Fullerene (C_{60}) immunoconjugates: interaction of water-soluble C_{60} derivatives with the murine anti-gp240 melanoma antibody†

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The first fullerene (C_{60}) immunoconjugates have been prepared and characterized as an initial step toward the development of fullerene immunotherapy (FIT).

The field of biomedicine offers a promising arena for new applications of fullerene materials.[1] Water-soluble C_{60} derivatives are now commonplace,[2] and the discovery that water-soluble C_{60} derivatives can cross cell membranes[3] and even produce transfection[4] has accelerated interest in using C_{60} for diagnostic and therapeutic medicine. Although fullerene toxicity is of some concern, several water-soluble C_{60} derivatives have shown acceptable cytotoxicity for drug-delivery applications.[5]

A number of water-soluble C_{60} derivatives have been suggested for various medical applications. These applications include neuroprotective agents,[6] HIV-1 protease inhibitors,[7] bone-disorder drugs,[8] transfection vectors,[4] X-ray contrast agents,[9] photodynamic therapy (PDT) agents,[10] and a C_{60}–paclitaxel chemotherapeutic.[11] In addition, endohedral metallofullerenes have demonstrated potential as radiopharmaceuticals[12] and MRI contrast agents.[13] Fullerene-based micelles have also been developed as a drug delivery system.[14] To date, however, only the bone-drug application has involved biological targeting of a C_{60}-based material,[8] even though drug targeting is a desirable, if not essential, component of all drug-delivery strategies.

There is now a large body of literature regarding the development of cell-targeted delivery of agents for imaging and therapeutic applications.[15] Growth factors, cytokines and antibodies have all been extensively studied for their abilities to deliver payloads to the surface and the cytoplasm of target cells. The antibody designated ZME-018 targets the gp240 antigen (also known as the high molecular weight melanoma-associated antigen, HMWMAA) found on the surface of >80% of human melanoma cell lines and biopsy specimens.[16] This antibody has previously been extensively used in clinical imaging trials[17] and for the delivery of toxins, cytokines and other therapeutic agents to melanoma cells *in vitro* and *in vivo*.[18] Immunoconjugates containing ZME-018 are rapidly internalized into melanoma cells in culture.[19] Moreover, these conjugates effectively localize into melanoma xenografts after systemic administration and demonstrate impressive cytotoxic effects against established tumors in orthotopic models.[20]

In this communication, we report the synthesis and characterization of a new water-soluble C_{60} derivative (Fig. 1a) designed to covalently attach to proteins such as ZME-018 as an initial step toward targeted fullerene immunotherapy (FIT). A single-drug chemotherapeutic agent such as a recently reported C_{60}–paclitaxel conjugate[11] might be employed for FIT, but the real advantage of FIT over other targeted therapeutic agents is the potential for the attachment of multiple (and possibly different) drugs to the C_{60} scaffold in order to create targeted, single-dose "drug cocktails".

Several reports have been published regarding C_{60} interactions with large biomolecules.[21] C_{60} derivatives have been developed to bind myoglobin,[21a] form electrostatic interactions with cytochrome c,[21b,c] induce protein clusters and complexes in human serum albumin,[21d,e] and enhance catalytic activity *via* conjugation with the serine protease, subtilisin.[21f] Finally, one study has reported the X-ray crystal structure of a C_{60}-specific monoclonal antibody.[21g] Together, these studies suggested to us the possibility of creating a C_{60}–antibody conjugate as a proof-of-principle step towards FIT.

Fluorescence spectroscopy and transient absorption spectroscopy have previously been used to detect dendritic C_{60} interactions with cytochrome c.[21c] These spectroscopic probes have the advantage of monitoring C_{60} without interference from the biomolecule. In particular, triplet → triplet (T–T) absorption provides a method to sensitively and selectively monitor C_{60} derivatives through their known spectral and kinetic signatures.[22] We therefore use transient and ground state absorption measurements to track the fullerene components in synthesized immunoconjugates.

The two C_{60} derivatives shown in Fig. 1 were used in this study. A monoadduct of C_{60}–SPDP (without the water-solubilizing

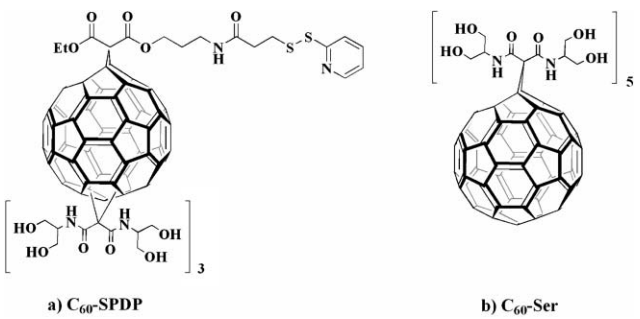

Fig. 1 The water-soluble fullerene (C_{60}) derivatives.

