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Recent advances in utilization of photochemical internalization (PCI) for efficient nano carrier mediated drug delivery

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Abstract. Despite recent progresses in nanoparticle-based drug delivery systems, there are still many unsolved limitations. Most of all, a major obstacle in current nanoparticle-based drug carrier is the lack of sufficient drug delivery into target cells due to various biological barriers, such as: extracellular matrix, endolysosomal barrier, and drug-resistance associated proteins. To circumvent these limitations, several research groups have utilized photochemical internalization (PCI), an extension of photodynamic therapy (PDT), in design of innovative and efficient nano-carriers drug delivery. This review presents an overview of a recent research on utilization of PCI in various fields including: anti-cancer therapy, protein delivery, and tissue engineering.

Keywords: photochemical internalization (PCI); photosensitizer; drug delivery system (DDS); nanoparticles; biological barriers

1. Introduction

Nanoparticle-based drug delivery system has emerged as a promising approach to improve therapeutic efficacy with minimal side effects (MuÈller *et al*. 2000, Probst *et al*. 2013). The first nanoparticle-based drug delivery system was lipid vesicles (now called liposome), which were described in the 1960s (Bangham *et al*. 1965, Farokhzad and Langer 2009). So far, there are over two dozen nanoparticle-based therapeutic products approved by Food and Drug Administration (FDA) for clinical use (Wagner *et al*. 2006, Zhang *et al*. 2007, Farokhzad and Langer 2009). Nevertheless, there are still many limitations to be solved, such as: low stability in physiological conditions, rapid release of cargo drugs, inherent toxicity of nanomaterials, and limited target specificity. Among them, a major obstacle in current nanoparticle-based drug carriers is the lack of sufficient drug delivery into target cells due to various biological barriers (e.g., extracellular matrix, endolysosomal barrier, and drug-resistance associated proteins). Low selectivity of drug-carriers is often compensated with dose-increasing. Thus repeated administrations of high drug

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doses are required, which could ultimately lead to drug resistance (Selbo *et al*. 2010).

To overcome the limitation, several research groups have utilized photochemical internalization (PCI), an innovative approach which stems from photodynamic therapy (PDT), in design of efficient drug delivering nano carriers. (Nishiyama *et al*. 2005, Park and Na 2012, Park *et al*. 2014, Yen *et al*. 2014). The PCI wasrecently developed at the Norwegian Radium Hospital as a method for performing light-induced cytosolic release of membrane-impermeable molecular therapeutics, entrapped in endocytic vesicles (Berg *et al*. 1999, Hogset *et al*. 2004, Selbo *et al*. 2010). In theological mechanism of PCI, light-activation of the photosensitizer results in reactive oxygen species (ROS)-mediated membrane of the cellular membrane or endolysosome vesicles, subsequently releasing drugs or genes into the cytosol (Selbo *et al*. 2010). In this way, combination of PCI with endosomally internalized nano-carriers that specifically release their cargo drugs in response to cytosolic conditions could be an effective approach for improving spatiotemporal control of their functions (Yen *et al*. 2014). In this review, we provide an overview of recent application of the PCI technique in nanoparticulate designs for efficient drug delivery.

2. Utilization of PCI in anticancer drug delivery

2.1 Design for overcoming endolysosomal barrier

Although nanoparticles-based anti-cancer drug delivery systems have shown promising results, they have some limitations against various biological barriers such as: skin, mucus, bloods, extracellular matrix and subcellular barriers (Lee and Na 2014, Park and Na 2015). Among them, the endolysosomal barrier is one of particularly important barriers in the delivery of chemotherapeutic agents by using nanoparticles. Endosomes are vesicles with an internal pH of approximately 5 and mature in a unidirectional manner from early to late lysosomes, before fusing with digestive enzyme containing lysosomes. The Since the endocytosis pathway is carried out by endosomes(Gruenberg and Van der Goot 2006, Varkouhi *et al*. 2011), anti-cancer drugs trapped in endosomes are typically trafficked into lysosomes and degraded (Whitehead *et al*. 2009). In this regard, various strategies based on functionalization of polymers and nanoparticles have been exploited to overcome the endolysosomal barrier (Funhoff *et al*. 2004, Kang and Bae 2007). Recently, Lee *et al*. reported a photochemically triggered cytosolic drug delivery system based on combining tumor-targetable pH-responsive hyaluronic acid (HA) nanoparticles (PHANs) with anticancer therapeutics (doxorubicin; DOX) (Lee and Na 2014). In design of this system, tumoral-receptor (CD44) targetable hyaluronic acid backbone was conjugated with a tertiary amine group containing polypeptidic pH-responsive moiety and photochemical agent, chlorin e6 (Ce6) (Fig. 1(a)). These PHANs encapsulated the anticancer drug doxorubicin (DOX@PHANs) by self-assembly and then internalized into cancer cells through CD44-receptor mediated endocytosis. Finally, the DOX@PHAN disruption by protonation of diisopropylethylamine (DIP) groups in acidic endolysosomes (~pH 5.0) and low-intensity laser irradiation (compared with laser intensity of PDT applications) stimulated the free photosensitizer to produce reactive singlet oxygen, which released DOX from endolysosomes into the cytosol of cancer cells by photochemical disruption of the endolysosomal membrane (Fig. 1(b)). Conclusively, in vitro cell and in vivo animal studies demonstrated that the treatment of DOX@PHANs with laser irradiation had powerful therapeutic efficacy regardless of laser dose (Fig. 1(c) and (d)). This promising platform may be useful vectors for overcoming biological barriers in cytosolic drug delivery applications.

