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COMMUNICATION

Site-specific crosslinking of annexin proteins by 1,4-benzoquinone: a novel crosslinker for the formation of protein dimers and diverse protein conjugates†

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Annexin V (1) specifically binds to phosphatidylserine on apoptotic and necrotic cells as well as certain cancer cells, making it an attractive vehicle for the delivery of therapeutically-relevant conjugates to such sites. The wild-type protein possesses a single thiol at Cys316, which is difficultly accessible to site-specific labeling by simple maleimides. By contrast, 1,4-benzoquinone site-specifically labels annexin V in minutes. The resulting conjugate (5) serves as an intermediate for crosslinking annexin molecules, which can be accomplished within hours either directly for linking annexin V-128 (19), or *via* an extended sequence involving the crosslinking of two units of (5) by the symmetrical α,ω-dithiol (20). Besides its ability to mediate protein dimer formation while retaining annexin V's ability to bind phosphatidylserine, (5) possesses classic 1,4-benzoquinone reactivity. Various nucleophiles and Diels–Alder dienes form adducts with (5) in reactions that may have general utility for the synthesis of novel biologically active entities. The present work presents the first example of thiol-specific crosslinking of proteins by 1,4-quinone-based methodology designed to exploit the reactivity of this versatile chemical entity.

The production of fusion proteins by recombinant methods has become an integral part of the repertoire of protein therapeutics.[1] By contrast, chemical methods designed to produce protein dimers and other multimers have been limited by slow kinetics, the need for metal catalysis, solubility considerations, and other factors that can compromise site-specific protein modification. However, some recent examples of coupling proteins that have been modified to undergo click chemistry have been reported.[2] Thiol groups, through the agency of maleimides, despite their limitations,[3] have also been key to labeling proteins site-specifically with a variety of small molecule entities,[4] as their superior nucleophilic reactivity in Michael reactions at pH's close to neutral, generally exceeds that of the more abundant amine nucleophiles of proteins.

Annexin V (**1**, AN-SH), an endogenous human protein containing a single cysteine thiol (Cys 316), binds tightly to apoptotic, necrotic, and cancer cells expressing phosphatidylserine on their surfaces.[5] Small molecule conjugates of this protein have been widely used in diagnostic imaging,[6,7] but few examples designed to exploit annexin-based therapeutics by chemical modification have been reported. Of particular interest is the challenge of coupling therapeutic proteins to annexin V to provide multimers that could complement or provide advantages over recombinant-based fusion protein technologies targeting cells expressing phosphatidylserine.

In this communication we report preliminary results on the use of 1,4-benzoquinone as a robust crosslinking agent, capable of mediating the formation of diverse conjugates, including protein dimers.

With respect to a program utilizing annexin V conjugates for drug delivery[8] by targeting them to tissues possessing sites of elevated apoptosis, we initially explored the feasibility of employing maleimides as potential site-specific crosslinking reagents.[9] However, even simple maleimide derivatives such as *N*-ethyl maleimide (**2**, NEM) fail to label this protein site-specifically at Cys 316. For example, when (**1**) at 10 μM is treated with (**2**) at 10 mM (under standard conditions used herein for both small molecule and protein thiol reactions, 0.1 M phosphate buffer, pH 8, 23°), for 30 minutes, a considerable percentage of (**1**) is multiply-labeled before the parent protein is completely consumed (Fig. 1). Given the propensity of thiols for Michael addition reactions, the possibility of exploiting 1,4-benzoquinone (**3**, BQ) and its derivatives as potential site-specific crosslinkers of annexin V was then explored. Nucleophilic additions to 1,4-benzoquinones are amply documented and can occur rapidly under a variety of conditions including aqueous media.[10–14] A limited number of protein reactions with BQ to form monoadducts have been reported with recent work emphasizing site-specific reactions,[15] including analytical applications.[16] Thus,

