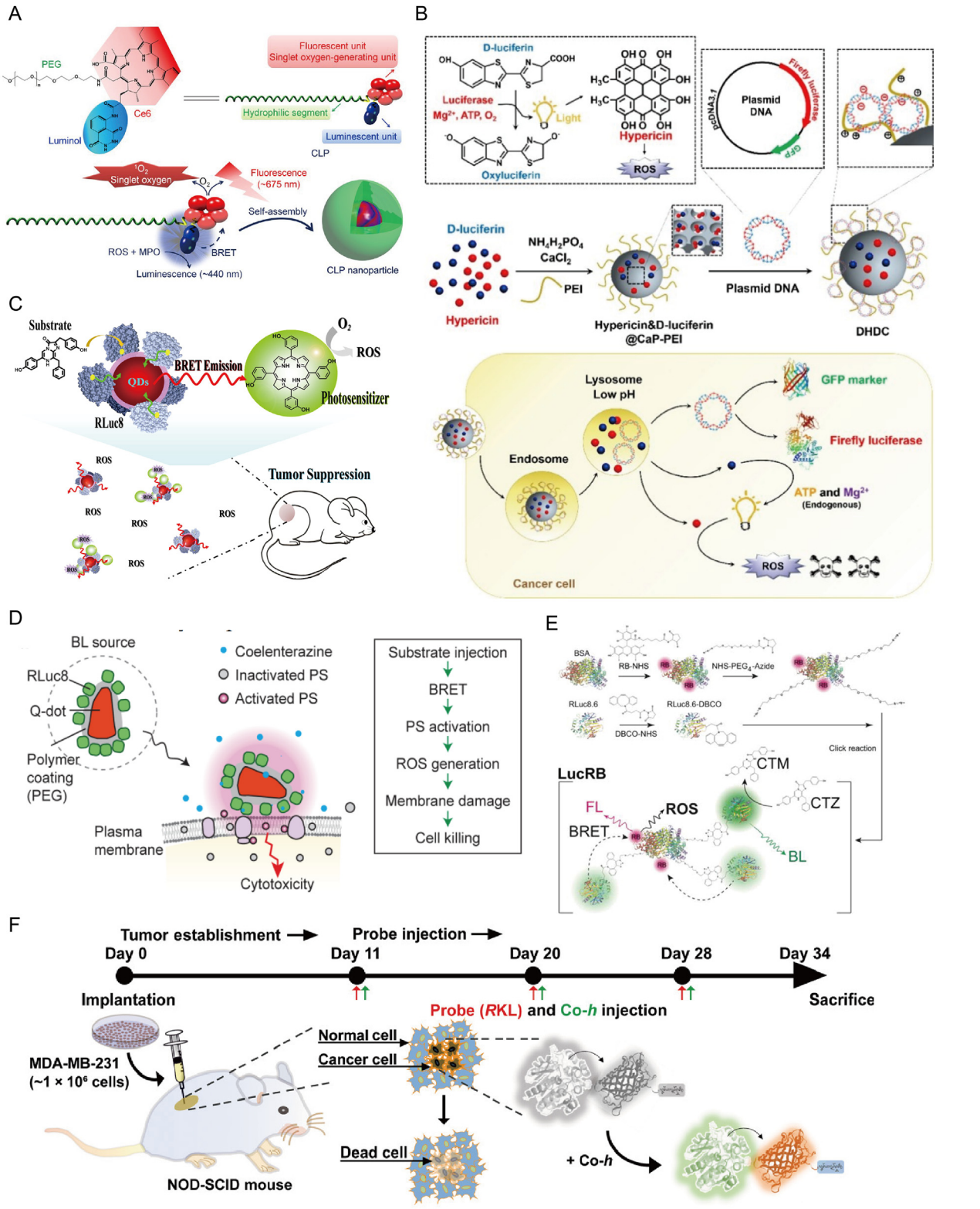

Figure 2 Examples of PDT using chemiluminescence and bioluminescence. (A) Luminol is linked with chlorine e6 (Ce6), and the construct self-assembles into nanoparticles that can be used for chemiluniescence-activated photodynamic therapy (PDT). Reproduced with permission from Ref. 47. Copyright © 2019 Wiley. (B) Polyethyleneimine-modified nano calcium phosphate encapsulating D-luciferin, hypericin, and a plasmid inserted with the Fluc gene is used for bioluminescence-mediated PDT. Reproduced with permission from Ref. 55. Copyright © 2021 Wiley. (C) Renilla luciferase-bound quantum dots (QD-RLuc8) emit 655 nm light due to bioluminescence resonance energy transfer, which can activate Foscan-loaded micelles producing ROS. Reproduced with permission from Ref. 57. Copyright © 2013 Elsevier. (D) QDs were coated with a PEG polymer encapsulating Rluc8. In the presence of coelenterazine, the construct self-illuminates and activates Ce6, leading to

The reagents and plasmids involved in the above-mentioned strategies need to be efficiently delivered to cancer cells to achieve improved PDT performance. Artificial nanomaterials are commonly used delivery vehicles to enhance PDT efficacy[18]. In recent years, advancements in synthetic biology have driven the development of engineered bacteria as drug delivery vehicles. Anaerobic bacteria prefer the hypoxic TME and actively colonize and proliferate at tumor sites[19]. As drug carriers, the accumulation of bacteria in the hypoxic TME is substantially higher than that of conventional abiotic carriers[20]. In addition, bacteria can stimulate an effective immune response to synergistically promote PDT results. Using the synthetic biology toolkit, bacteria can be further programmed to produce specific drugs or target certain types of cells[21].

In this review, we present a comprehensive overview and analysis of the methodologies employed to overcome the constraints associated with antitumor PDT, with a particular emphasis on endogenous approaches. Strategies involving the endogenous production of excitation light, photosensitizers, and O_2 are discussed in detail, with particular emphasis on the biogenesis of these components. In light of recent advancements in synthetic biology, the application of genetically engineered bacteria in PDT has also been discussed. Finally, we highlight the potential of engineered bacteria not only as effective drug carriers but also as producers of excitation light, photosensitizers, and O_2.

2. Excitation light for PDT

Photosensitizers need to be excited by light with matching wavelengths to generate ROS for antitumor PDT[22,23]. Upon the absorption of photons, the photosensitizer undergoes a transition from the ground singlet state to a higher-energy excited state, which subsequently initiates a photochemical reaction *via* two distinct mechanisms. In Type I PDT, the photoactivated photosensitizer engages in direct interactions with adjacent biological substrates, including nucleic acids, proteins, lipids, or water molecules, leading to the production of free radicals. On the other hand, Type II PDT involves the transfer of energy from the excited photosensitizers to molecular oxygen in the vicinity, resulting in the generation of $^1O_2$, which is considered as the primary therapeutic ROS[24−28].

For optimal results, it is imperative to align the emitting wavelength of the light source with the absorption spectrum of the photosensitizer. Furthermore, meticulous modulation of light energy is essential to enhance ROS generation while mitigating potential damage to healthy tissues[29]. The excitation light wavelength of most photosensitizers falls within the optical window of biological tissues and is therefore not ideal for deep tumor treatment due to limited penetration depth[30,31]. Numerous strategies have been devised to overcome the aforementioned constraints.

2.1. *External light source*

To address the issue of light penetration in deep tissues, various wire-based methodologies have been devised to facilitate the transmission of external light. These approaches include the utilization of light-emitting diodes and fiber optics coupled with lasers[32]. Another promising approach involves the use of light conversion nanomaterials, which can be used to convert external light or radiofrequency signals into localized light internally, enabling wireless phototherapy. UCNPs are nanoparticles that are doped with rare-earth elements and possess the ability to convert NIR radiation to UV−Vis light. When employed in PDT, UCNPs transform externally applied NIR light into localized UV−Vis light, thus enabling the activation of photosensitizers. UCNP-based PDT shows better performance in treating deep-seated tumors[33]. For instance, a UCNP $NaYF_4$:Yb^{3+}, Er^{3+} was constructed for PDT application, which can be excited by 980 nm NIR light and emit 660 nm light[34,35]. The UCNP was coated with mesoporous silica and loaded with the photosensitizer zinc phthalocyanine. The emission light from NIR-activated UCNPs excited zinc phthalocyanine, producing ROS to eliminate cancer cells[36]. Similar strategies have been explored using different UCNPs and photosensitizers pair[37], demonstrating good outcomes in overcoming the poor tissue penetration challenge in conventional PDT[38,39]. The depth of treatable tumors through NIR-activated PDT can be further enhanced with the adjunctive application of biocompatible implants[40].

X-ray is a source of ionizing radiation with no penetration depth limitation in human tissue. X-ray can be used to excite scintillating nanoparticles which emit UV−Vis light for the activation of photosensitizers[41]. Micheletto et al.[42] combined LaF_3:Tb^{3+} with the protein photosensitizer KR (KillerRed) to perform X-ray activated PDT. Under irradiation, the emission light from LaF_3:Tb^{3+} with a wavelength of around 580 nm activates the photosensitizer. X-ray responsive PDT is a promising treatment strategy for deep-seated tumors, but the safety issue of X-ray needs to be addressed with caution.

2.2. *Internal light source*

Light sources, including Cerenkov radiation luminescence and chemiluminescence, not requiring external irradiation are alternative options for antitumor PDT. CR[43] luminescence has a wavelength range of 250−600 nm, which is generated by positron-emitting radionuclides[44]. Kotagiri et al.[45] employed CR luminescence as an internal light source for the activation of the photosensitizer TiO_2 nanoparticles. The excited TiO_2 nanoparticles generated hydroxyl and superoxide radicals, which effectively impeded tumor proliferation and significantly extended the survival of tumor-bearing mice. Chemiluminescence produces light from chemical reactions, also requiring no external irradiation[46]. For example, luminol is a commonly used substrate for chemiluminescence, which can luminescence after a reaction with H2O2 catalyzed by horseradish peroxidase. The luminescence from luminol around 450 nm was utilized to excite the photosensitizer Ce6 for PDT. The chemiluminescence induced PDT exhibited *in vitro* cytotoxicity against human lung cancer cells and inhibited the tumor development *in vivo*[47] (Fig. 2A).

cytotoxicity. Reproduced with permission from Ref. 49. Copyright © 2015 Ivyspring. (E) BSA conjugated with both RLuc8.6 and Rose Bengal can produce ROS *via* BRET in the presence of the luciferin substrate (BSA: bovine serum albumin, RB: Rose Bengal). Reproduced with permission from Ref. 58. Copyright ©2017 Royal Society of Chemistry. (F) A fusion protein of RLuc8.6 and KillerRed is constructed which generates ROS when coelenterazine-h is provided. Injection of the fusion protein and coelenterazine-h resulted in strong luminescence and killed cancer cells in the xenograft mouse model established using MDA-MB-231 cells (RKL: fusion of Rluc8.6 and KillerRed, Co-h: coelenterazine-h). Reproduced with permission from Ref. 59. Copyright © 2020 Wiley.