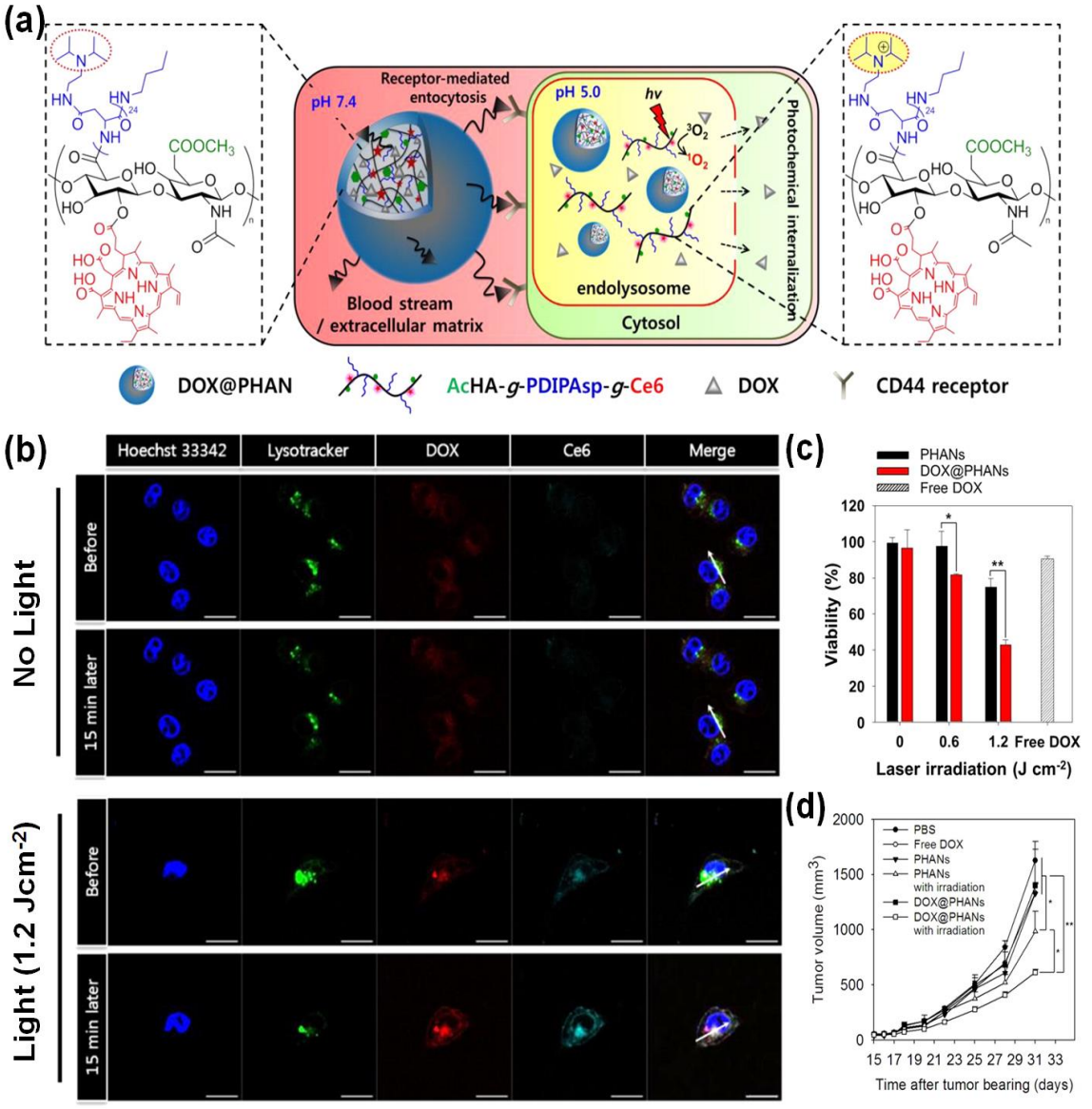

Fig. 1 PCI-mediated targeted cytosolic delivery of anti-cancer drugs. (a) Schematic representation of doxorubicin-loaded pH-responsive hyaluronic acid nanoparticles (DOX@PHANs). (b) Photochemically triggered endolysosomal escape behavior. Photographs captured by confocal microscopy with live cell imaging system of DOX@PHANs-treated HCT-116 cells with or without laser irradiation. In each panel, Lysotracker (endolysosome) is green, the cell nuclei stained with Hoechst 33342 are blue, DOX is red, and Ce6 is cyan (scale bar: 20 μm). (c) In *vitro* cytotoxicity assays in HCT-116 cells treated with free DOX, PHANs, and DOX@PHANs (DOX and Ce6, 0.1 μg mL^{-1} each), with or without laser irradiation (n=3, *P<0.05, **P<0.005). (d) In vivo tumor therapy of a subcutaneous tumor model injected systemically with various treatments (dose: 2.0 mg DOX and 2.0 mg Ce6 per kg body weight, laser power: 100 J cm^{-2}, n=5, *P<0.05, **P<0.005). (Reprinted with permission from Ref. Lee and Na (2014). Copyright 2014 American Chemical Society)