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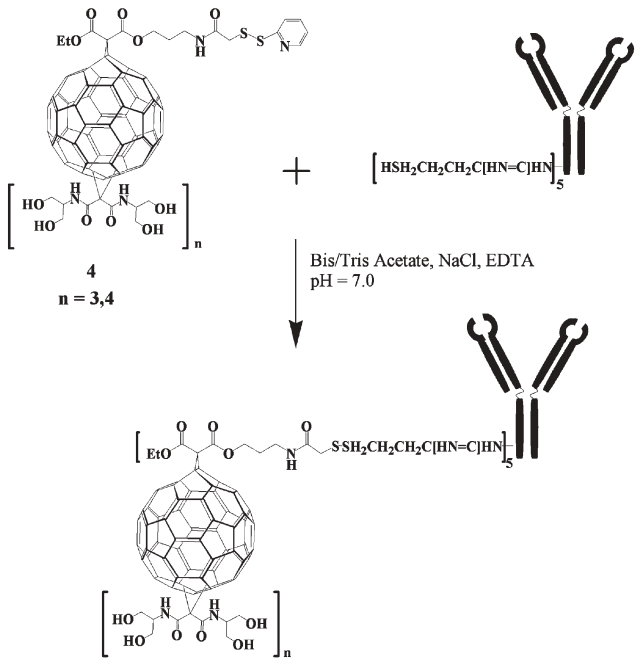

Scheme 1 Schematic representation showing the formation of the C_{60} immunoconjugate from C_{60}–SPDP (C_{60} and antibody figures not to scale).

malonodiserinolamide groups of Fig. 1a) was first prepared (see ESI†) to test the feasibility of attaching the cross-linker, *N*-succinimidyl-3-(2-pyridyldithio)propionate (or SPDP),[23] to C_{60}. Water-solubilizing malonodiserinolamide groups were then first attached to C_{60}, followed by the SPDP moiety in Fig. 1a to provide a cross-linking agent for the ZME-018 antibody. A water-soluble derivative of C_{60}–SPDP was found to be necessary to allow an interaction with the antibody.

Coupling of the C_{60}–SPDP to the antibody (for ratios of 1 : 1, 5 : 1 and 10 : 1) was then accomplished by reacting ZME-018 with 2-iminothiolane, which added an average of five thiol groups to the F_c fragment,[24] each of which can form a new disulfide bond with the SPDP sidearm of C_{60}–SPDP (Scheme 1). The coupling was performed in a salt solution to minimize fullerene aggregation.[21d] Products were purified by size-exclusion chromatography and then examined by transient absorption spectroscopy (ESI†). As shown in Fig. 2a, the C_{60} core's 690 nm T–T spectral signature was clearly present with intensities reflecting the reactant ratio. However, it was unclear whether covalent bonds had formed between C_{60}–SPDP and ZME-018. Therefore, the related water-soluble C_{60}–Ser derivative (Fig. 1b),[2b] was substituted for C_{60}–SPDP in the reaction schemes with ZME-018 (10 : 1 C_{60}–Ser : ZME-018). To our surprise, results for the C_{60}–Ser derivative mirrored those of C_{60}–SPDP. This implies that C_{60}–(ZME-018) conjugate formation may not require covalent bond formation.

Our quantitative characterization began with BioRad protein assays, which showed that the concentration of ZME-018 in the chromatographically purified samples was 0.40 μM for C_{60}–SPDP–(ZME-018) and 0.36 μM for C_{60}–Ser–(ZME-018) (see ESI†). To find the corresponding fullerene concentrations in these conjugates, we used UV-vis spectroscopy. At 440 nm, the molar absorptivity of C_{60}–Ser far exceeds that of ZME-018. The conjugate's measured 440 nm absorbance (ESI†) directly showed a C_{60}–Ser concentration of 15 μM, implying that the ratio (C_{60}–Ser) : (ZME-018) was 42 : 1.[25] Spectral analysis of the C_{60}–SPDP–(ZME-18) conjugate was more complex because absorption bands of C_{60}–SPDP at 440 nm are not intense enough for determining concentrations <20 μM and at lower wavelengths (<350 nm) there is an overlap of absorption bands from the antibody. To account for this, we first prepared a reference solution containing only 0.40 μM ZME-018. As shown in Fig. 2b, this solution has significant absorption at 282 nm. We then added C_{60}–SPDP until the absorbance of the mixture near 282 nm matched that of the C_{60}–SPDP–(ZME-18) immunoconjugate known to contain a 0.40 μM concentration of antibody. The upper traces in Fig. 2b show spectra of this mixture and the conjugate. From the amount of C_{60}–SPDP used to prepare the matching mixture, we deduced a C_{60}–SPDP concentration of 6 μM in the conjugate, corresponding to a (C_{60}–SPDP) : (ZME-018) molar ratio of 15 : 1.