Bioluminescence is a type of chemiluminescence observed in living organisms that has been investigated in PDT as well.

A typical bioluminescence system is composed of a luciferase enzyme and a luciferin substrate for the enzyme. Bioluminescence systems have been identified across various organisms, including fungi, bacteria, insects, and certain marine organisms. In order to overcome the challenges associated with conventional PDT regarding light delivery, researchers have explored the utilization of bioluminescence as an internal light source alternative. This involves investigating the ability to introduce luciferase proteins or luciferase genes to tumor sites, enabling the endogenous production of light in the presence of luciferins. By ensuring that the bioluminescence spectrum overlaps with the absorption spectrum of the photosensitizer and that they are in close proximity, it becomes possible to excite the photosensitizer and generate ROS for PDT[48]. Three bioluminescence systems (*i.e.*, FLuc, Rluc, and NLuc) have been tested for PDT[49].

2.2.1. *Firefly luciferase bioluminescence in PDT*

The firefly luciferase (FLuc) bioluminescence system is the first bioluminescence system discovered and is also the most studied and used system. It was initially isolated from the firefly *Photinus pyralis*. The FLuc protein, with a molecular weight of 61 kDa, catalyzes the biochemical reaction between D-luciferin, O_2, and ATP. The Fluc luminescence exhibits a maximum emission wavelength of 558 nm[50]. Fluc bioluminescence can function as an endogenous light source within tumors, thus solving the problems associated with delivering external light to deep tissues. In 1994, Carpenter et al.[51] reported Fluc bioluminescence-mediated PDT using hypericin as the photosensitizer. The PDT effectively inactivated the equine infectious anemia virus, demonstrating that the light from Fluc bioluminescence can induce PDT. In 2003, Fluc bioluminescence induced PDT was reported in cells[52]. Theodossiou et al.[52] transfected the FLuc gene into mouse embryonic fibroblast cells and treated the cells with the photosensitizer Rose Bengal (RB). PDT was induced by the addition of the luciferin substrate, which led to 89% cell death. This study suggested that bioluminescence can be an endogenous light source for antitumor PDT. However, this result was challenged by a study performed by Schipper et al.[53] in 2006 showing that the FLuc bioluminescence system cannot produce enough photons to achieve potent photodynamic toxicity. The observed disparity between the outcomes obtained by the two groups may be attributed to the different concentrations of D-luciferin employed in their respective experimental procedures (500 μmol/L by Theodossiou et al.[52] and 20 μmol/L by Schipper et al.[53]). Since then, accumulated efforts have been devoted to improving the performance of Fluc bioluminescence-mediated antitumor PDT.

The poor overlap between the bioluminescent spectrum and the photosensitizer absorption spectrum hinders effectiveness of PDT. Yang et al.[54] converted the 558 nm light from Fluc bioluminescence to 635 nm through a BRET (bioluminescence resonance energy transfer) process between Fluc and carbon dots. The wavelength of the emission light from carbon dots overlapped well with the photosensitizer PPIX. The distance between the bioluminescence source and the photosensitizer is another factor limiting PDT efficacy. Confining Fluc proteins and photosensitizers in close proximity contributes to a more efficient energy transfer between them. A polylactic-glycolic acid nanoparticle was loaded with the photosensitizer RB and then bioconjugated with FLuc proteins. The system allowed a more efficient BRET between the Fluc protein and RB, exhibiting enhanced antitumor PDT performance[48]. Nevertheless, the low delivery efficiency of Fluc protein limits the intensity of bioluminescence. An alternative approach is to deliver the gene encoding Fluc instead of Fluc proteins. A nanomedicine was developed by encapsulating D-luciferin, hypericin, and a plasmid inserted with the Fluc gene in polyethyleneimine-modified nano calcium phosphate[55] (Fig. 2B). The nanomedicine collapsed in the lysosome of tumor cells, releasing the plasmid. The Fluc gene carried by the plasmid was abundantly expressed in tumor cells, leading to the accumulation of FLuc proteins. The endogenously expressed Fluc proteins reacted with the released D-luciferin to generate bioluminescence. This strategy can increase the concentration of FLuc protein at tumor sites, thereby enhancing the intensity of bioluminescence and thus stimulating a stronger PDT.

2.2.2. *Renilla luciferase bioluminescence in PDT*

Renilla luciferase (RLuc) is a 36 kDa protein found in sea pansy *Renilla reniformis*. RLuc reacts with its enzyme substrate coelenterazine to generate bioluminescence in the presence of O_2. Compared to FLuc, RLuc is smaller in size and does not require ATP to luminesce. RLuc bioluminescence exhibits emission at 480 nm. RLuc has been extensively investigated as a luminescent reporter in mammalian cells. RLuc8 and RLuc8.6 are brighter and more stable mutants derived from the original RLuc protein. RLuc8 demonstrates a 4-fold increase in bioluminescent intensity and a marginal redshift in its emission, relative to RLuc. RLuc8.6 has an emission wavelength of 535 nm, which is redshifted by 55 nm relative to RLuc. The RLuc substrate coelenterazine is less soluble and less stable than D-luciferin and is prone to autoxidation[56].

To convert the light from RLuc bioluminescence to light with excitation wavelengths of common photosensitizers, quantum dots have been used as the intermediate energy transmitter. The RLuc8 protein has undergone a process of bioconjugation, resulting in its linkage to a quantum dot that emits at 655 nm. When coelenterazine is present, the bioluminescence of RLuc8 activates the fluorescence of the quantum dots, which in turn can stimulate the photosensitizer Foscan for PDT[57] (Fig. 2C). The ROS produced led to 50% eradication of A549 tumor cells *in vitro*, along with a substantial retardation in tumor progression in an *in vivo* model. Similarly, quantum dots were conjugated with RLuc8 to achieve bioluminescence-mediated excitation of Ce6[49] (Fig. 2D). The system was therapeutically effective against a variety of cancer cells and metastatic tumors as well. It exhibited better therapeutic effects on deep tumors compared to a system activated by external laser irradiation. RLuc8.6 has redshifted emission that overlaps with the absorption spectrum of the photosensitizer RB. A covalent conjugate of RLuc8.6 and RB was constructed to form a BRET pair[58] (Fig. 2E). The conjugate underwent additional coupling with cell-penetrating peptides in order to enhance its internalization efficiency into cancer cells. Upon the introduction of coelenterazine, the conjugate exhibited photodynamic toxicity, resulting in a 75% decrease in viability of colon cancer cells. To avoid the risks for the toxicity of chemical photosensitizers and nanomaterials, KillerRed as a protein photosensitizer was fused with RLuc protein for PDT[59] (Fig. 2F). The fusion protein was subsequently conjugated with a leading peptide for the purpose of selectively targeting cancer cells. The resulting protein construct demonstrated the ability to specifically recognize and enter breast cancer cells. It exhibited the capacity to induce PDT-mediated cancer cell death within tumor xenograft mouse models when coelenterazine was present. Notably, these findings highlight that

bioluminescence, which is a relatively low-intensity light source, is able to efficiently induce antitumor PDT *in vivo*.

2.2.3. *Luciferase nanoLuc−furimazine bioluminescence in PDT*

NanoLuc (NLuc) is a luciferase protein derived from a deep-sea shrimp *Oplophorus gracilirostris* luciferase after several rounds of mutagenesis. It is smaller than both Fluc and RLuc, with a molecular weight of 19.1 kDa. NLuc catalyzes the reaction involving furimazine, its substrate, leading to the emission of bioluminescent light with a wavelength of 456 nm in the presence of O_2. The emission of NLuc is blue-shifted by approximately 20 and 100 nm relative to RLuc and FLuc[60]. The luminescence produced by NLuc is ∼150-fold greater than that of FLuc or RLuc[61]. However, the substrate furimazine is more expensive than D-luciferin and coelenterazine. The maximum emission wavelength of NLuc at 460 nm matches the absorption wavelength of the protein photosensitizer miniSOG[62,63]. The NLuc and miniSOG were encoded in one plasmid and expressed as a fusion protein[64]. The phototoxicity of miniSOG stimulated by NLuc bioluminescence was comparable with that of miniSOG excited by LED. As the construct is genetically encodable, it can potentially be delivered to any tissue site to treat deep and metastatic tumors.

3. Photosensitizers for PDT

The photosensitizer constitutes a triad of indispensable components for PDT, wherein it absorbs excitation light and subsequently generate cytotoxic ROS[65]. Photofrin, a photosensitizer authorized by U.S. Food and Drug Admistration (FDA) in 1993, emerged as the first photosensitizing agent approved for application in PDT. Since the approval of photofrin, more than twenty PDT reagents have been commercialized and hundreds more are in clinical trials or preclinical studies[66]. Photosensitizers are mainly classified into organic photosensitizers and inorganic photosensitizers[67,2]. The requirements for an ideal photosensitizer include: 1) high stability at room temperature, good solubility and pharmacokinetics, and nontoxicity in the absence of photoactivation; 2) high extinction coefficient within the optical range characterized by minimal tissue absorption; 3) significant singlet oxygen quantum yield and an extended lifetime of trilinear excited state.

3.1. *Organic photosensitizer*

To date, four generations of organic photosensitizers have been developed. The initial cohort of photosensitizers encompasses chiefly hematoporphyrin and its derivatives as the primary compounds. Photofrin is a mixture of hematoporphyrin derivatives that can be excited by 630-nm light, showing inhibitory effects on breast, colon, prostate, head, and neck cancers[68]. Most of the 1st generation photosensitizers are faced with limitations such as long metabolic cycles in the human body, high accumulation in skin, and the need for patients to avoid strong light for weeks. The 2nd generation photosensitizers mainly include chlorine, bacteriochlorin analogues, phthalocyanines, and protoporphyrin IX (PPIX) compounds[69]. Foscan, NPe6, Levulan, and Metvix are 2nd generation photosensitizers that have been clinically approved with inhibitory effects on lung, skin, and bladder cancer. Compared with the 1st generation, 2nd generation photosensitizers are improved in terms of homogeneity, tumor selectivity, quantum yield, and reduced accumulation in the skin[70]. However, the efficiency of PDT therapy with 2nd generation photosensitizers is hindered by the hydrophobicity and self-aggregation of the reagents in biological media[71]. The 3rd generation photosensitizers use nanoparticles (*e.g.*, liposomes, micelles, dendrimers, and mesoporous silica) as drug carriers; and the 4th generation are photosensitizers loaded in metal−organic frameworks[72]. These two types are developed to enhance the uptake by tumors and attenuate the off-target release of photosensitizers. They exhibit promising outcomes in preclinical studies, but their long-term biosafety is still under scrutiny[73].