2.2 Design for overcoming multi-drug resistance in cancer

Drug resistance remains one of the primary obstacles to effective cancer chemotherapy and is responsible for the high recurrence rate and treatment failure in more than 90% of patients with metastatic cancer (Lage 2008, He *et al*. 2011). P-glycoprotein (P-gp)-mediated resistance has been described as a key contributing factor underlying multidrug resistance (MDR) (Ling 1992, Meng *et al*. 2010). The overcoming of the MDR in cancer has continuously been a hot topic in anti-cancer therapy. Although many strategies have been investigated, including the use of excipients that reduce the function of ABC transporters, or chemical P-gp inhibitors (Bansal *et al*. 2009, Kievit and Zhang 2011, Park *et al*. 2014), they accompanied high risk because systemic inhibition of P-gp may lead to severe adverse effects. This is because P-gp has an important physiological function of protecting many types of normal tissues such as brain, mammary gland, testis, and papillary dermis (Cordon-Cardo *et al*. 1989, Robey *et al*. 2009). In this regard, localized P-gp inhibition methodology based on PCI has been investigated as a new strategy to improve the therapeutic efficacy against drug-resistant cancers (Gillmeister *et al*. 2011, Selbo *et al*. 2012). The mechanism of PCI is based on cellular membrane disintegration by (ROS)-induced lipid peroxidation of laser exposed cells. (Berg *et al*. 1999, Nishiyama *et al*. 2005, Selbo *et al*. 2012). Therefore, the PCI-incorporated anti-cancer drug delivery systems could be expected to enhance significantly therapeutic effects in drug-resistant cancer cells and effectively reduce systemic toxicity. Very recently, Park *et al*. reported singlet-oxygen producible polymeric (SOPP) micelles based on photosensitizer conjugated amphiphilic copolymers to circumvent drug resistance in cancer, by applying PCI (Park *et al*. 2014). The photosensitizer conjugated amphiphilic copolymer was synthesized from facile conjugation of chlorin e6 (Ce6) and pluronic F127 (PF127) (Fig. 2(a)). The SOPP micelles were prepared through the self-assembly of Ce6-PF127 conjugates in an aqueous solution (Fig. 2(b)). Upon irradiation of laser in a particular section of the cancer, the photosensitizer moiety caused singlet-oxygen-mediated cellular membrane damage (Fig. 2(c)), which exhibited significant enhancement of chemotherapeutic efficiency in P-gp expressing drug-resistant cancer cells (HCT-8) due to enhancedcell membrane permeability and accelerated accumulation of the anti-cancer drug via singlet-oxygen-mediated cellular membrane damage (Fig. 2(d)). These properties of SOPP micelles indicate that ROS producible nanocarriers can be applied to the design of advanced drug delivery system to overcome drug resistance in cancer.

3. Utilization of PCI in gene delivery

Gene therapy has great promises as a therapeutic approach for serious acquired and inherited diseases such as cancer, Parkinson's disease, cardiovascular diseases and genetic disorders (Evans and Robbins 1995, Dachs *et al*. 1996). The delivery of therapeutic DNA can be accomplished using a variety of vectors, including viral-mediated vectors and synthetic vectors (Davis 2002, Zhang and Godbey 2006). Cationic polymers, such as poly-l-lysine, polyethylenimine (PEI) and polyamidoamine, are major types of synthetic vectors, and they have been extensively studied due to their ability to condense DNA into a nano-sized polyplex, which can protect them from extracellular enzymatic degradation and facilitate interaction with the extra-cellular membrane (Godbey *et al*. 1999, Kwoh *et al*. 1999, Lemkine and Demeneix 2001, Zhong *et al*. 2007). However, low gene transfection efficiency is a major problem of conventional polycationic-based gene delivery systems, mainly because of insufficient in vivo stability, low targeting efficacy, and

