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Photodynamic activity of viral nanoparticles conjugated with C_{60}

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Abstract

The development of viral nanoparticles (VNP) displaying multiple copies of the buckyball (C_{60}) and their photodynamic activity is described. VNP-C_{60} conjugates were assembled using click chemistry. Cell uptake and cell killing using white light therapy and a prostate cancer cell line is demonstrated.

Photodynamic therapy (PDT) is a minimally invasive focal therapy, less invasive than surgery and resulting in fewer adverse effects compared to systemic chemotherapy.[1] In PDT, a photosensitizer is delivered systemically to the tumor and locally activated by a probe containing a low-power light source and optical fibers. Management of localized cancers using PDT holds great promise and is undergoing clinical tests. A wide array of photosensitizing drugs has been developed, such as porphyrins[2] and phthalocyanine dyes.[3] Novel materials currently being explored include the buckyball (C_{60}) and its derivatives.[4] Upon irradiation of C_{60} with UV or white light, the molecule is excited to a triplet state, which can directly interact with oxygen to generate reactive singlet oxygen. The fullerene triplet can also be reduced by biological reductants to give the fullerene radical anion; electron transfer from the latter to oxygen generates the superoxide radical and ultimately produces highly reactive hydroxyl radicals through dissociation of hydrogen peroxide.[5] Either event leads to cell death. C_{60} is a highly hydrophobic material, and it has been reported that colloidal aggregates induce toxicity in human cells.[6] A delivery vehicle is therefore required to further develop this material for *in vivo* PDT. Several approaches toward the solubilisation and stabilization of C_{60} in aqueous media have been developed; these include C_{60}-micelle and liposome nanocomposites.[7]

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Nevertheless, tissue-specific delivery of C_{60}-conjugates to cancerous cells *in vivo* remains challenging. Therefore we turned toward the development of a bio-inspired nanotechnology, specifically viral nanoparticles (VNPs) loaded with C_{60} drug. Although the use of VNPs as carriers for the delivery of therapies is widely discussed, only two reports have been published describing the use of VNPs in PDT. Staphylococcus-targeted ruthenium-CCMV conjugates have been developed to treat bacterial infections,[8] and MS2 particles loaded with porphyrins have been targeted to T-cells using receptor-specific aptamers as candidates for PDT in the treatment of leukemia.[9] VNPs can be produced in large scale using molecular farming or fermentation. VNPs are highly multivalent and amendable through genetic engineering and chemical modification.[10] Hundreds of copies of drugs, imaging moieties, or targeting ligands can be displayed on the exterior or interior VNP surfaces. We have previously shown that VNPs modified with targeting ligands can be effectively targeted to prostate tumors.[11] VNPs can be used as carriers for delivery of chemotherapies[12] and photosensitizers.[13] In this study, we turned toward the development of bacteriophage Qβ-C_{60} conjugates as PDT agents for treatment of prostate cancer. The 30 nm-sized icosahedral capsid has $T = 3$ symmetry and is formed by 180 copies of a single coat protein. Qβ displays 720 reactive Lys side chains, 4 each per 180 identical coat protein units.

Qβ was modified with Oregon Green 488 (O488, Invitrogen) and C_{60} at solvent-exposed surface lysines. *N*-hydroxysuccinimide (NHS) ester chemistry was used to conjugate O488 and an azide ligation handle (azido $(PEO)_4$ propionic acid succinimidyl ester, Invitrogen) to Qβ (Scheme 1).