3.2. *Inorganic photosensitizer*

Compared with organic photosensitizers, inorganic nanoparticle photosensitizers have higher light conversion efficiency, higher $^1O_2$ quantum yield, and better stability. The TiO_2 nanoparticle is an inorganic photosensitizer that responds to UV irradiation[74]. Metallic nanoparticles, including silver, gold, and platinum nanoparticles, exhibit the capability of generating $^1O_2$ when exposed to light irradiation (398−530 nm)[75]. Additional inorganic photosensitizers encompass nanoparticles based on metal sulfides (such as copper sulfide and molybdenum sulfide)[76], as well as nanoparticles based on carbon (including graphene and carbon dots)[77]. Although with better photostability than most organic photosensitizers, the long-term toxicity and biosafety of inorganic photosensitizers need to be thoroughly investigated by more *in vivo* studies. Barriers to the clinical use of inorganic photosensitizers mainly include the unclear biocompatability, their slow clearance in bodies, and the tendency to aggregate and bind biomolecules in biological media[78].

The organic and inorganic photosensitizers discussed above are chemically synthesized and supplied through injection or oral administration. Efforts have been made to optimize the stability, solubility, and specificity of photosensitizers to ensure that they are not modified or degraded in circulatory systems and that sufficient amounts can be targeted to tumors. Recently, genetically encodable photosensitizers that can be biosynthesized endogenously at tumor sites have received growing attention.

Such photosensitizers are biosynthesized in cells, thus having better biocompatibility than exogenously provided photosensitizers that are chemically synthesized. On-site synthesis of photosensitizers also helps to address the issues related to the stability of PDT reagents during delivery. Using different inducible promoters, the biogenesis of genetically encoded photosensitizers can be precisely regulated temporally and spatially. Genetic tools can be used to modify the photosensitizers, for example, specific localization peptide sequences can be easily fused to protein photosensitizers to target specific organelles of tumor cells. Genetically encodable photosensitizers include protein-based photosensitizers and small molecule-based photosensitizers.

3.3. *Genetically encodable protein photosensitizers*

3.3.1. *Different protein photosensitizers*

KillerRed is a dimeric protein with a molecular weight of 54 kDa that originates from anm2CP, a chromoprotein initially identified in hydrozoan species. KillerRed exhibits a structural similarity to GFP and possesses a chromophore composed of the amino acids Gln-Tyr-Gly[79]. The chromophore absorbs green light (540−580 nm) and emits red fluorescence (maximum emission at 610 nm), meanwhile producing ROS. The KillerRed exhibits a phototoxic effect that is greater than that of other fluorescent proteins by 1000-fold[80].

KillerRed demonstrated strong inhibitory effects against both bacterial and mammalian cells under green light irradiation. KillerRed proteins mainly produce phototoxicity *via* Type I PDT, and thus can be applied in hypoxic TME[81]. *Via* genetic engineering, fusion proteins of KillerRed with linked localization peptides were constructed to target specific organelles or cellular compartments in cancer cells. It has been observed that the delivery of KillerRed to different organelles leads to varied PDT treatment outcomes. For instance, delivery of KillerRed to mitochondria and lysosomes triggers apoptosis, while PDT induced by KillerRed in the plasma membrane results in necrosis[82].

In spite of the efficacious PDT outcomes and the genetic editability exhibited by KillerRed, there exist inherent limitations that hinder the *in vivo* application of KillerRed. The primary impediment to the *in vivo* utilization of KillerRed is attributed to the limited tissue penetration depth stemming from the short wavelength of the excitation light. In addition, KillerRed requires dimerization to function, which may interfere with the properties of genetically engineered KillerRed fusion proteins. To address these limitations, KillerRed derivatives (*e.g.*, SuperNova, KillerOrange, and mKillerOrang) containing selected mutations have evolved.

SuperNova is a monomeric phototoxic protein derived from KillerRed containing a total of six mutations. SuperNova exhibits similar optical properties and comparable phototoxicity to KillerRed. SuperNova proteins do not dimerize; therefore SuperNova fusion proteins can be easily designed[83,80]. KillerOrange is a KillerRed mutant with a blueshifted emission spectrum[84]. It absorbs light at 455 and 514 nm and emits orange fluorescence, producing ROS with a quantum yield of 0.42. The chromophore of KillerOrange consists of tryptophan instead of the tyrosine in KillerRed. The phototoxicity of KillerOrange was demonstrated in both *E. coli* cells and HEK 293 cells. It was also observed that when KillerOrange and KillerRed are used simultaneously, the two proteins do not interfere with each other in both bacterial and mammalian cells. A monomeric variant of KillerOrange, mKillerOrange, was constructed by introducing a tyrosine-to-tryptophan mutation in SuperNova[85]. The mKillerOrange protein has similar optical properties as KillerOrange, but does not require dimerization to function.

MiniSOG (mini Singlet Oxygen Generator) is a protein photosensitizer that has been developed through mutagenesis of the LOV2 domain, which is a component of the blue light photoreceptor known as phototropin 2[86]. MiniSOG is a 15.3 kDa monomeric protein, much smaller than 54 kDa KillerRed, thus allowing easier expression and exerting less influence in the physiology of cells. Unlike KillerRed and KillerOrange, miniSOG uses bound flavin mononucleotide (FMN) as the chromophore, producing green fluorescence (500 nm) and ROS upon irradiation by blue light (448 nm). It was reported later that miniSOG produces singlet oxygen less than originally expected and also generates superoxide *via* a Type I photochemical reaction[87]. As a result of the absence of FMN within mammalian cells, the supplementation of FMN from an external source becomes necessary to perform PDT utilizing miniSOG[88].

The miniSOG structure was mutated to tailor the optical and photodynamic properties. The targeted mutagenesis of glutamine[103] in miniSOG to leucine resulted in a more phototoxic photosensitizer SOPP with a higher $^1O_2$ quantum yield, also named miniSOGQ102L[89−91]. miniSOG2 was a miniSOG mutant obtained by directed evolution. Seven mutations were introduced in miniSOG2, four of which are on residues in the binding pocket for FMN. These four mutations substantially enhanced the $^1O_2$ quantum yield of miniSOG2 to 0.47[92].

3.3.2. *PDT with protein photosensitizers*

The localization of the protein photosensitizer is a critical factor that governs the different damage pathways elicited by PDT. In recent times, a variety of promising methodologies have emerged pertaining to the transportation of purified protein photosensitizers or genes encoding such photosensitizers to tumors.

3.3.2.1. Delivery of purified protein photosensitizers. Protein photosensitizers can be expressed in cells, usually bacterial cells, and purified to homogeneity. The purified proteins are then delivered to tumors for PDT treatment. In order to protect the protein photosensitizers and augment the selectivity of transportation, nanocarriers are frequently employed.

KillerRed was expressed and isolated from *E. coli* cells for PDT in three leukemia cell lines (*i.e.*, K562, NB4, and THP-1). Under irradiation (400−700 nm, 80 mW/cm^2, 20 min), KillerRed induced apoptosis of leukemia cells in a concentration-dependent manner[93] Liang et al.[94] covalently coupled KillerRed to UCNPs to construct a nanoparticle−protein complex responsive to NIR light. Under 980 nm NIR irradiation, the UCNPs emitted green light to excite KillerRed. The KillerRed-mediated PDT efficiently eradicated breast cancer cells *in vitro* and also demonstrated successful deep tumor therapy *in vivo* (Fig. 3A). To improve the tumor targeting specificity of KillerRed, a liposome and cancer cell membrane hybrid complex was developed as a delivery vehicle. A fusion protein of KillerRed linked to a membrane-localization signal peptide was expressed in 4T1 cancer cells. The cell membrane was extracted with KillerRed anchored and hybridized with liposomes. The lipo-complex prepared in this study demonstrated a cancer-targeting efficiency that was 3.3 times greater than that of a control liposome. Moreover, it effectively suppressed the growth of primary tumors and inhibited lung metastasis *in vivo*[95]. MiniSOG has also been expressed as fusion proteins for correct localization in target cells[96]. Mironova et al.[97] conjugated miniSOG with a peptide capable of selectively binding to the human epidermal growth factor receptor 2 (HER2). The fusion protein specifically targeted HER2-positive cancer cells as it could be internalized *via* receptor-mediated endocytosis. Upon irradiation with 1 W/cm^2 white light for 10 min, the miniSOG derivative exerted strong and highly specific phototoxic effects on HER2-positive breast cancer cells.

3.3.2.2. Delivery of genes encoding protein photosensitizers using viral vectors. KillerRed and miniSOG are genetically encodable, thereby their genes can be delivered to tumors enabling endogenous biogenesis of the photosensitizers. This strategy will accumulate more photosensitizers at tumor sites than a single shot of purified proteins, thus allowing sustainable PDT treatment[98] (Fig. 3B). Viruses can be genetically engineered to target and eliminate tumor cells. FDA has approved genetically modified viruses as anticancer treatments. Viral vectors have been extensively used to deliver genes into mammalian cells, due to their high transfection efficiency. Both KillerRed and miniSOG have been successfully delivered to tumor cells using viral vectors.