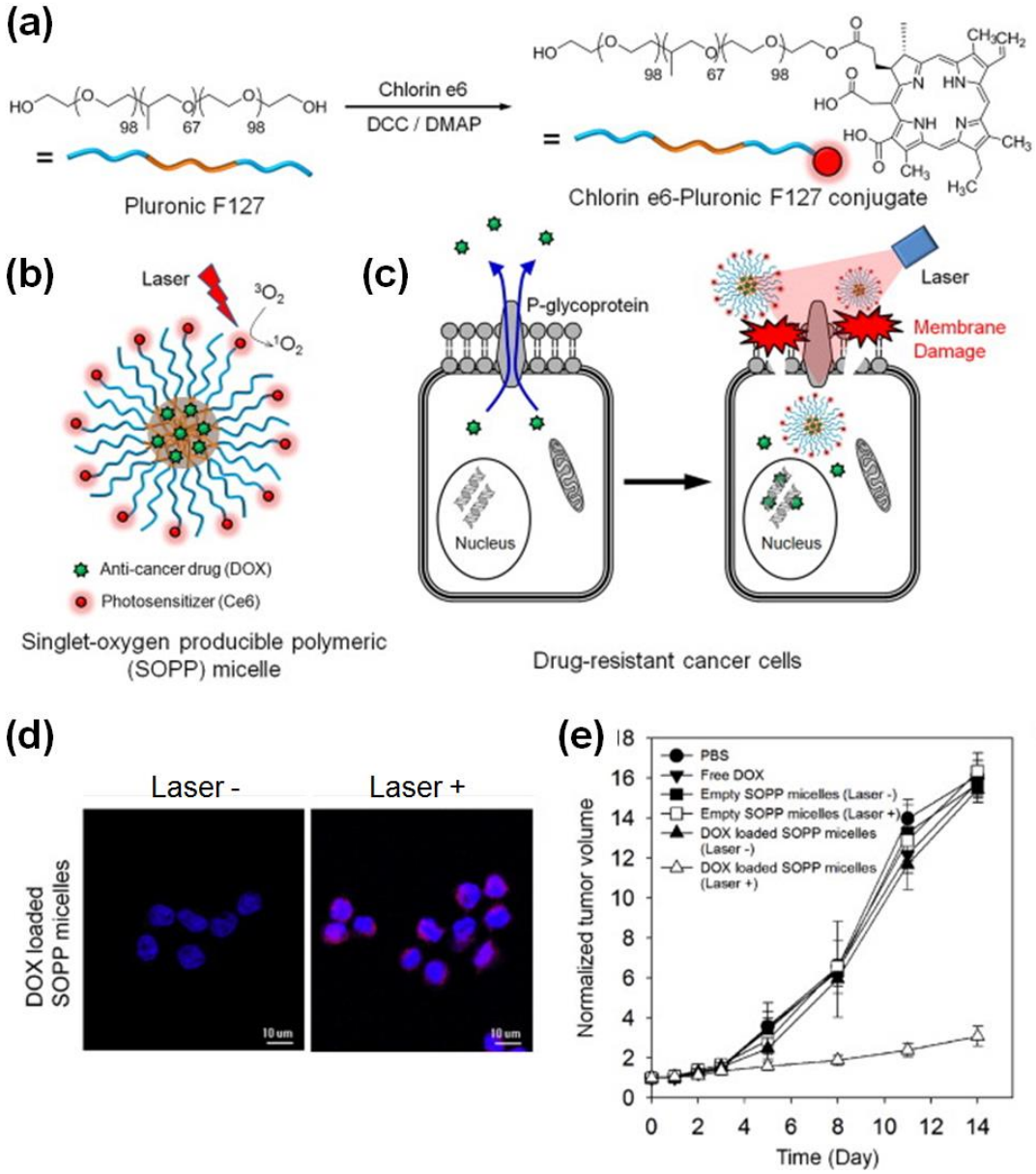

Fig. 2 Singlet-oxygen producible polymeric (SOPP) micelles for overcoming drug-resistance in cancer. (a) Synthetic route to PS conjugated amphiphilic copolymer (Ce6-PF127 conjugate), (b) Schematic illustration of formation of the SOPP micelle and their singlet-oxygen generation upon laser irradiation, (c) Schematic illustration of strategy for overcoming drug-resistance in cancer cells via singlet-oxygen-mediated cellular membrane damage, (d) CLSM image of HCT-8 cells treated with DOX-loaded SOPP micelles with or without laser irradiation (DOX and nucleus were appeared as red and blue color, respectively), and (e) In vivo anti-tumor efficacy of DOX-loaded SOPP micelles in drug-resistant tumor bearing mice after intravenous injection of different formulations (dose of DOX: 2 mg/kg and/or dose of Ce6: 0.75 mg/kg) involving DOX-loaded SOPP micelles with or without laser irradiation with a 670 nm fiber coupled laser system (laser power: 100 J/cm^2, 150 mW/cm^2 for 11 min, n=4) (Reprinted with permission from Ref. Park, Park *et al*. (2014). Copyright 2014 Elsevier B.V.)

endosomal escape. Especially, degradation of DNA under harsh acidic pH conditions in the endo/lysosomal vesicle is a major hindrance for efficient gene delivery. PCI has been studied to improve intracellular delivery of DNA using amphiphilic photosensitizers, such as TPPS4, PTTS2a, and AlPcS2a, dendrimers and multifunctional photosensitizers. Hogset *et al*. have explored the potential use of amphiphilic photosensitizers (AlPcS2a) to deliver exogeneous genetic material into the cytosol (Hogset *et al*. 2000). To demonstrate that photochemical treatment can be used to enhance the efficiency of gene transfection in a light-dependent manner, the THX cell and human melanoma cell line were photo-chemically transfected with plasmid encoding green fluorescence protein (GFP)/poly(L-lysine) complexes and AlPcS2a, before undergoing light irradiation. After PCI treatment, a specific induction of GFP expression was found in THX cells, as expected, and after 5 min of light treatment, GFP expression levels increased from 2.5% to 65% compared with nonirradiated cells, representing a more than 25-fold potentiation of transfection after the PCI treatment.