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) was used to conjugate a propargyl-O-PEG-C_{60} derivative[14] (Scheme 1 and Supporting Information). The reaction was purified and analysed by size exclusion chromatography (SEC) (Fig. 1A). Fractions corresponding to labeled, intact particles (9–15 mL) were collected and concentrated with 10 kDa cut-off centrifugal filters. SEC indicated covalent attachment of C_{60} and O488 to Qβ particles (Fig. 1A). Native particles elute at 10.2 mL from the column, compared to 9.7 mL for Qβ-C_{60} and 10.1 mL for Qβ-O488. The shift suggests that Qβ-C_{60} were larger in size; this is as expected, since C_{60} measures approximately 1 nm in diameter. Attachment of O488 was validated based on the co-elution of O488 (Abs at 496 nm) with the Qβ particles (Abs at 260 nm and 280 nm). O488 conjugation was determined using a Nanodrop spectrophotometer (Fig. 1B) and the characteristic extinction coefficient of O488 at 496 nm of 70,000 M^{-1} cm^{-1}. Qβ and Qβ-C_{60} were labeled with 110±10 dyes each. C_{60} conjugation was monitored using native and denaturing SDS gel electrophoresis. After separation, the gels were photographed using AlphaImager (Biosciences) imaging system after staining with Coomassie Blue (Fig. s 1C+D). In native gels, Qβ particles change mobility based on charge and size. Mobility toward the anode increased upon azide conjugation; converting the amine group of Lys side chains into an azide results in decreased positive charge of the particles, thus enhancing the electrophoretic mobility toward the anode. Electrophoretic mobility is slowed down upon C_{60}-conjugation due to increased size of the complex; this is in good agreement with SEC data. In denaturing gels, heating of the sample and presence of the anionic detergent would likely separate any non-covalent complex formation between Qβ and C_{60}. Thus, the lower mobility band indicates successful covalent conjugation. Quantification of C_{60} moieties per Qβ particle was determined based on density analysis and ImageJ band analysis tool (http://imagej.nih.gov/ij). It was estimated that 60±10% C_{60} drugs were attached to Qβ (Fig. S1). Non-specific binding of C_{60} to Qβ was not observed; this was tested by mixing Qβ and $C_{60,}$ while omitting the coupling reagents, followed by analysis on native and denaturing gels.

Fluorescently-labeled Qβ-C_{60} formulations were evaluated in tissue culture using human prostate cancer cells. Since invasive surgery at the prostate can be associated with various

complications and chemotherapy induces many adverse effects, PDT is a desired alternative for the treatment of prostate cancer. We chose PC-3 cells, a highly metastatic cell line, because it is a well-characterized model for prostate cancer, and we have already developed methods to target and image PC-3 derived tumor xenografts in animal models.[11] First cell interactions were evaluated using confocal microscopy to assess whether conjugation of C_{60} affected cellular uptake. Live PC-3 cells were incubated with 5 μg of Qβ-O488 and Qβ-O488-C_{60} at 37°C, 5% CO_2 for 3 h. Post incubation, cells were washed thoroughly, fixed, cell membranes were stained using wheat germ agglutinin (WGA) conjugated with Alexa Fluor 555 (WGA-A555) (Invitrogen), and cell nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (MP Biomedicals). Confocal images were obtained using Olympus FluoView™ FV1000 LSCM and data processed using ImageJ software. We had previously shown that Qβ and Qβ-C_{60} complexes are taken up by breast cancer cells,14 and we show here that the VNP delivery vehicle is also taken up by prostate cancer cells (Fig. 2A). Instead of being widely distributed throughout the cytoplasm, the punctate pattern indicates uptake via endocytosis and targeting to the endolyososmal compartment; the cell interaction is consistent with data reported in the literature.[12e,15] Further, uptake is inhibited at 4°C thus further supporting internalization through an energy-dependent endocytosis mechanism. Our data indicate that the display of multiple copies of hydrophobic C_{60} drugs does not inhibit prostate cancer cell uptake.

Next, drug efficacy was tested using white light therapy. Triplicates of Qβ, C_{60}, and Qβ-C_{60} in 100 μL of medium were added to live cells in increasing amounts, matching the concentrations of Qβ and C_{60} to Qβ-C_{60}, respectively. Following incubation for 3 h, the medium was removed, the cells were washed with saline, and 100 μL of fresh medium was added. The cells were returned to the incubator for 24 h to allow bound particles to be internalized. A mirror was then used to reflect white light from a Proxima DP1000x projector onto the cells in a 6 by 8 in. rectangle at a dose of 2 mW/cm^2 (Fig. 2B). The cells were irradiated for 1 h (6.4 J/cm^2). The cell plate was then incubated at 37°C for 96 h. Cell viability was measured using an XTT cell proliferation assay kit (ATCC). Cell viability data are shown in Fig. 2C. Toxicity of Qβ was not apparent in the dark, indicating that the biological nanomaterial is not cytotoxic, even at high concentrations (0.42 μM, i.e. 1.67×10^9 particles/cell). Data indicate increased photodynamic activity of the Qβ-C_{60} *versus* free drug compound when used at 25 μM drug concentration. Up to 70% of PC-3 cells treated with the Qβ-C_{60} formulation were killed using white light therapy, while only 50% of cells treated with propargyl-O-PEG-C_{60} alone were found dead ($p < 0.05$), thus indicating an advantage of delivering C_{60} drugs to cancer cells using VNPs.