To achieve selective expression of KillerRed in cancer cells, a recombinant adenovirus vector, named TelomeKiller, was constructed by placing the KillerRed gene under the control of a human telomerase reverse transcriptase promoter. This promoter is upregulated in most cancers but not in normal cells. Intratumoral administration of TelomeKiller exhibited potent anti-tumor effects by attenuating the proliferation of human rectal cancer cells and impeding the regional lymph node metastasis in a HCT116 xenograft tumor model, facilitated by the application of green

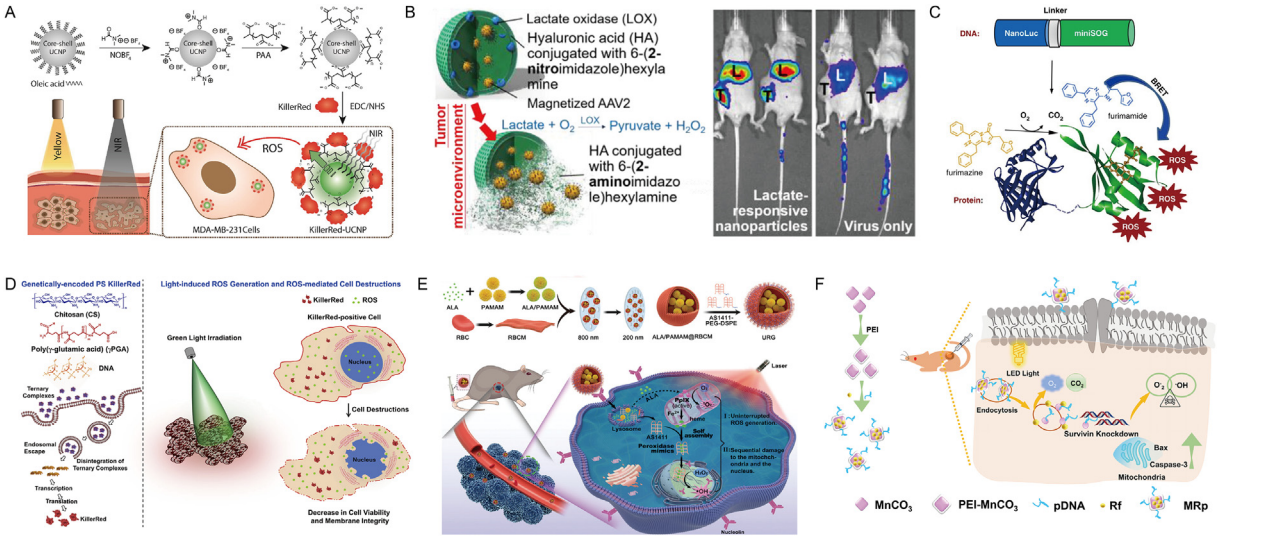

Figure 3 Examples of PDT using protein photosensitizers and small-molecule natural product photosensitizers. (A) UCNPs are employed to enhance the depth of PDT treatment under NIR excitation by coupling with KillerRed through EDC/NHS chemistry (PAA: poly(acrylic acid), EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS: *N*-hydroxysuccinimide). Reproduced with permission from Ref. 94. Copyright © 2017 Elsevier. (B) Virus AAV2 encoding KillerRed is loaded in lactate responsive nanoparticles to achieve tumor microenvironment targeting PDT (AAV2: adeno-associated virus serotype 2). Reproduced with permission from Ref. 98. Copyright © 2018 ACS publications. (C) The fusion of NanoLuc luciferase and miniSOG allows energy transfer from bioluminescence to miniSOG, generating ROS to kill cancer cells. Reproduced with permission from Ref. 103. Copyright © 2022 Springer Nature. (D) A complex consisting of chitosan and poly(γ-glutamic acid) is constructed to enhance the delivery of a plasmid carrying KillerRed gene. KillerRed is expressed intracellularly and induces green light-triggered cell destruction (CS: chitosan, γPGA: poly(γ-glutamic acid)). Reproduced with permission from Ref. 104. Copyright © 2014 Elsevier. (E) 5-ALA loaded PAMAM dendrimers are wrapped with RBCMs and surface modified with DNA aptamer AS1411. 5-ALA released from the resulting materials can be transformed into heme in cells, which forms peroxidase mimics with the AS1411 to produce ROS (5-ALA: 5-aminolevulinic acid, PAMAM: polyamidoamine, RBCMs: red blood cell membrane). Reproduced with permission from Ref. 115. Copyright ©2022 Wiley. (F) A nanocomposite consisting of $MnCO_3$, riboflavin, and pDNA is constructed to treat triple-negative breast cancer. It decomposes under TME, releasing Mn^{2+}, O_2, and riboflavin for PDT and CDT, as well as pDNA to suppress the expression of survivin gene for enhanced tumor destruction (Rf: riboflavin, pDNA: plasmid DNA). Reproduced with permission from Ref. 116. Copyright © 2021 Springer Nature.

light irradiation[99]. Intravenous injection of viral vectors faces more challenges, as the immune system tends to clear the viruses. A magnetic field-responsive viral vector was developed by chemically conjugating iron oxide nanoparticles to a recombinant adeno-associated virus carrying the KillerRed gene[100]. The magnetic vector was administered intravenously through the tail vein of mice bearing xenografted H1975 tumors, and its localization to tumor sites was facilitated by a magnetic field. KillerRed was abundantly expressed in tumors and significantly inhibited tumor growth upon illumination at 561 nm for 20 min.

Lentiviruses can be engineered to be integration vectors that insert the carried gene into chromosomes of target cells to construct stable expression cells. Different miniSOG constructs targeting varied cellular components (*i.e.*, mitochondria, chromatin, and cell membrane) were expressed in tumor cells using lentiviral vectors[101]. A derivative of miniSOG that specifically targets the cell membrane demonstrated the highest level of photoactivated cytotoxicity against HeLa cells. Nevertheless, the lentiviral vector failed to manifest substantial therapeutic efficacy in murine models, presumably attributed to the limited tissue permeability of blue light and the inefficient delivery of FMN cofactors[101]. As NLuc bioluminescence overlaps with the absorption of miniSOG, a lentiviral vector hosting a fusion construct of NLuc and miniSOG was developed. The DNA encoding NLuc-miniSOG fusion protein was injected into BT-474 tumor cells bearing mice. The NLuc substrate furimazine and the FMN precursor riboflavin were provided intravenously every day. A tumor growth inhibition coefficient exceeding 67% was achieved after the treatment, suggesting bioluminescence can be an effective light source for *in vivo* PDT using miniSOG as the photosensitizer[102,103] (Fig. 3C).

3.3.2.3. Delivery of genes encoding protein photosensitizers using nonviral vectors. Due to the concern about genotoxicity related to viral vectors, nonviral vectors are also developed to deliver genes for protein photosensitizers. Cationic polymers, such as chitosan, can be carriers for negatively charged DNA molecules. To facilitate the DNA release and improve the transfection efficiency, polyglutamic acid was incorporated into chitosan to deliver a plasmid hosting the KillerRed gene. HEK293 cells transfected with the polymer vehicles could express KillerRed and got inhibited under green light exposure. The effect was negligible after eight days, indicating that cells did not undergo genetic modification[104] (Fig. 3D). In addition, guanidine modified pullulan[105,106], polyethyleneimine polymer[104,107], and DNA tetrahedron[108] have also been used to optimize the delivery efficiency of KillerRed gene. Nonviral vectors commonly mediate transient transfection of genes, and thus the expression of protein sensitizers is temporal, and the

toxicity associated with the proteins spontaneously decreases over time.

3.4. Genetically encodable small molecule-based photosensitizers

There are a variety of small-molecule photosensitizers discovered that are natural products biosynthesized by plants, microbes, and mammalian cells. For example, the commonly used photosensitizer hypericin can be biosynthesized by species of the genus *Hypericum*, and some fungi as well. Endogenous small molecules with phototoxicity have also been reported in human cells, such as biogenic 3-hydroxy pyridine derivatives in human skin cells that respond to UV irradiation[109−111]. These small-molecule photosensitizers can be endogenously biosynthesized like protein photosensitizers but are smaller in size and better diffused.

PPIX is a ubiquitous natural porphyrin compound found in living organisms. PPIX absorbs light with a wavelength of 380−650 nm and generates ROS to kill cancer cells[112−114]. The utilization of the red light for *in vivo* stimulation of PPIX is a prevalent practice owing to its better tissue permeability characteristics. Verteporfin, an analogue of PPIX, has been approved by FDA as a PDT reagent. Another photosensitizer for PDT that has obtained approval from the FDA is 5-aminolevulinic acid (5-ALA), which acts as the precursor for PPIX. PPIX can be biotransformed from 5-ALA *via* a cascade of enzymatic reactions in tumor cells. After supplementation of 5-ALA, PPIX accumulates preferentially at tumor sites. Under laser irradiation, PPIX produces ROS to eradicate tumor cells. Heme is the downstream product of PPIX, which slows the conversion from 5-ALA to PPIX through negative feedback. To alleviate the inhibitory effect of heme, 5-ALA was co-delivered with a DNA aptamer which self-assembled with heme to form peroxidase mimics[115] (Fig. 3E). Combination of PPIX PDT and the effects of peroxidase mimics led to 80% regression of tumors in B16F10-tumor-bearing mice.

Riboflavin[116] (Fig. 3F), also known as vitamin B2, is a photosensitive natural product biosynthesized in plants and microorganisms (Fig. 3D). Under 430−440 nm light irradiation, riboflavin emits green fluorescence and transfers energy to O_2 or H_2O_2 producing ROS. PDT using riboflavin as the photosensitizer has been tested in 4T1 cells[117], SCC-13 cells[118], and HCT116 cells[119]. A crucial shortcoming of riboflavin photosensitizer is that its excitation wavelength falls in a range with poor penetration in tissues. UCNPs were employed for the purpose of converting NIR radiation into localized UV−blue light, to induce the excitation of riboflavin. NIR-activated PDT using riboflavin led to 90% tumor growth inhibition in SK-BR-3 breast tumor xenografts[120]. Another factor hindering the PDT performance using riboflavin is its limited cellular uptake. The derivative can enter tumor cells more efficiently by conjugating riboflavin with cell-penetrating peptides, thus improving the PDT outcomes[121].

In addition to the widely studied biosynthesized photosensitizers PPIX and riboflavin, pterin derivatives and tryptophan derivatives have also been investigated as potential endogenous photosensitizers. Pterins are endogenous photosensitizers that are ubiquitous in all domains of life. They produce singlet oxygen under UV light irradiation. Pterin derivatives have been successfully applied to PDT treatment of pancreatic cancers[122]. The endogenously synthesized tryptophan-derived photoproduct, 6-formylindolo(3,2-*b*) carbazole, has been employed in PDT for the eradication of skin cancer cells[123,124].

4. Solutions to hypoxia in PDT

Hypoxia is an intrinsic feature of TME that makes tumors resistant to conventional antitumor treatments including chemotherapy, radiotherapy, and PDT[125,126]. For PDT, especially Type II PDT, the availability of O_2 around photosensitizers affects the yield of cytotoxic ROS. Photodynamic reactions result in the depletion of O_2, which worsens the issue of hypoxia and ultimately hampers the efficacy of PDT[4]. Various approaches have been employed to mitigate the hypoxia-related constraints encountered during PDT.

4.1. Non-enzymatic approach

Delivery of exogenous O_2 to tumors is a direct approach to alleviating tumor hypoxia. Hyperbaric oxygen therapy increases the level of dissolved O_2 in plasma, thereby increasing the amount of O_2 transported to tumors and improving the ROS production in PDT[127,65]. Hemoglobins can bind O_2 and therefore have been combined with PDT reagents to deliver O_2 for PDT enhancement[128].