Although the PCI using amphiphilic photosensitizer accomplished enhanced transfection by irradiation, delivery vectors and photosensitizers have to be administered simultaneously as a single component in to target cells for systemic PCI mediated gene delivery. Nishiyama et al. designed a PCI mediated gene transfection method using a polyion complex (PIC) micelles, which is a combination of the dendrimer phthalocyanine- poly (ethylene glycol)-blockpoly (L-lysine) (DPs-PEG-PLL) micelles and pDNA-PEG-PLL micelles (Nishiyama *et al*. 2006). In their research, they developed DPs as a new type of photosensitizer, which possess a center phthalocyanine molecule surrounded by a second generation of aryl ether dendrons with 32 carboxyl groups and have a high efficacy of singlet oxygen production. The DPs allowed the formation of PIC micelles through an electrostatic interaction with the PEG-PLL block copolymers. When the PCI-mediated enhancement of the transfection was observed in HeLa cells,the result was the PCI system using PIC micelles achieving 100-fold photochemical enhancement of the transgene expression.

More recently, Park *et al*. reported a photo-activatable ternary complex (PTC, Fig. 3(a)) which has multi-functionality for targeted photo-mediated shRNA delivery (Park *et al*. 2013). The PCI system using PTC was designed to protect nucleic acid from degradation under physiological conditions, tumor-specific targeting, and rupturing of the endosomes. This PTC consists of a polyethyleneimine/EGFR shRNA complex core and multifunctional shielding materials (MSM), which is a photosensitizer-conjugated chondroitin sulfate. The PCI-mediated gene transfer with the PTC resulted in a significant EGFR mRNA silencing efficacy (≈80% knockdown), in *vitro*, due to the endosomal rupturing ability of photosensitizer (Fig. 3(b)). With this great endosomal disruption capability, PTCs showed increased intracellular uptake due to the CD44 receptors expressed on tumor cells and exhibited serum stability due to the ligand receptor interaction between chondroitin sulfate and hyper-structural shielding of the negative-charged MSM. From these results, it is hypothesized that the PCI using PTCs could successfully control the gene expression in vivo. To demonstrate the potential of the PTC for in vivo PCI-mediated gene transfer, the PTCs incorporating EGFR shRNA were injected to Balb/C mice, followed by laser irradiation. As expected, the PTCs incorporating EGFR shRNA and laser treated mice showed significantly reduced tumor volumes (Fig. 3(c), (d)). In contrast, only PTCs incorporating EGFR shRNA, PTCs incorporating scramble shRNA, and the laser treated group showed marginal tumor growth inhibition.

