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Cell-Specific Nanopatform-Enabled Photodynamic Therapy for Cardiac Cells

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Keywords

Photodynamic therapy; Targeted ablation; Arrhythmia; Nanoparticle; Methylene blue

Introduction

Over the last decades, various cardiac ablation technologies and procedures have been developed for patients with drug-resistant cardiac arrhythmias. It is now widely accepted that in selected patient populations, catheter ablation is an advantageous alternative to lifelong pharmacologic treatment.¹⁻³ Ablation consists of delivering physical energy locally to specific myocardial regions to abolish arrhythmogenic tissue. Regardless of the energy employed, be it radiofrequency energy, cryoenergy, laser, or ultrasound, ablation techniques are limited by the non-specific nature of the resultant cellular damage. Myocytes perpetuating the arrhythmia experience similar damage to that of bystander cells, such as fibroblasts, adipocytes or neurons. This can result in complications such as atrioesophageal fistula, pulmonary veins stenosis, or coronary artery injury.⁴⁻⁸ In addition, the lack of cellular discrimination increases the required energy for ablation and can prolong procedure times.

Photodynamic therapy

Photodynamic therapy (PDT) consists of a chemical reaction whereby a photosensitizer is activated by light energy and releases reactive oxygen species⁹ (Figure 1). PDT includes two stages. First, the photosensitizing agent is administered and accumulates in the tissue passively or by active targeting using targeting agents like an antibody or a peptide. Then, the photosensitized tissue is exposed to light at a wavelength that coincides with the

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absorption spectrum of the photosensitizing agent which, upon illumination, becomes excited. With photodynamically efficient photosensitizers, this leads to an energy transfer to molecular oxygen (available in cells) and to the generation of reactive oxygen species (ROS), mainly singlet oxygen ($^1\text{O}_2$). The subsequent oxidation of the cell's lipids, amino-acids and proteins induces necrosis and/or apoptosis of the tissue. As ROS, due to an extremely limited lifetime and diffusion length, have a much localized toxicity, their release leads to irreversible but exquisitely restricted cellular damage and tissue necrosis. Thus the damage induced by PDT is confined to the cells that have been photosensitized, while adjacent non-photosensitized cells remain unaffected.¹⁰ The recent development of nanoplateforms has enabled conjugating photosensitizers as well as targeting moieties to hydrogels in such a way that *targeted, cell-specific PDT* has been made available for a variety of applications.¹¹⁻¹⁴ However, the efficiency of implementing nanoplateform-enabling PDT to target specifically a cardiac cell population has not been tested. Also, photodynamic therapy has the ability to be spatially specific, as only the areas illuminated are receiving therapy, and other regions remain untreated. Here, we present the proof-of-principle for a novel targeted cardiac ablation technology that could possibly achieve cell and spatial specificity. We present preliminary results *in vitro* demonstrating in cardiac cells in culture that nanoplateform-enabled targeted PDT is achievable. This method consists of myocyte-specific targeted delivery of photodynamic therapy (PDT)-enabled nanoparticle platforms (NPs). The cell type selectivity is achieved through conjugation of a myocyte-specific target agent-Cardiac targeting peptide (CTP)¹⁵ onto the NP's surface (Figure 2). In addition, the spatial specificity is achieved by photodynamic ablation that enables local confinement of the therapeutic effect, minimizing adverse damage to adjacent non-targeted cells and tissues.

Methods

Synthesis of Methylene Blue (MB) incorporated polyacrylamide (PAA) nanoparticles

Nanoparticles synthesis and characterization has been done as detailed in our recent publication.¹⁶ CTP has the sequence APWHLSSQYSRT to which we added cysteine (C) to the N-terminus to make possible its conjugation with our nanoplateform (MB PAA NPs). Its phototoxic capability is determined as previously reported.¹⁶

Nanoparticles conjugation

Polyacrylamide (PAA) nanoparticles were covalently linked to photosensitizer, methylene blue (MB)- MB PAA NP,¹⁶ and the surface is conjugated with polyethylene glycol (PEG) and CTP (Figure 2). Advantages of having MB covalently linked to the NP matrix are (i) to prevent MB dimerization, which would otherwise interrupt energy transfer from MB to oxygen to generate $^1\text{O}_2$.¹⁷ (ii) to prevent the conversion of MB to the photo-inactive leuco-isomer form,²⁷ by the action of reductases enzyme and (iii) to prevent the leaching of MB out of the NP.