H_2O_2 is more abundant in tumors compared to normal tissues, at concentrations of ∼50−100 μmol/L, which can be decomposed to provide O_2 *in situ*. Gold, platinum, and manganese dioxide nanoparticles have been explored as nanozymes to catalyze the transformation of H_2O_2 to O_2. These nanozymes have demonstrated the capability to alleviate tumor hypoxia while augmenting the efficacy of PDT[129]. Hemin, an iron-containing porphyrin compound, exhibits catalase-like characteristics that enable it to facilitate the breakdown of endogenous H_2O_2. Hemin has been co-delivered with Ce6 by DNA aptamer nanopolymers[130] (Fig. 4A), M1 macrophage vesicles[131], and nanographene oxide[132] for PDT treatments. Hemin could alleviate the hypoxia *via* breaking down H_2O_2 both *in vitro* and *in vivo*, significantly improving antitumor PDT performance[133]. Hemin can be converted to the photosensitizer PPIX after losing the bound iron, and thus hemin can be a photosensitizer precursor as well. A thermally carbonized hemin nanoparticle was prepared to function as the catalase-like nanozyme and photosensitizer. The nanoparticle acted as a catalyst in the conversion of H_2O_2 into O_2 and •OH through Fenton reactions, thereby alleviating hypoxic conditions and generating ROS. Due to the high temperature during preparation, hemin lost the iron ion bound to the porphyrin ring, resulting in a porphyrin derivative that could generate $^1O_2$ upon photoactivation. The nanoparticle derived from hemin demonstrated enhanced inhibitory efficacy against 4T1 breast cancer cells both *in vitro* and *in vivo*, surpassing the inhibitory effects of conventional PDT[134].

4.2. Enzymatic approach using catalase

Catalase, a natural protein, functions as a catalyst for the enzymatic breakdown of H_2O_2 into water and oxygen. Its application presents a potential strategy for ameliorating hypoxia within tumors. Intertumoral vascular heterogeneity hampers the diffusion of catalase proteins, therefore various delivery vehicles have been constructed to deliver purified catalase proteins into tumors[135]. For example, catalase and alginate dialdehyde were assembled onto $CaCO_3$ nanoparticles with RB loaded as the photosensitizer. Catalase decomposed H_2O_2 to produce O_2 *in situ*, increasing O_2 concentration and promoting the production of $^1O_2$, thus enhancing the efficacy of PDT against MCF-7 breast cancer cells. This early work demonstrated that effective delivery of active catalase proteins can be achieved using nanocarriers and can

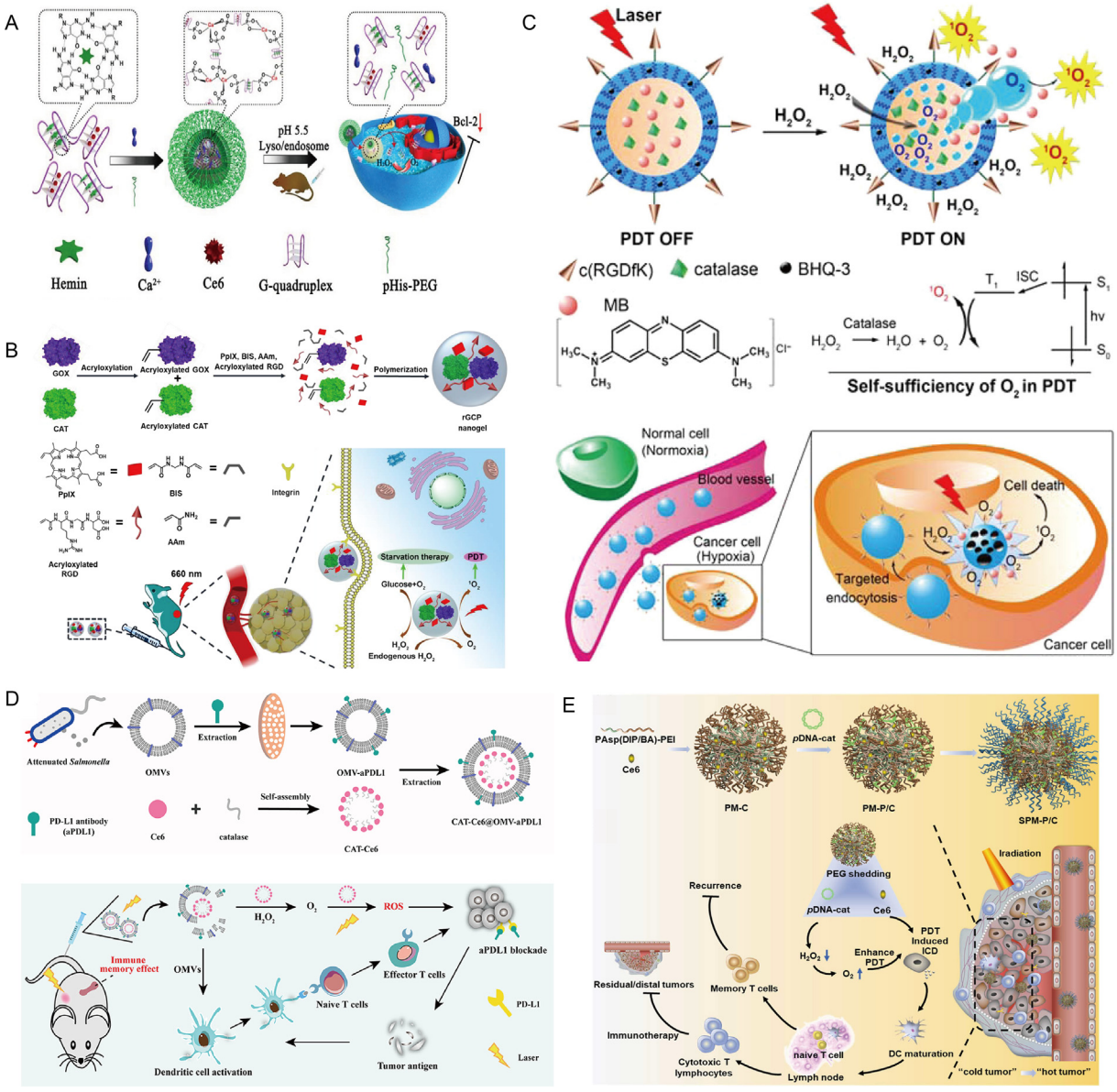

Figure 4 Examples of PDT that employ strategies to alleviate hypoxia in tumor microenvironment. (A) A coordination polymer is formed using Ca^{2+} and AS1411 aptamer, with Ce6 and hemin incorporated. Hemin alleviates hypoxia at tumors, improving the performance of PDT mediated by Ce6 (Bcl-2: B-cell lymphoma 2). Reproduced with permission from Ref. 130. Copyright © 2018 ACS publications. (B) GOX, CAT, and photosensitizers are encapsulated with the cancer cell targeting tripeptide Arg-Gly-Asp in nanocarriers to enhance the uptake and accumulation at tumor sites. GOX and CAT generate oxygen from glucose *via* cascade reactions, alleviating hypoxia at tumor sites. (GOX: glucose oxidase, CAT: catalase). Reproduced with permission from Ref. 137. Copyright © 2022 Elsevier. (C) Nanoparticles containing catalase and MB are coated with BHQ-3 loaded PLGA to quench excited photosensitizers. The catalase reacts with H_2O_2 present in the tumor microenvironment (TME) to generate O_2, which disrupts the PLGA shell leading to the release of BHQ-3 to activate PDT. Additionally, the O_2 attenuates hypoxia in TME to enhance PDT performance (MB: methylene blue, BHQ-3: black hole quencher-3, c(RGDfK): pentapeptide targeting cancer cells). Reproduced with permission from Ref. 138. Copyright © 2015 ACS publications. (D) Bacterial outer membrane vesicles are used as carriers for the co-delivery of Ce6 and catalases (OMV-aPDL1: PD-L1 antibody modified-attenuated Salmonella outer membrane vesicles). Reproduced with permission from Ref. 143. Copyright ©2022 DovePress. (E) A pH-responsive nanomedicine, comprising Ce6 core, pDNA-cat, and PEG shell, targets tumor cells and mediates effective PDT and antitumor immunity. Catalase expressed from pDNA-cat alleviates hypoxia and enhances ROS generation (pDNA-cat: plasmid expressing catalase). Reproduced with permission from Ref. 144. Copyright © 2022 Elsevier.

facilitate PDT treatment[136]. The diffusion distance of $^1O_2$ in cells is limited, and thus precise delivery of catalases to the treatment sites is helpful. The cancer cell targeting tripeptide Arg-Gly-Asp[137] (Fig. 4B) and cyclic pentapeptide Arg-Gly-Asp-D-Phe-Lys[138] (Fig. 4C) have been encapsulated with catalase proteins in nanocarriers to enhance the uptake and accumulation of catalases at tumor sites. TME responsive delivery vehicles have also been constructed to specifically transport catalases to tumors.

Nanomaterials, such as chitosan[139], $CaCO_3$[140], and metal−organic frameworks[141], that are responsive to low pH can disintegrate more rapidly in the acidic TME to release the encapsulated catalases to tumor cells. Catalase proteins coated by cancer cell membrane can be protected from attacks by immune cells and their circulation time in blood can be prolonged to ensure that catalases are effectively retained in tumors[142]. Bacterial outer membrane vesicles have also been explored as carriers for catalases due to the multiple bacterial components capable of modulating and stimulating tumor immune responses. When bacterial outer membrane vesicles were used to deliver catalases and photosensitizers, PDT was enhanced by the O_2 generated by catalases and synergized with immunotherapy induced by the components from bacteria[143] (Fig. 4D).

Despite all sorts of vehicles, the catalase proteins accumulated in tumors are much less compared to the total administered dose. Efforts have been undertaken to enhance the accumulation of catalases within tumors by delivering the catalase gene and subsequently expressing the protein *in situ* at specific tumor locations. A pH sensitive nanomedicine was constructed to internalize the photosensitizer Ce6 and plasmids hosting catalase gene into tumor cells[144] (Fig. 4E). The catalase gene expression was driven by a cytomegalovirus (CMV) promoter. Intratumoral injection of the nanomedicine to 4T1 tumor-bearing mice extended their survival to 46 days, compared with less than 31 days when not supplied with catalase plasmids. Delivery of catalase gene instead of purified proteins solved the problem of low catalase delivery efficiency.