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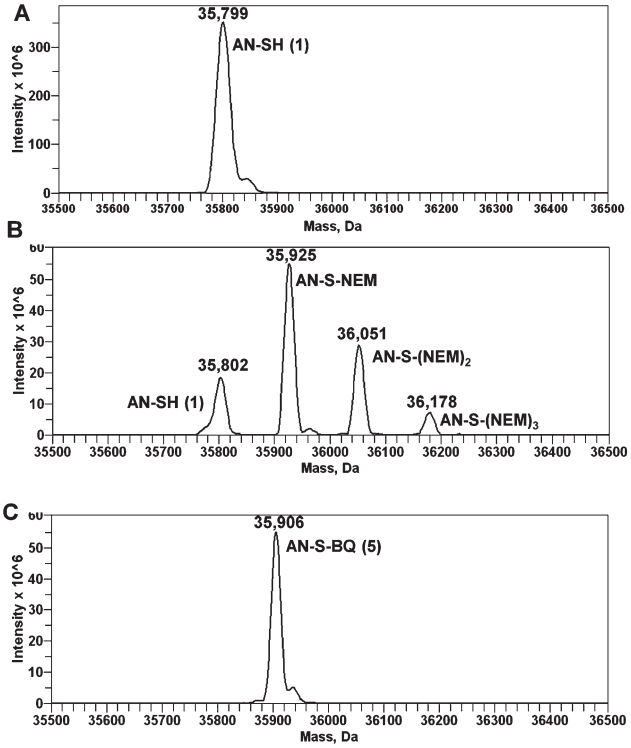
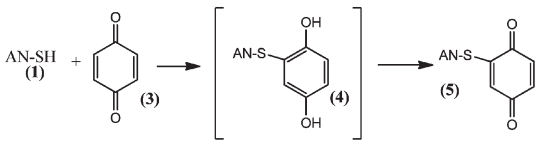


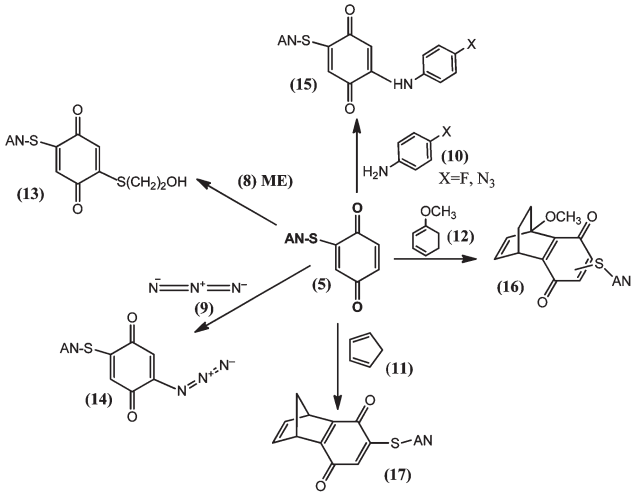
Fig. 1 Comparison of reactions of annexin V (1) with *N*-ethyl maleimide (2) and 1,4-benzoquinone (3). Mass spectra of A. annexin V; B. reaction of annexin V with (2) showing peaks due to multiple additions of (2); C. reaction of annexin V with 1,4-benzoquinone (see text for experimental conditions.) Raw data were collected using a Finnegan LCQ MS ion trap with electrospray (ESI) ionization. Biomolecule charge deconvolution was accomplished with ProMass (Novatia).



Scheme 1 Formation of the annexin V-benzoquinone conjugate (**5**).

Li *et al.* demonstrated that when certain tri-substituted 1,4-quinones and naphthoquinones were incubated with yeast iso-1 cytochrome C, they became site-specifically bound to Cys-102 through thioether bond formation. They also reported that model thioethers of these quinones formed by the addition of 2-mercaptoethanol, were vulnerable to thiol exchange reactions. Implicit in these observations is the notion that such displacement reactions could place limits on the possibility of successively linking thiol proteins to 1,4-benzoquinones in tandem reactions.

By contrast with the reaction of NEM, annexin V can be fully converted by BQ to a 1 : 1 adduct. Although adduct formation can be carried out at near-stoichiometric conditions, it is convenient to employ BQ in large excess to rapidly drive the reaction to completion. Thus when annexin V (**1**, 10 μM) is treated with BQ (**3**, 1 mM) for 4–5 minutes, the parent annexin V mass shifts to a single mass of 36 906 Da (Fig. 1). Under these conditions, excess BQ serves as an oxidant of the dihydroquinone product (**4**) to generate the quinone conjugate, AN-S-BQ (**5**) (Scheme 1).



Scheme 2 Small molecule reactions of the conjugate AN-S-BQ (**5**). The regiochemistry of the condensation reactions has not been established. The adducts are depicted arbitrarily in their oxidized, quinone forms.

Quantitative conversion of AN-SH to the Cys 316 disulfide (**6**, 10 μM) by the thiol-specific protecting group 2,2′-dipyridyl disulfide (**7**, 3 mM) at pH 7, blocks the addition of 1,4-benzoquinone (**3**) under the above conditions, supporting the notion of a thiol-specific reaction of (**1**) and indicating significantly lower reactivity of the protein's amino groups at pH 8.