Extraction of primary cardiac myocytes and fibroblasts

Adult rat ventricular myocytes and fibroblasts were isolated as detailed previously.^{18, 19} All animal experiments were approved by the Unit for Animal Laboratory Medicine (ULAM) of the University of Michigan.

In vitro PDT on adult rat ventricular myocytes and fibroblasts co-culture

PDT experiments were performed with an Olympus IX-70 microscope equipped with the Perkin Elmer UltraVIEW confocal imaging system and an argon-krypton Laser light source-647 nm laser beam, 1-mm diameter, 500 μW .

(i) Non-targeted MB PAA NPs were added to a co-culture of adult rat ventricular myocytes and fibroblasts, at 0.5 mg/mL NP concentration. Then, cells were Laser illuminated for 30 minutes.

(ii) Similarly, targeted MB PAA NPs (CTP-conjugated) were added to a co-culture of adult rat ventricular myocytes and fibroblasts, at 0.5 mg/mL NP concentration. After 1 hour of incubation, unbound NPs were washed 3 times and cells were Laser illuminated for 30 minutes.

(iii) The photodynamic effect on cell viability was quantified using a commercial live/dead cell assay (Invitrogen, USA) which consists of calcein acetoxymethyl (calcein AM) and propidium iodide (PI).²⁰ Live cells have intracellular esterases that convert non-fluorescent, cell-permeable calcein AM to intensely green fluorescent calcein. The fluorescent cleaved calcein is retained within cells. In contrast, damaged cells have ruptured membranes which allow PI to enter these cells and bind to nucleic acids. Once bound to nucleic acids, PI produces a bright red fluorescence, seen only in cells with a damaged membrane, i.e. in damaged/dead cells. Thus, a calcein AM and PI mixture in PBS buffer was used to differentiate live cells (green) from dead cells (red).²⁰

Results

The size of the produced particles was determined by two methods: Scanning Electron Microscopy (SEM) imaging, in the dry phase, and dynamic light scattering (DLS), in the wet phase. The SEM showed a nearly homogeneous size distribution around a diameter of 20 nm (Figure 3A), while the hydrodynamic diameter determined by DLS showed a distribution around 50 nm, as depicted in the NP size histogram (Figure 3B). The fact that the PAA nanoparticles exhibited a swelled-up size in the wet phase, in comparison with the dry phase, is a characteristic of hydrogels. The photodynamic efficacy was confirmed by measuring $^1\text{O}_2$ production with a $^1\text{O}_2$ sensitive fluorescent probe, anthracene-9,10-dipropionic acid (ADPA).³¹ The MB PAA NPs exhibited a reduction in ADPA fluorescence intensity, with a rate constant of $k=0.064 \text{ s}^{-1}$, indicating the generation of singlet oxygen during continuous illumination at 647 nm (Figure 3C).

In vitro experiments were conducted in co-cultured isolated adult rat ventricular myocytes and fibroblasts: first, to establish the feasibility of our approach that cardiac cells (both myocytes and fibroblasts) are susceptible to PDT and second, to show if PDT-NPs could be selectively delivered to the cardiac myocytes, resulting in myocyte selective cell death. In the first set of cellular experiments, cells were treated by PDT in a medium containing 0.5 mg/mL *non-targeted* MB PAA NPs (meaning NPs without CTP) in the presence of live/death indicator reagents, Calcein AM for live cells and propidium iodide (PI) for dead cells. Upon illumination with a weak 647 nm laser beam (1 mm in diameter, 500 μW), the myocytes exhibited rapid morphological changes from a rod-like shape to a random shrunken shape (after 1 min) while the fibroblasts showed slightly delayed morphological changes (after 5 min) and, yet, both cell types exhibited progressively increasing PI uptake (fluorescence) and vanishing calcein staining, indicating cell death (Figure 4A, 4B, and 6A). This observation showed that both myocytes and fibroblasts are sensitive to the oxidative damage by PDT, as both cell types rapidly experienced cell death after illumination. Importantly, cell death was only observed inside the illuminated region (Figure 4C), evidencing that the cell death was indeed from PDT (see online supplement, video 1).