Catalases require endogenous H_2O_2 as the substrate to generate O_2. As the reaction catalyzed by catalase will rapidly consume H_2O_2, gradually no more O_2 will be produced therefore the tumor hypoxia is only temporarily relieved. Other alternative strategies have been explored, for instance, the rate of O_2 uptake by tumor cells can be reduced by inhibiting the mitochondrial respiration[145,146], or photosynthetic microbes that are capable of producing O_2 can be used in combination with PDT reagents.

5. Bacteria in PDT

Bacteria including *Streptococcus*, *Salmonella*, *Escherichia*, *Clostridium*, *Bifidobacterium*, and *Lactococcus* have been explored for use in antitumor treatment[21,147]. Poor vascularization at tumor sites hinders the effective delivery of antitumor drugs and conventional nanomaterial drug carriers[148] Anaerobic or facultative anaerobic bacteria can migrate from the bloodstream to tumor areas due to their self-propulsion and preference for the hypoxic TME[149,150]. Bacteria administered intratumorally or intravenously accumulate substantially more in tumors than in other tissues[151]. This high targeting ability to tumor tissue makes bacteria promising delivery vehicles for antitumor reagents[152]. Xiao et al.[147] developed a biohybrid antitumor platform from the anaerobic bacterium *Bifidobacterium infantis* and DOX-loaded bovine serum albumin nanoparticles. The biohybrid exhibited a 4-fold higher cumulative concentration of DOX at the tumor site than in mice treated with drugs not carried by the bacteria. After treatment, the treated group exhibited significantly reduced tumor volume compared to the control groups, and the survival duration of the mice was notably extended (Fig. 5A). *Clostridium butyricum* has been investigated as an oncolytic bacterium for use in antitumor therapy. It has recently been engineered with an aggregation-induced emission photosensitizer (TPApy) to improve the melanoma treatment. TPApy was incorporated into the peptidoglycan of bacteria and released upon bacterial death. The engineered *C. butyricum* colonized and proliferated in the hypoxic TME of melanoma, ablating the tumor. Hypoxia was attenuated as the ablation continued, which killed the obligate anaerobe *C. butyricum*, leading to the release of photosensitizers and ensuing PDT. PDT effectively treated the marginal areas of the tumor and helped achieve complete ablation of malignant melanoma[153] (Fig. 5B).

Furthermore, apart from the tumor-targeting ability, the immunogenic nature of bacteria further synergizes to bolster the antitumor effects[154]. Components of bacteria, such as peptidoglycan and lipopolysaccharide, can be recognized by pattern recognition receptors expressed on dendritic cells, leading to the subsequent initiation of an immune response. For example, nanomedicines encapsulated within outer membrane vesicles derived from Gram-negative bacteria demonstrate enhanced immunostimulatory potential, leading to significant suppression of tumor growth and metastasis[155]. Synthetic biology tools can genetically modify bacteria to meet the needs of customized antitumor therapies. For instance, virulence factors of bacteria may cause damage and therefore need to be addressed carefully. Attenuated *E. coli* and *Salmonella* strains have been constructed by deleting the msbB virulence factor gene, which is responsible for the myristoylation of lipid A. When used for antitumor therapy, such genetically engineered strains are less virulent but retain the ability to colonize tumor sites[156]. Genetic tools can also empower bacteria with new functionalities that can be used to develop precision medicines. With delicate design, engineered bacteria are capable of biosynthesizing small molecules and proteins with therapeutic activity. Bacteria expressing cytokines and interferons, such as TNFα, IL-2, CCL21, and IFNs, have been constructed for antitumor therapy, demonstrating promising outcomes[157], and when combined with PDT can lead to synergistic results[158].

There have been several reviews discussing the application of bacteria in antitumor treatments. In the reviewed applications, bacteria are primarily used as drug carriers or therapeutic factories. For detailed reviews on this topic, see Refs. 149, 159 and 160. In this section, we discuss the potential of engineered bacteria as a solution to the limitations of PDT through the endogenous production of light, photosensitizers, and oxygen.

5.1. *Endogenous production of light by bacteria*

PDT activated by bioluminescence has demonstrated potency in treating deep tumors. Luciferase is a genetically encodable protein that can be heterologously expressed in bacteria. Yang et al.[161] recently transformed a plasmid containing the FLuc gene into an attenuated *S. typhimurium* to construct a bioluminescent strain Luc-S.T.$_{\Delta ppGpp}$ (Fig. 5C). Bioluminence was enriched in tumors after intratumoral administration of Luc-S.T.$_{\Delta ppGpp}$ along with luciferin. The bioluminescence generated at tumor sites can activate photosensitizer Ce6 to produce ROS, effectively inhibiting the growth of CT26 cancer cells in mice. The efficacy of PDT activated by bioluminescent Luc-S.T.$_{\Delta ppGpp}$ surpassed that of PDT activated by externally applied high-energy LED illumination. No evidence of tumor recurrence was observed within a 60-day period. The frequency of effector memory T cells in treated mice was twice as high as in healthy mice, indicating that the treatment induced a potent immune memory effect that could prevent tumor recurrence. Apart from FLuc, alternative

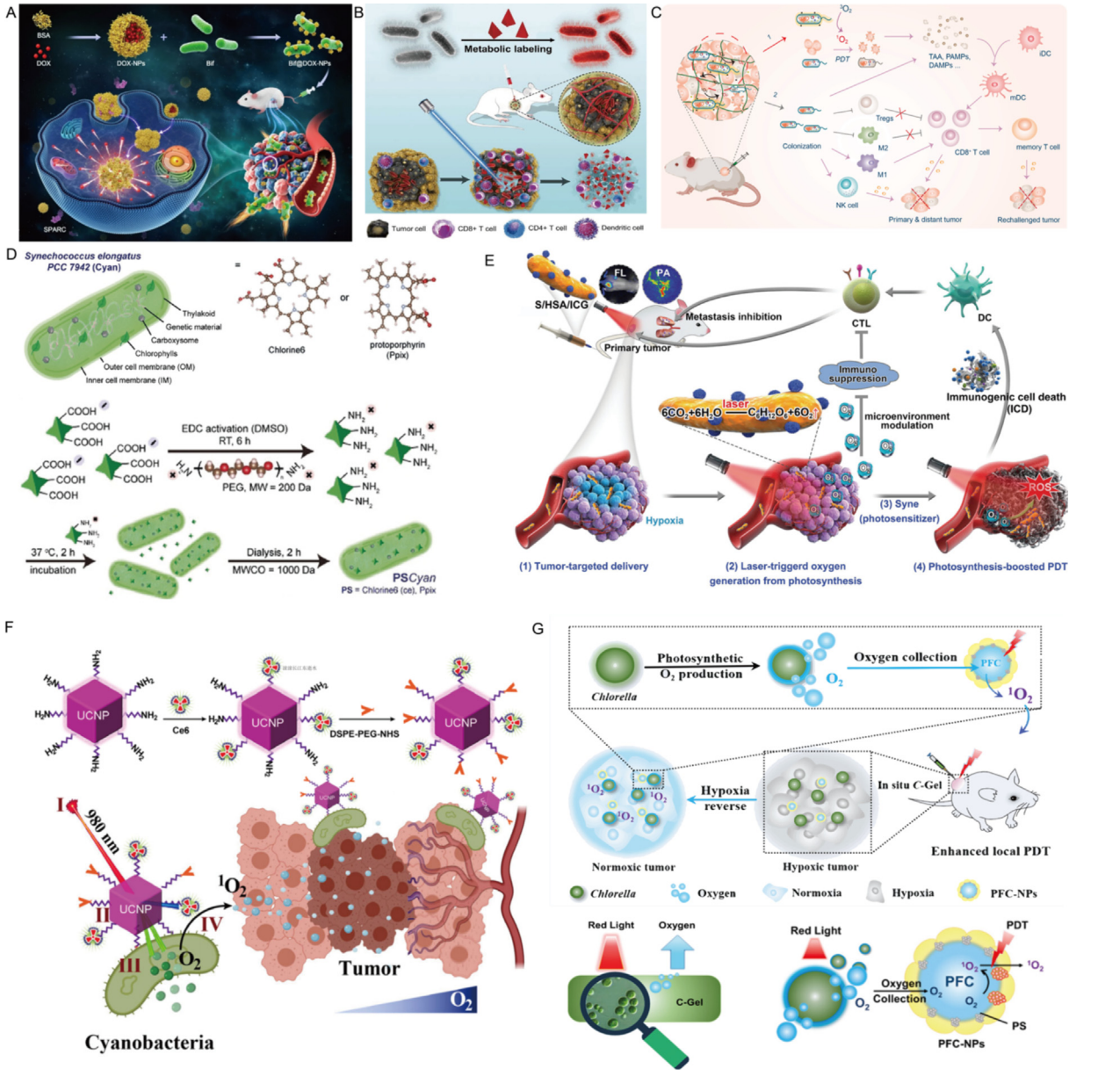

Figure 5 Examples of PDT using engineered bacteria and schematic representation endogenous production of PDT components by genetically modified bacteria. (A) *Bifidobacterium infantis* is employed as a carrier to transport DOX to breast tumors. *B. infantis* loaded with DOX containing nanoparticles targets the hypoxic regions of tumors and increases the drug accumulation, leading to a prolonged survival of tumor-bearing mice (Bif: *Bifidobacterium infantis*, DOX: doxorubicin). Reproduced with permission from Ref. 147. Copyright © 2022 Springer Nature. (B) *C. butyricum* is labeled with AIE photosensitizer modified D-Alanine. The labeled bacteria produce singlet oxygen to eliminate cancer cells under light irradiation (AIE: aggregation-induced emission). Reproduced with permission from Ref. 153. Copyright © 2022 Wiley. (C) *S. typhimurium* is transformed with a plasmid expressing FLuc, which emits bioluminescence in the presence of luciferin serving as the light source for PDT. The engineered bacteria additionally stimulate the immune response to enhance the effectiveness of the antitumor therapy (TAAs: tumor associated antigens, DAMPs/PAMPs: damage/pathogen associated molecular pattern). Reproduced with permission from Ref. 161. Copyright © 2022 Elsevier. (D) *S. elongatus* hybridized with photosensitizers Ce6 or PPIX exhibit enhanced PDT efficiency due to its capability to produce O_2 *in situ*. Reproduced with permission from Ref. 164. Copyright ©Copyright 2020 Wiley. (E) ICG-encapsulated nanoparticles are attached to *S. elongatus via* amide bonds. Photosynthesis-boosted PDT is achieved using this platform. Reproduced with permission from Ref. 166. Copyright © 2020 Wiley. (F) *S. elongatus* integrated with UCNPs can convert NIR light to visible light to induce photosynthesis in *S. elongatus* and to excite Ce6 for PDT. Reproduced with permission from Ref. 168. Copyright © 2021 Springer Nature. (G) Chlorella is co-delivered with perfluorocarbon nanoparticles loaded with Ce6. Upon exposure to laser light, the O_2 produced by Chlorella can be absorbed by the nanoparticles to enhance the antitumor PDT (C-Gel: Chlorella-gel, PFC: perfluorocarbon). Reproduced with permission from Ref. 170. Copyright © 2021 Elsevier.