Besides the increased photodynamic activity of VNP-C_{60} compared to free drug, the nanoparticle carrier offers further advantages, i.e. multivalent formulations can be designed. Qβ-C_{60} displays 60 C_{60} moieties, i.e. 660 reactive Lys side chains remain available for further functionalization with, for example, targeting ligands such as the peptide bombesin,[11] and chemotherapies.[12e] The synergistic effect from the multivalent display of targeting ligands, along with the increased activity seen here from drug delivery via a carrier suggest the development of targeted combination therapies has the potential to greatly enhance the effectiveness of PDT. Furthermore, it should be noted that propargyl-PEG-C_{60} had low solubility in buffered solutions (precipitation was noted at 100 μM concentration in 20% DMSO/buffer mixtures). By visual inspection, the 25 μM concentration used for the *in vitro* studies was the highest concentration without noticeable precipitation. Conjugation to Qβ increased the solubility such that there was no precipitation at a concentration of 4.2 mg/mL Qβ-C_{60}, which corresponds to an concentration of 100 μM C_{60}.

Conclusions

We have successfully demonstrated conjugation of C_{60} to Qβ using click chemistry as a highly efficient method for the development of biocompatible therapeutic agents for PDT. We have shown that using Qβ as a scaffold enhances cellular internalization of the C_{60} by prostate cancer cells, resulting in greater therapeutic efficacy. In addition, we found that the propargyl-O-PEG-C_{60} derivative alone in the absence of light therapy also did not result in cell toxicity. However, its insolubility in water detracts from its potential as a PDT drug by itself. Overall, Qβ-C_{60} is a promising platform for PDT, with additional sites for functionalization with additional biomedically relevant moieties, such as targeting ligands or additional drugs. Future work will explore *in vivo* applications of Qβ-C_{60} for targeted and combination therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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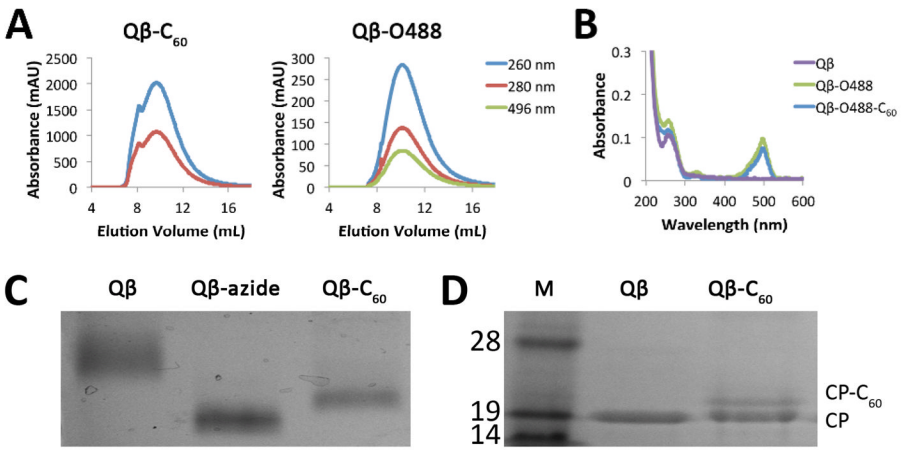


Fig. 1.

(A) SEC of Qβ-C_{60} and Qβ-O488. Intact particles elute at approximately 10 mL. The co-elution of O488 (496 nm) validates attachment. (B) UV/visible spectra of Qβ, Qβ-O488, and Qβ-O488-C_{60}. (C) Native agarose gel. (D) Qβ and Qβ-C_{60} proteins separated on a denaturing 4–12% SDS-PAGE. M=SeeBlue Plus2 molecular weight marker. Approximately 33% of the Qβ coat proteins (CPs) are labeled with C_{60} as estimated by densitometric analysis using ImageJ software.