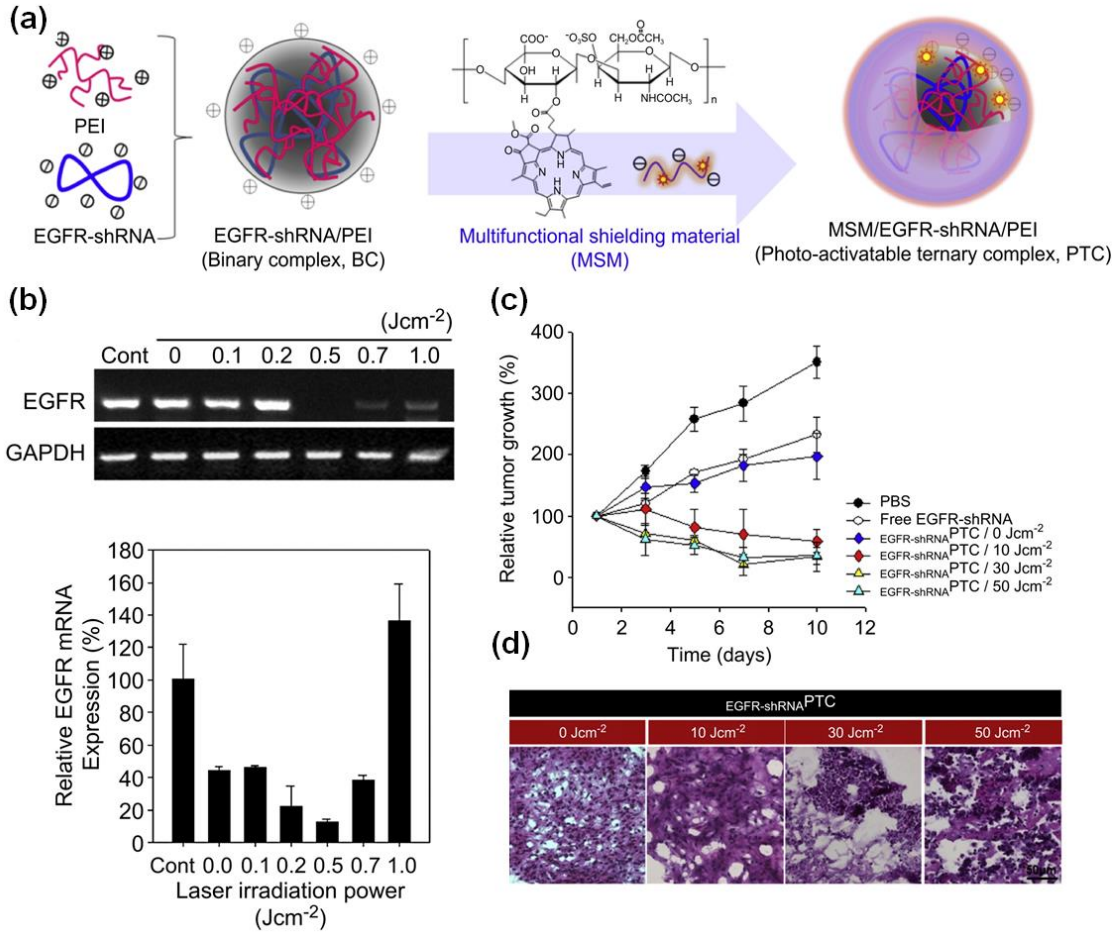

Fig. 3 Photo-activatable ternary complex (PTC) for efficient gene delivery. (a) Chemical structure of the multifunctional shielding material (MSM) composed of a photosensitizer (PS) and acetylated chondroitin sulfate (CS) and a schematic view of the photo-activatable ternary complexes (PTCs). (b) In vitro EGFR gene-silencing effect of PCI treatment using PTCs. The EGFR mRNA levels were measure RT-PCR and real-time PCR. (c) The tumor growth inhibition by PCI treatment with TPCs. The HCT116 cells were injected subcutaneously into BALB/c mice. Mice with established tumors were treated by intratumoral injections of PTC incorporating EGFR shRNA with 0-50 J/cm^2 of laser irradiation. (d) Frozen sections of the tumor tissues isolated from the mice after the PCI treatments were stained with hematoxylin and eosin (H&E). (Reprinted with permission from Ref. Park *et al*. (2013). Copyright 2013 Elsevier B.V.)

4. Utilization of PCI in protein (immunotoxin) delivery

Proteins have been viewed as promising therapeutic agents in tumor therapy. In particular, Immunotoxins have been reported to be capable of producing desirable therapeutic effects by inducing apoptosis of tumor cells (Pastan *et al*. 2006). Immunotoxin has been reported as an extremely toxic protein because it inhibits protein synthesis by causing an irreversible with only one or a few molecule (Johannes and Decaudin 2005). Despite the high toxicity of immunotoxin,

clinical application of toxins has been very circumscribed due to its limited penetration. In this combination, ligand conjugated immunotoxins have been utilized primarily, which in turn allowed for the immunotoxin to bind to tumor cells (Johannes and Decaudin 2005). However, this did not suffice for effective therapy because nanoparticle based protein carriers underwent enzymatic degradation due to the endolysosomal barrier. Consequently, overcoming the intracellular endolysosomal barrier had been a major task that could enable effective macromolecule mediated tumor therapy. PCI has recently been viewed as an effective means of bypassing the endolysosomal barrier and internalizing macromolecules into the tumor cell cytosol (Berg *et al*. 1999). As mentioned earlier, photosensitizers have a predisposition to emit ROS, such as singlet oxygen, when irradiated under certain wavelengths of light. Using this ROS produced, PCI effectively aids macromolecule internalization by peroxiding the endocytic vesicle membrane, and thus evades the endolysosomal process entirely, after endocytosis (Berg *et al*. 1999). Contemporarily, research is being focused on improving macromolecule delivery in a site specific and efficient manner, which produces desired therapeutic effects in vivo. Recently, Yip *et al*. has reported a protein delivery method, which combined a targeting moiety and PCI for enhanced therapeutic effects. Cetuximab-saporin, which has ribosome inactivating protein (ROP) capabilities, was conjugated with the epidermal growth factor (EGF), which in turn allowed tumor site targeting for EGF receptor positive human cancer cell lines (HCT-8). Furthermore, PCI was also applied to aid cellular internalization of Cetuximab-saporin (Yip *et al*. 2007). Cetuximab-saporin was prepared by biotinylation, and LumiTrans (TPPS2a, meso-tetraphenylporphine) was used as photosensitizer for PCI. Fluorescence microscopy confirmed that protein uptake was higher in EGF receptor positive cell lines (HCT-8), while EGF receptor negative cell lines (MES-SA) showed no cellular uptake (Fig. 4(a)), and cell viability studies showed that tumor cell apoptosis was dependent on the intensity of PCI (Fig. 4(b)). In short, the study well portrayed the effectiveness and potential of protein mediated tumor therapy when PCI was incorporated to aid its delivery.

5. Utilization of PCI in tissue engineering

Stem cell therapies have had much attention in the treatment of various human diseases, such as neurodegenerative disorders, cancer, and cardiovascular diseases (Rossi and Cattaneo 2002, Segers and Lee 2008, Park *et al*. 2014). However, the multipotent nature of of stem cell differentiation remains an unsolved problem and has limited the utilization of cell therapy and tissue engineering protocols (Zhou and Freed 2009). Therefore, directing the uncontrolled differentiation of stem cells in a lineage-specific manner is required. To this end, various nanotechnology using strategies have been extensively explored including cell shape control and administration of exogenous proteins, chemicals, and genes (Marklein and Burdick 2010, Park *et al*. 2011). Among them, genetically triggered methodology holds great potential for differentiation of stem cells into specific lineages. There are several methods for effective gene delivery to stem cells, including electroporation, microinjection, ultrasound, viral, non-viral, and liposome-mediated transfection methods (Otani *et al*. 2009, Madeira *et al*. 2010, Park, Yang et al. 2011). Although the non-viral systems have the advantage of being non-pathogenic, non-immunogenic and less toxic, their transfection efficiency in stem cells is limited due to poor internalization and poor endosome escape of the transfected genes; less than 10% transfection efficiency was reported for gene delivery in H9 human embryonic stem cell (hESC) lines (Green *et al*. 2008). Therefore, it is