In a different set of experiments, the CTP conjugated MB-PAA NPs were incubated with the adult rat ventricular myocyte and fibroblast co-culture for 1 hour and then unbound NPs were washed out thoroughly. PDT was again performed by illuminating an about 1 mm

diameter area with a 647 nm red laser (500 μ W) for about 30 minutes, in the presence of live/dead indicator reagents (see above). During this time period, the myocytes progressively exhibited morphological changes, and showed uptake of PI, a dead cell indicator, as well as a progressive loss of Calcein-AM, a live cell indicator. On the other side, while all myocytes exhibited major changes, leading to rapid cell death, none of the cardiac fibroblasts were affected, indicating that this targeted PDT achieved nearly complete cell specificity (Figure 5A, 5B and 6B). The binding of CTP conjugated MB PAA NPs to myocytes was easily confirmed by imaging the fluorescence of MB dye within NPs (Figure 5C). In contrast, negligible binding towards the fibroblasts was detected (See online supplement, video 2).

Note that while the excitation maximum of the MB PAA NP is around 665 nm, a sharp line width, 647 nm laser light was used (because of limitations in the laser optics setup). Thus, it is likely that the PDT efficiency would have increased or that the required illumination time would have been briefer or an even weaker light source would have sufficed, if an optimal wavelength light source had been employed.

Discussion

Here, we demonstrate the proof-of-principle for developing a cell- and spatially-specific ablation technique encompassing the synergistic implementation of two agents, both conjugated with a biodegradable nanoparticle: a myocyte-targeting peptide (CTP), and a photodynamic therapy-enabling photosensitizer, methylene blue.

We demonstrated that CTP-MB-NPs have the unique capability to specifically attach to myocytes and not to fibroblasts and to induce cell-specific death upon local laser light delivery, followed by local release of ROS. This is exemplified by the markedly decreased number of viable myocytes in the areas illuminated, while the number of healthy fibroblasts stays constant after illumination (Figure 5 and Online movie 2). As we see it, this cell-selective therapy, developed initially for cancer, may represent an innovative concept to overcome some of the current limitations of cardiac ablation.

Nanotechnology and PDT in cardiac electrophysiology

In general, photodynamic therapy has the ability to be spatially specific, as only the areas illuminated are receiving therapy, and other regions remain untreated. To our knowledge, the only study to have implemented PDT for cardiac ablation is the one by Ito A, et.al., in which the authors used talaporfin sodium as a photosensitizer agent injected intravenously in rats to demonstrate that electrical conduction blocks may be created upon epicardial illumination.²¹ Also, Dr. Miyoshi et al. indeed recently presented an abstract to the American College of Cardiology Scientific Sessions demonstrating that after intra-venous injection of the photo-sensitizer talaporfin and introduction of an intra-cardiac Laser light delivery catheter, a cavo-tricuspid isthmus conduction block is readily obtained.²² However, it should be noted that in these studies, PDT was *not targeted* and damage likely occurred in all cardiac cell types in the region illuminated. In addition, the fact that talaporfin non-specifically binds to all organs, including the skin, would hamper clinical applicability, as talaporfin skin deposition may lead to sunburn sun exposure.^{23, 24} This problem is less severe with the use of targeted NPs.

In comparison, we show in our experiments that PDT with CTP-MB-NPs enabled selective myocyte cell death without damaging adjacent fibroblast cells, even when the latter were at a nearly zero-distance from dying myocytes. More generally, we foresee that our approach may be advantageous over current ablation energies which induce damage to myocytes as well as to bystander cells such as fibroblasts, adipocytes or neurons.²⁵

Advantage of nanocarriers over CTP-methylene blue conjugates

To implement two different compounds, CTP and methylene blue, conjugated to the same nanocarrier represents a unique advantage over using such agents separately or without a nanocarrier. The advantages are: (i) to maximize the likelihood of myocytes attachment as multiple CTP molecules are conjugated on each nanoparticle, (ii) to increase the ratio methylene blue/CTP so as to modulate PDT effects. In comparison, a methylene blue- CTP conjugate would be far more limited by a 1:1 receptor-peptide binding ratio. Instead, nanoparticles deliver significantly higher amount of methylene blue and maximize the likelihood of obtaining cell death reliably.