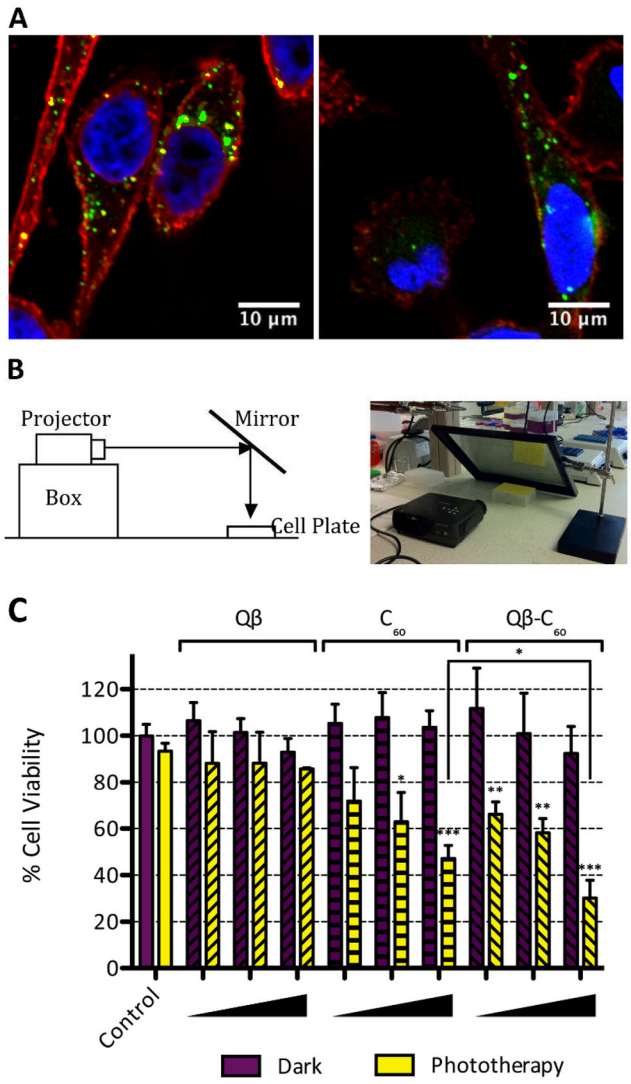
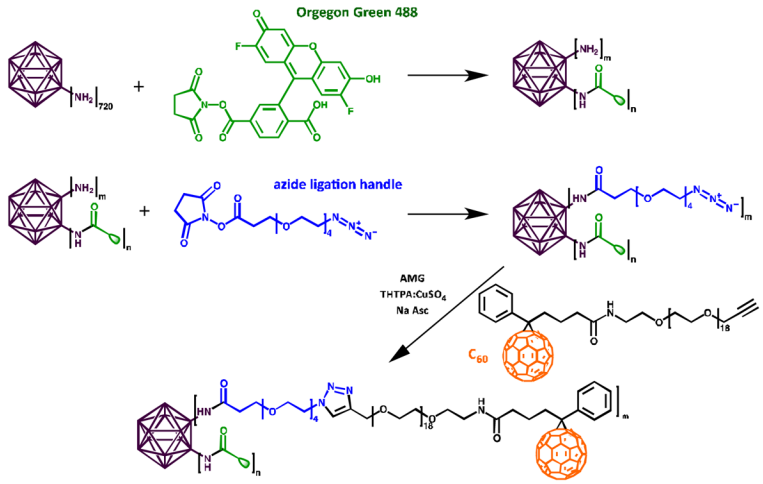


Fig. 2.

(A) Confocal microscopy of PC-3 cells treated with Qβ-O488 (left) and Qβ-O488-C_{60} (right) show internalization (green). The nucleus was stained with DAPI (blue), and the cell membrane was stained with Alexa Fluor 555-labeled wheat germ agglutinin (red). (B) Experimental set-up for white light PDT. (C) Cell viability assay indicates cell killing after an hour of phototherapy. Three concentrations were tested: 1, 10, and 25 μM C_{60} (corresponding to 0.0167, 0.1667, and 0.4167 μM Qβ). Statistical comparisons were made to the dark control and significance is indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). There was a significant difference between the levels of cell killing of Qβ-C_{60} compared to free C_{60} at 25 μM drug concentration ($p<0.05$).



Scheme 1.
Bioconjugation of O488 and C_{60} to Qβ.