Table 1 Recent examples of treatments involving biogenesis of PDT components.

Type	Photosensitizer source	Light source	Oxygen source	Cell type	Ref.
Biogenesis of photosensitizer	MiniSOG expressed from lentiviral vector in HeLa cells	External 465 nm laser	–	Cervical cancer (HeLa cells)	101
	MiniSOG expressed from lentiviral vector in SK-BR-3 cells	Bioluminescence of NanoLuc	–	Breast cancer (SK-BR-3 cells)	102
	MiniSOG expressed from lentiviral vector lentiviral in BT-474 cells	Bioluminescence of NanoLuc	–	Breast cancer (BT-474 cells)	103
	MiniSOG expressed from plasmid in *E. coli*	External 465 nm laser	–	Breast cancer (SK-BR-3)	96
	MiniSOG expressed from plasmid in *E. coli*	External 458 nm laser	–	Breast cancer (SK-BR-3 cells)	97
	KillerRed expressed from adenovirus vector in H1975 cells	External 561 nm laser	–	Lung cancer (H1975 cells)	98
	KillerRed expressed from adenovirus vector in H1299, HCT116, or HT29 cells	External green light	–	Lung cancer and colon cancer (H1299, HCT116, HT29 cells)	99
	KillerRed expressed from adenovirus vector in MCF7 cells	External 561 nm laser	–	Breast cancer (MCF7 cells)	100
	KillerRed expressed from plasmid in 4T1 cells	External 532 nm laser	–	Breast cancer (4T1 cells)	95
	KillerRed expressed from plasmid in *E. coli*	External 400–780 nm white light	–	Leukemia (K562, NB4, THP1 cells)	93
	KillerRed expressed from plasmid in *E. coli*	External 586 nm laser	–	Breast cancer (MDA-MB-231 cells)	94
	KillerRed expressed from plasmid in *E. coli*	External green light	–	Ovarian cancer (SKOV-3 cells)	81
	KillerRed protein delivered in DNA nanostructures	External 610 nm laser	–	Cervical cancer (HeLa cells)	108
Biogenesis of light	Hypericin	Firefly luciferase expressed from plasmid in HeLa cells	–	Cervical cancer (HeLa cells)	55
	Rose Bengal	Firefly luciferase expressed from plasmid in 3T3 cells	–	Embryonic fibroblast (3T3 cells)	52
	Chlorin e6	Firefly luciferase expressed from plasmid in *Salmonella typhimurium*	–	Colon cancer (CT26 cells)	161
	KillerRed	Renilla luciferase expressed from plasmid in *E. coli*	–	Breast cancer (MCF7 cells)	59
	Rose Bengal	Renilla luciferase expressed from plasmid in *E. coli*	–	Colon cancer (CT26 cells)	58
	MiniSOG	NanoLuc expressed from a plasmid in *E. coli*	–	Breast cancer (SK-BR-3 cells)	62–64
Biogenesis of O_2	Chlorin e6	External 660 nm laser	Photosynthetic Cyanobacteria	Breast cancer (4T1 cells)	164
	Rose Bengal	External 640 nm laser	Photosynthetic *Synechococcus elongatus*	Breast cancer (4T1 cells)	165
	Chlorophyll	External 650 nm laser	Photosynthetic Spirulina platensis	Breast cancer (4T1 cells)	169
	Chlorin e6	External 660 nm laser	Photosynthetic Chlorella	Colon cancer (CT26 cells)	170

luciferases, namely RLuc and NLuc, also hold potential for expression in genetically engineered bacteria.

5.2. *Endogenous production of photosensitizers by bacteria*

Endogenously biosynthesized photosensitizers can overcome the shortcomings in applications of exogenous chemically synthesized photosensitizers, such as low uptake efficiency and off-target toxicity. Genetically engineered bacteria expressing photosensitizers can swim to tumors and accumulate photosensitizers there. Such bacteria are similar to weapons that can generate explosives automatically and strike targets precisely. Yan et al.[162] constructed an engineered bacterium (KR-*E. coli*) by expressing photosensitizer KillerRed in *E. coli*. KR-*E. coli* injected intratumorally or intravenously targeted and proliferated at tumor sites. Within one day, the fluorescence from KillerRed covered the entire tumor, demonstrating that KillerRed was abundantly expressed in the tumor. Upon light irradiation, the KR-*E. coli*-treated mice underwent efficient PDT, leading to the complete disappearance of tumor within a few days. No tumor recurrence was observed within 2 months. In addition to protein photosensitizers, small-molecule photosensitizers widely used in PDT, such as hypericin and riboflavin, are natural products that can be biosynthesized. Recently, studies have demonstrated the production of hypericin in repurposed *Cladosporium fulvum*. The gene *claM* was deleted to accumulate emodin, which is the precursor for hypericin biosynthesis. Heterologous expression of RugG, a substrate-promiscuous fungal P450 monooxygenase, in the mutant strain led to the production of emodin bianthrone derivatives, which can be transformed to hypericin *via* subsequent nonenzymatic reactions. The engineered *C. fulvum* strain was capable of biosynthesizing hypericin with a titer of 43.1 mg/L[163]. We envision that a similar genetic circuit can be constructed in bacteria to produce hypericin inside the tumor for PDT.

5.3. *Endogenous production of oxygen by bacteria*

Cyanobacteria are prokaryotes that can perform photosynthesis to generate O_2. Application of *Synechococcus elongatus* PCC 7942 (*S. elongatus*) in PDT with photosensitizer Ce6[164] (Fig. 5D) and RB[165] demonstrated enhanced production of $^1O_2$ and improved treatment outcomes. *S. elongatus* has also been combined with nanosized photosensitizers for antitumor PDT[17]. For example, *S. elongatus* was engineered by attaching ICG-encapsulated nanoparticles to its surface *via* amide bonds[166] (Fig. 5E). Following the intravenous administration of the engineered bacteria, mice were subjected to illumination using a 660 nm laser to facilitate photosynthesis, and an 808 nm laser to excite ICG. *S. elongatus* produced O_2 under 660 nm irradiation, resulting in the production of more ROS, which completely eliminated the primary tumor. This photosynthesis-boosted PDT not only effectively reverses the hypoxic TME but also strongly triggers a systemic antitumor immune response that prevents tumor relapse and suppresses metastatic progression in murine models of metastatic triple-negative breast cancer. By integrating *S. elongatus* with UCNPs, the biohybrid can harvest energy from NIR light[167]. Under NIR irradiation, UCNPs converted NIR light to visible light to induce photosynthesis in *S. elongatus* and to excite Ce6 for PDT[168] (Fig. 5F). *Arthrospira platensis* (also named as *Spirulina platensis*) is another cyanobacterium that is biocompatible, safe, and is commercially available as a nutritional supplement. Biohybrids of *A. plantensis* were constructed by modifying the bacterial cells with magnetic Fe_3O_4 nanoparticles[169]. The biohybrids could be guided by magnetic field to target tumors precisely and generated

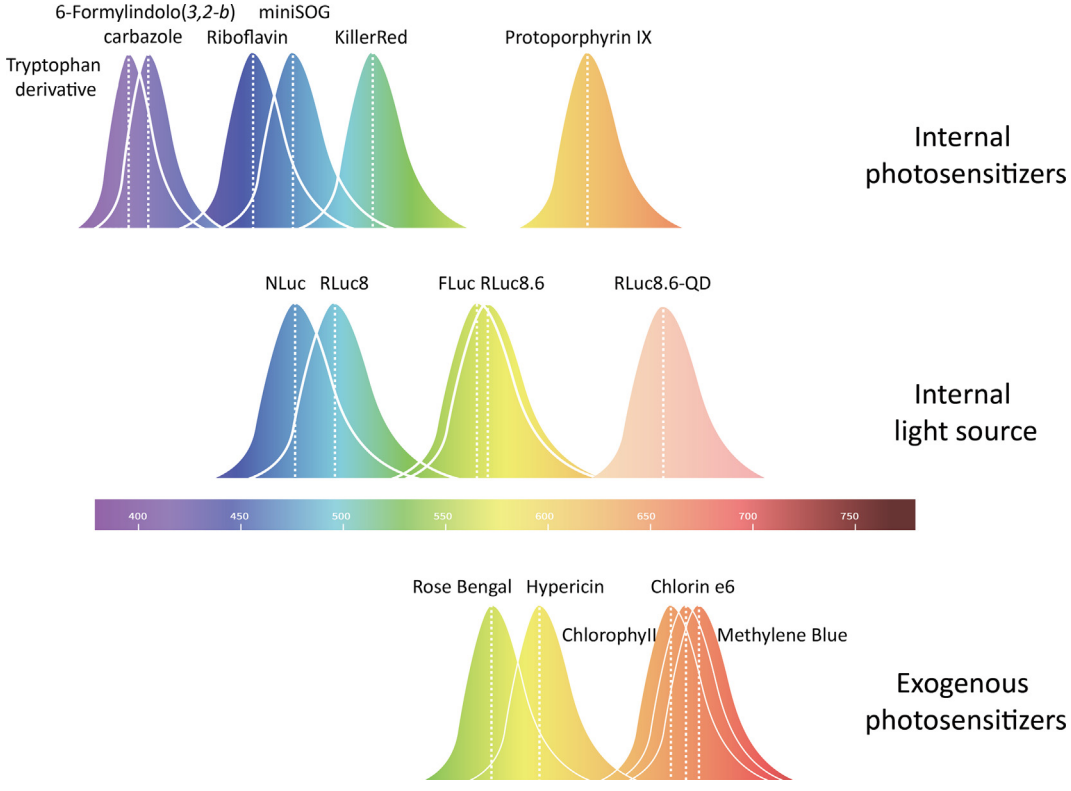

Figure 6 Schematic diagram showing the excitation spectra of photosensitizers and emission spectra of bioluminescence.