Enzyme-linked immunosorbent assay (ELISA) binding curves using antigen-positive cells as targets gave mid-points of 1.2 nM for the C_{60}–SPDP–(ZME-018) immunoconjugate, 26 nM for the C_{60}–Ser–(ZME-018) immunoconjugate, and 724 nM for a non-specific, isotype-matched murine IgG antibody used as a control (ESI†). Amazingly, the C_{60}–SPDP–(ZME-018) conjugate demonstrated binding midpoints similar to the non-conjugated ZME-018 antibody (mid-point of 0.46 nM), even though 8% (by weight) of the immunoconjugate is fullerene. However, the non-covalently bound C_{60}–Ser–(ZME-018) conjugate, consisting of 17% (by weight) fullerene, exhibited a much lower affinity than C_{60}–SPDP–(ZME-018). Regardless, the C_{60}–Ser–(ZME-018) conjugate was still a factor of 30 more effective in binding the target than was the control.

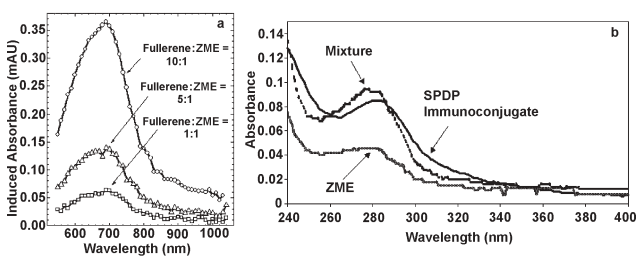

Fig. 2 (a) T–T spectrum of C_{60}–SPDP–(ZME-018) immunoconjugate prepared with three different ratios of fullerene to antibody, after chromatographic purification. (b) UV absorption spectra of 0.40 μM ZME-018, the C_{60}–SPDP–(ZME-018) immunoconjugate (chromatographically purified), and an unreacted mixture of the two components.

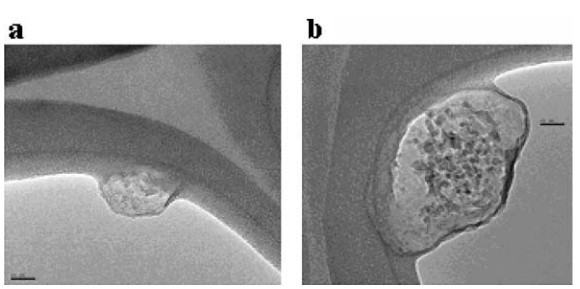

Fig. 3 TEM images of (a) ZME-018 antibody and (b) C_{60}–Ser–(ZME-018) immunoconjugate. The scale is the same for both frames; scale bar length is 20 nm. The solid curved feature in the image is the lacy carbon grid material.

To visualize the two C_{60} immunoconjugates, TEM images of both were obtained on a lacy carbon grid. Comparative images of the ZME-018 antibody and the immunoconjugate are shown in Fig. 3 (An image of C_{60}–SPDP–(ZME-018) and experimental details are presented in the ESI†). Fig. 3 shows that the free antibody appears to have a clear globular structure ~60 nm in diameter, whereas the image of the C_{60}–Ser immunoconjugate is also globular but 4–5 times larger in diameter. In addition, the C_{60}–Ser immunoconjugate image reveals numerous dark spots scattered throughout the structure that can be attributed to small aggregates of C_{60}–Ser, ~2–5 nm in diameter. The larger C_{60}–Ser–(ZME-018) size may reflect disruption of hydrogen bonding networks inside the antibody or some aggregation effect.

The above experiments confirm that covalent bond formation is not necessary to form immunoconjugates of water-soluble C_{60} derivatives with an antibody, and that antibody to antigen binding is not significantly reduced for high C_{60} : antibody molar ratios (15 : 1). Future studies will explore the cancer cell biology of these new C_{60} immunoconjugates, as well as immunoconjugates derived from other fullerene-based nanostructures that have the potential for targeted imaging and therapy in medicine.[11,13,26,27]

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