Perspectives for CTP-MB-NPs-enabled applications

Another advantage of implementing therapeutic nanoplatforms is the high versatility of these carriers to be conjugated to various optional targeting agents for distinct cardiac ablation applications. In fact, any other targeting moieties (antibodies, peptides, etc.), functional dyes or bioactive agents may be readily implemented with these nanoplatforms.^{26, 27} Our targeting moiety (CTP) and the photosensitizer (MB) were selected for their specific functional capabilities (PDT) and a specific targeting of myocytes. However, one may consider that the best cell type to be targeted for clinical efficacy may be fibroblasts or other cardiac cell types. Thus, we foresee the implementation of targeting moieties specific to adult human cardiac fibroblasts and to other human cardiac cell types (e.g. Purkinje cells or cardiac neurons) known to be involved in cardiac arrhythmias perpetuation. Finally, a similar approach may also help deliver anti-arrhythmic drugs in a cell-specific manner. As an example, ventricular pro-arrhythmic effects of common anti-arrhythmic drugs represent a major limitation of atrial fibrillation management,^{28, 29} and atrial-specific pharmacological agents are highly desirable.^{30, 31} Targeted biodegradable nanoparticles which release drugs upon illumination³²⁻³⁴ may be implemented to release an anti-arrhythmic drug only to atrial myocytes. Such highly selective drug administration would drastically reduce the global dose, and thus any potential side effects.

Limitations

Key experiments to establish feasibility in vivo are warranted. In particular, aspects such as endocardial light delivery, illumination time, lesion depth and width will have to be carefully evaluated. Recently, encouraging results have been presented by Miyoshi et al. demonstrating that after intra-venous injection of the photo-sensitizer talaporfin and introduction of an intra-cardiac Laser light delivery catheter, a cavo-tricuspid isthmus conduction block is readily obtained.²² It should be mentioned, however, that in this work, the lesions formed were not cell-specific as talaporfin was delivered equally to all cardiac cell types. Also, it appears increasingly clear that cardiac fibroblasts and myofibroblasts are key players in cardiac arrhythmias pathophysiology.³⁵ Thus, fibroblast-specific ablation may represent a crucial development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

AF	Atrial fibrillation
PDT	Photo dynamic therapy
NP	Nanoplatfrom/Nanoparticle
CTP	Cardiac targeting peptide
PAA	Polyacrylamide
MB	Methylene blue
ADPA	Anthracene-9, 10-dipropionic acid
PI	Propidium iodide
DLS	Dynamic light scattering
SEM	Scanning Electron Microscopy
ROS	Reactive oxygen species

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Figure 1.
Schematic of the prepared nanoparticle

Figure 2.
Principle of Photodynamic therapy

Figure 3.

(A) Scanning Electron Microscopy (SEM) imaging in dry phase showing homogeneous NP size distribution. (B) NP size distribution by Dynamic light scattering (DLS) in wet phase. (C) Singlet oxygen generation. The CTP-conjugated MB PAA NPs exhibited a reduction in fluorescence of ADPA at a wavelength of 410 nm as shown by the dotted arrow, indicating the generation of singlet oxygen during continuous illumination at 647 nm.

Figure 4. Susceptibility of cardiac cells to PDT (Non targeted PDT)

(A) Co-culture of adult rat ventricular myocytes (rod shaped) and fibroblasts (flat irregular shape) before illumination and after illumination (B) showing significant morphological changes and PI uptake are seen in *both cell types*. (C) View of the area of illumination (circled area) illustrating that both cell types received PDT. (D) Schematic showing that both cell types underwent cell death after non-targeted PDT.

Figure 5. Myocyte-specific ablation by CTP targeted-NPs

(A) Co-culture of adult rat ventricular myocytes (rod shaped) and fibroblasts (flat irregular shape) before illumination and after illumination (B), showing significant morphological changes and PI uptake in *myocytes only*. (C) Confocal fluorescence image showing the selective binding of targeted-NPs to only myocytes. (D) Schematic showing that only myocytes underwent cell death after targeted PDT.

Figure 6. Quantification of PI fluorescence uptake and cell size changes

(A) Non-targeted PDT experiment: Both cell types exhibited a progressive increase in PI fluorescence uptake (left panel) and a decrease in cell size (right panel).

(B) Targeted-PDT experiment: Fibroblasts were unaffected while myocytes showed PI uptake (left panel) and decrease in cell size (right panel).