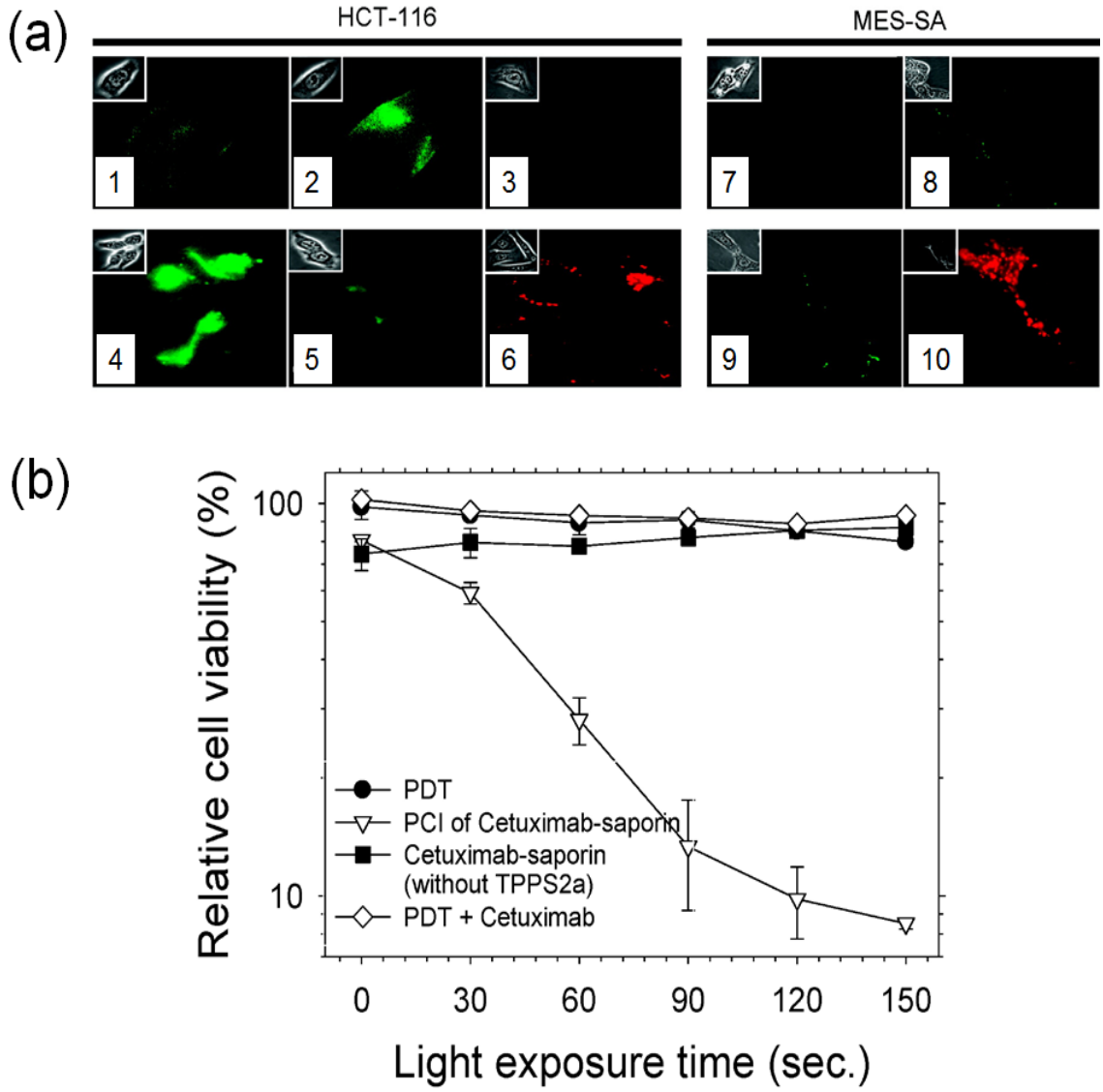

Fig. 4 Utilization of PCI in protein (immunotoxin) delivery. (a) Fluorescence microscopy of HCT-116 incubated with 100 nM Alexa488-cetuximab. The cells were analyzed after 30 min on ice (1); 30 min on ice, then 3 h chase in drug-free medium for at 37℃ (2); no treatment (3); 18 h incubation at 37℃, then 4 h chase in drug-free medium at 37℃ (4) or as in 4 but Alexa488-cetuximab coincubated with 2 μM cetuximab (5). In panel 6, HCT-116 cells were incubated with 1 μg/mL TPPS2a for 18 h, then 4 h chase in drug-free medium at 37℃. (7-10) MES-SA cells were incubated with 100 nM Alexa488-cetuximab after 30 min on ice (7), with Alexa488-cetuximab for 18 h followed by a 4 h chase at 37℃ (8), or as in panel J8, but coincubated with 2 μM cetuximab (9). (10) The cells were incubated with 1 μg/mL TPPS2a for 18 h followed by a 4 h chase in drug-free medium at 37℃. (b) Light dose-dependent effects of PDT affecting the relative viability of DU-145 cells, PCI of 3 pM cetuximab-saporin, 3 pM cetuximab-saporin without TPPS2a, or 6 nM cetuximab in combination with PDT. (Reprinted with permission from Ref. Yip *et al*. (2007). Copyright 2007 American Chemical Society)