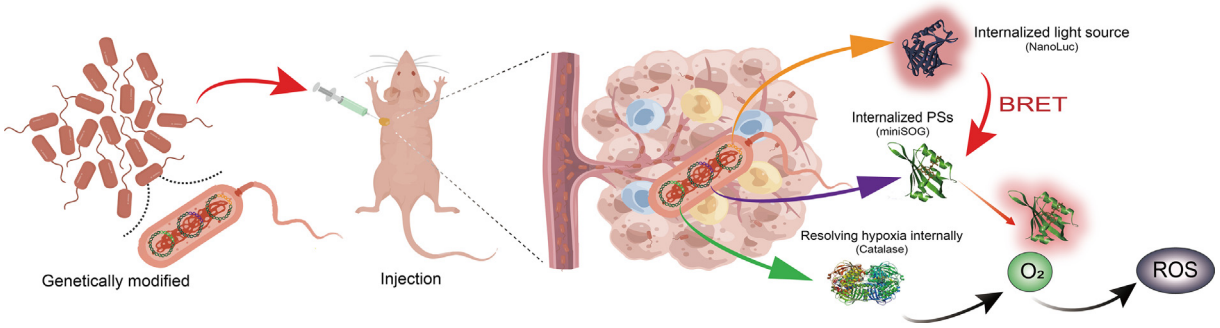

Figure 7 Schematic representation of the PDT treatment process mediated by the light, photosensitizer and oxygen simultaneously generated by genetically engineered bacteria.

O_2 *in situ* to boost the antitumor PDT effects. No long-term toxicity was observed after the treatment using *A. plantensis* biohybrids. In addition to cyanobacteria, Chlorella is a genus of single-celled algae that has been developed as O_2 suppliers for PDT. Chlorella has a size of 2−10 μm and is capable of photosynthesis. Wang et al.[170] established a sustainable PDT platform using Chlorella (the species was not disclosed in the paper) and perfluorocarbon nanoparticles (Fig. 5G). Alginate-encapsulated Chlorella cells were injected into mice harboring CT26 colon cancer in order to generate O_2 in response to 660 nm light exposure. Perfluorocarbon nanoparticles that can absorb O_2 were used to load and deliver Ce6. With the photosynthesis of Chlorella, the killing rate of tumor cells was enhanced by approximately 63%. Besides O_2 producers, Chlorella cells also simulated antitumor immune responses by promoting dendritic cell activation.

In addition to photosynthetic bacteria, other bacteria can be genetically modified to acquire the capability to produce O_2 in tumors. As discussed in section 4.2, the catalase enzyme can breakdown endogenous H_2O_2 at tumor sites to produce O_2. Catalase enzymes can be hydrolyzed and cleared by human proteases during delivery, thereby requiring the use of nanocarriers for additional protection. *In situ* production of catalase enzymes at tumor sites by genetically engineered bacteria helps to solve the stability issue of enzymes during transport. *E. coli* was engineered by transforming a plasmid containing the catalase gene and further loaded with polydopamine-encapsulating Ce6. Under irradiation, Ce6 was excited to produce ROS, and the PDT performance was enhanced by the O_2 produced by the catalase enzyme expressed in *E. coli*. The system effectively treated malignant tumors and exhibited no significant biotoxicity[171]. In another similar system, Ce6 was replaced by black phosphorous-derived photosensitizing agents. The catalase enzyme expressed by *E. coli* enhanced the antitumor PDT mediated by black phosphorous as well[172]. In these examples, catalase production was achieved *via* a plasmid-based expression system. The plasmid relied on antibiotic selection to persist within bacterial cells, with the potential for loss over time in the absence of antibiotics. To ensure a sustainable and enduring therapeutic approach, it will be essential to integrate the gene cassette expressing and regulating catalase into the bacterial genome.

6. Conclusions and outlook

PDT is an extensively investigated non-invasive therapy for cancer, involving tripartite fundamental components: excitation light, photosensitizers, and oxygen. Photosensitizers necessitate excitation through light of a specific wavelength and subsequent interaction with O_2 to produce ROS. PDT has been a clinically approved treatment option for over three decades, first gaining approval for use in treating bladder cancer. Since then, PDT has demonstrated its efficacy in treating diverse cancers such as breast, head and neck, pancreatic, cervical, and brain cancers. PDT's foremost advantage lies in its non-invasiveness, setting it apart from conventional tumor treatments. For instance, sunlight-based PDT has been approved as an actinic keratosis treatment in Europe. Furthermore, PDT can complement existing therapies, enhancing their effectiveness. PDT has been approved for advanced head and neck squamous cell carcinoma patients when other treatment options are not feasible. Despite its success in numerous clinical trials, PDT has yet to realize its full potential in current clinical practice and is not regarded as the primary standard-of-care treatment for most cancer types. Effective implementation of antitumor PDT encounters diverse obstacles, such as penetration limitations of external light sources, low delivery efficiency and off-target effects of photosensitizers, poor tumor distribution of photosensitizers, and lack of O_2 in the hypoxic TME. These obstacles collectively lead to a prominent issue faced by PDT in clinical practice: PDT alone often yields only partial and incomplete tumor destruction, resulting in tumor regrowth and progression. Overcoming this challenge necessitates a multifaceted approach involving the resolution of the aforementioned obstacles and the integration of PDT with complementary treatment modalities.

These obstacles have been the main focus of studies aimed at enhancing the effectiveness of antitumor PDT. For example, X-ray has been used as the excitation light for PDT, which has better tissue penetration but is more restrictive in terms of dose usage. Upconversion nanoparticles can convert external NIR light to localized UV−Vis light for excitation of photosensitizers, but synthesizing them on a large scale is generally challenging and expensive. For the improvement of photosensitizers, different generations of photosensitizers have been developed to enhance their toxicity and solubility profiles. Inorganic and organic nanomaterial delivery vehicles have also been constructed to deliver more photosensitizers to tumors in a targeted manner. Aggregation-induced emission (AIE) photosensitizers represent a class of chemical compounds that have higher photostability and can overcome the aggregation-induced quenching commonly encountered by conventional photosensitizers. Notably, AIE photosensitizers with Type I PDT mechanism have been developed, exhibiting enhanced efficiency within hypoxic tumors[173]. In

order to mitigate hypoxia within tumors, a commonly employed approach entails the administration of catalase enzymes to tumor locations to facilitate the breakdown of endogenous H_2O_2 into O_2. Nanoparticles with catalase-like activity, including gold nanoclusters and MnO_2 nanoparticles, can generate O_2 *in situ* to improve PDT performance.

These approaches have shown promising outcomes, however, the three components for PDT still need to be delivered externally, which discourages sustainable treatment and often requires multiple doses to improve efficacy. In this review, we examine the advancements made in an alternative strategy involving the internal production of light, photosensitizers, and oxygen (Table 1). Our emphasis lies in using bioluminescence as the excitation light source, employing encodable proteins and small molecules as photosensitizers, and using enzymes or photosynthetic microbes as sources of oxygen. Bioluminescence can act as an endogenous light source that does not require external light and thus resolves the problem of light transmission. The most commonly used bioluminescence in PDT is produced by firefly luciferases. The luciferase gene was delivered to tumor cells in one shot, and the protein could be expressed thereafter under the control of promoters for ongoing treatment. Similarly, tumor cells were transfected with the gene encoding the catalase enzyme, resulting in the expression of the catalase enzyme that produced O_2 endogenously and continuously. Genetically encodable photosensitizers such as KillerRed can be expressed endogenously at tumor sites as well. For example, a plasmid carrying the KillerRed gene was transfected into HEK293 cells with high efficiency. KillerRed protein was expressed and produced ROS under green light, inducing the apoptosis of tumor cells[104]. This approach avoids problems associated with photosensitizer delivery efficiency and enables continuous *in situ* production of photosensitizers, thus not requiring repeated drug administration. The excitation spectra of common endogenous photosensitizers and emission spectra of bioluminescence are summarized in Fig. 6.

Bacteria have been extensively explored in antitumor therapy owing to their capacity for targeting tumors and eliciting immune responses. Using synthetic biology tools, bacteria can be further engineered to endogenously produce bioluminescence, photosensitizers, and oxygen inside tumors. Bacteria transformed with a plasmid carrying the FLuc gene were successfully applied to *in vivo* antitumor treatment. Bioluminescence was generated in the presence of luciferin to activate photosensitizers. Such a system requires the luciferase substrates to be supplied externally. Some luciferase substrates have solubility and off-target effect issues, and require multiple doses to enhance therapeutic efficacy. Subsequent efforts can be directed towards the advancement of self-sufficient PDT systems by genetically encoding both luciferase and luciferin, thereby negating the need for exogenous substrates.

Attempts have been performed to heterologously express protein photosensitizers in bacteria for antitumor PDT. Engineered bacteria expressing KillerRed enhanced the accumulation of photosensitizers in tumors and eliminated tumor cells *in vivo*. Further investigation is needed to explore the excretion and localization of photosensitizers produced by bacteria within tumor sites. Tagging of localization peptide sequences to such protein photosensitizers can help to better target them to tumor cells. The toxicity of endogenous photosensitizers within bacterial cells also needs to be carefully monitored to prevent premature death of bacterial cells prior to treatment. There have been no studies on the employment of engineered bacteria producing small molecule photosensitizers in antitumor PDT. With advances in synthetic biology, heterologous expression of natural products in bacteria is feasible. Small-molecule photosensitizers such as hypericin have been expressed in engineered microbes. In the future, the use of bacteria to produce small-molecule photosensitizers *in situ* will provide an alternative strategy for the endogenous production of protein photosensitizers.

Endogenous production of light, photosensitizers, and oxygen is a promising strategy to enhance antitumor PDT. As a widely studied antitumor drug delivery vehicle, bacteria can undergo genetic engineering to form an integrated platform for the internal production of the three components of PDT at tumor sites (Fig. 7). This would eliminate the need for exogenous light and ensure a continuous supply of photosensitizers and oxygen during PDT. The rich synthetic biology toolbox offers a wealth of genetic parts and devices to support the design and construction of such bacteria. To maximize the generation of ROS, careful programming of the production and distribution of the three components is crucial. Nonetheless, issues such as engineered bacteria−host interactions, as well as biosafety risks, must be prudently considered. Despite these challenges, the use of engineered bacteria holds great promise for overcoming current limitations of antitumor PDT.

In summary, endogenous production of light, photosensitizers, and oxygen is a promising strategy for overcoming the obstacles in Type II PDT. With the continuous progress of synthetic biology, engineered bacteria emerge as valuable allies in realizing this objective.

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Author contributions

Conceptualization, Yihan Wu; Data curation, Lin Yu, Zhen Liu; Project administration, Jinliang Liu, Xiaohui Zhu; Validation, Wei Xu, Kai Jin; Writing − original draft, Lin Yu, Zhen Liu; Writing − review & editing, Kai Jin, Lin Yu, Xiaohui Zhu, Yihan Wu, Yong Zhang. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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