necessary to develop a gene transfection method for effective and safe gene transfer to stem cells. Recently, Park *et al*. reported a photosensitizer-induced gene delivery method for effective gene delivery to human mesenchymal stem cells (hMSCs) (Park and Na 2012). Under laser irradiation, the photosensitizers can transfer energy from the ground singlet state to the excited triplet state, leading to the generation of singlet oxygen, which can highly react with unsaturated lipid. In these regards, authors hypothesized that effective gene delivery to hMSCs could be facilitated by a photosensitizer that i) enhances cell membrane permeability and facilitated the internalization of a gene or gene carrier into hMSCs and ii) promotes rapid endosomal escape of the gene, leading to enhanced gene expression in hMSCs (Fig. 5(a)). As shown in Fig. 5(b), the protein and mRNA expression of enhanced green fluorescent protein (EGFP) in hMSCs treated with 0.9 J/cm^2 irradiation increased 9.8- and 8.7-fold compared with non-irradiated hMSCs, respectively, which could be attributed to enhanced internalization and improved endosomal escape by PCI effect. In addition, to monitor osteogenic induction by photosensitizer-induced transfection, the Runx2 (an osteogenic induction gene) was delivered to hMSCs with or without laser irradiation, and von Kossa staining was performed after 3 weeks (Fig. 5(c)). Consequently, osteocalcin, a late marker of osteoblast differentiation, was strongly stained (17.3-fold compared with control hMSCs cultured in osteogenic induction medium). These results clearly demonstrate that photosensitizer-induced gene delivery system has potential applications in practical stem cell therapy.

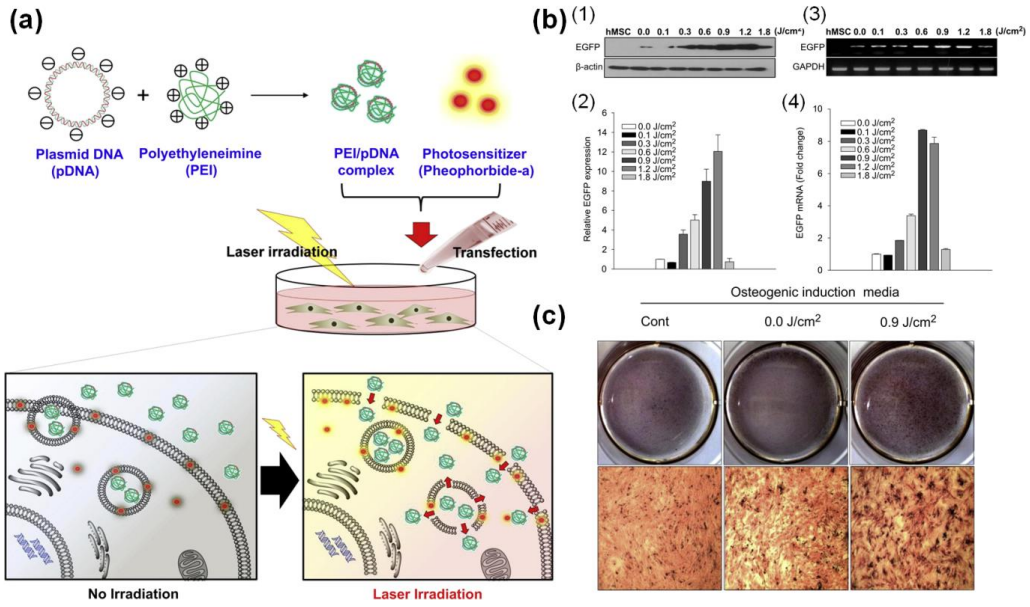

Fig. 5 PCI-mediated gene delivery in stem cell therapy. (a) Schematic representation of PS-mediated transfection: For the PS-induced transfection, a PEI/pDNA complex mixed with PS was treated with stem cells. Under laser irradiation, the PS-mediated cellular membrane damage facilitated the internalization and endosomal escape of gene complexes, (b) Evaluation of PS-mediated transfection efficacy in hMSCs: (1) Western blot analysis, (2) Quantitative analysis of western blot, (3) Gel electrophoresis of RT-PCR products, and (4) RT-qPCR analysis., and (c) Evaluation of osteogensis of hMSCs by PS-induced transfection: Histological images of 2D culture for 3 weeks. The hMSCs were stained with von Kossa. (Reprinted with permission from Ref. Park and Na (2012). Copyright 2012 Elsevier B.V.)

6. Conclusions

In this review, we have reviewed the recently advanced utilization of the PCI technique in design of nano-carriers for efficient drug delivery. PCI is a promising technology which enables efficient transcellular delivery of nanomedicines in various fields including anti-cancer therapy, protein delivery, and tissue engineering. Based on recent research data in combining nanotechnology and the PCI technique, it could be observed that therapeutic efficiency of conventional drugs or nanomedicines improved due to PCI-mediated drug delivery. We believe that this PCI-assisted drug delivery system can be extended for applications in a wide range of areas to improve human healthcare.

Acknowledgments

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