In 0.1 M phosphate buffer AN-S-BQ (**5**) exhibits classical 1,4-benzoquinone reactivity,[17–19] forming diverse adducts, to varying degrees, with small molecule nucleophiles and dienes (Scheme 2, Fig. 2). (See ESI† for detailed experimental conditions leading to condensation products with masses consistent with theory.) Thus, (**5**, 10 μM) reacts rapidly and quantitatively with simple thiols such as 2-mercaptoethanol (**8**, 10 mM) at 23 °C, pH 8, in 0.1 M phosphate buffer within 10 minutes. In contrast to the reactions with the protein conjugate (**5**), analogous small molecule reactions of BQ reported by Katritzky *et al.*[13] are apparently more difficult to control and involve multiple additions of thiol. AN-S-BQ also adds nitrogen nucleophiles such as azide anion (**9**) and anilines such as 4-aminophenyl azide (**10**, X = N_3), producing adducts that could serve as substrates in click chemistry protocols. Diels–Alder reactions with cyclopentadiene (**11**) and 1-methoxycyclohexa-1,3-diene (**12**) are facile, with unoptimized conversions exceeding 60% after 20 minutes, at 4 °C. On the other hand, 1,3-dipolar cycloadditions to AN-S-BQ with phenyl azides are not observed at ambient conditions, overnight, in line with quinone kinetic precedents.[20,21]

The observations of facile Diels–Alder reactions, which feature highly oriented transition states,[22] strongly suggest that the ring in (**5**) must be in quinone form; despite the macromolecular substituent, at least two contiguous positions of the ring in (**5**) must be unencumbered for the Diels–Alder reactions to occur. As well, the redox properties of the system are confirmed, since after treatment with ascorbic acid, a known reductant of 1,4-quinones,[23] the solution originally containing (**5**), is unable to undergo such Diels–Alder reactions.

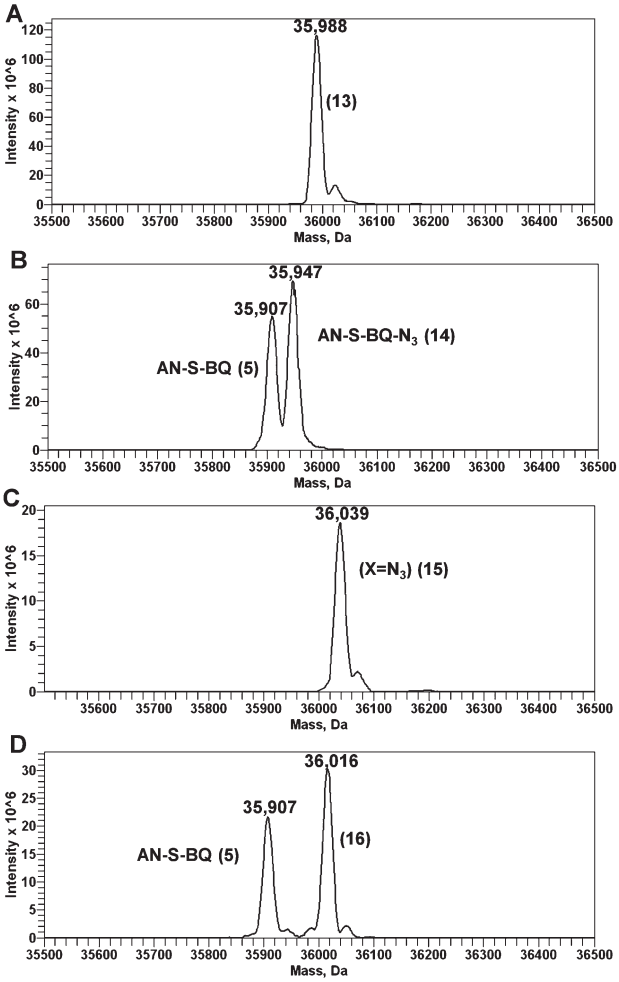
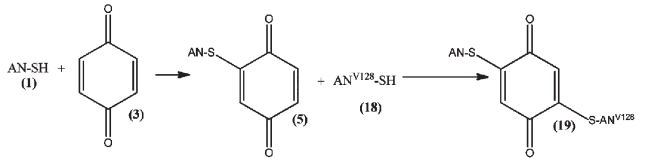


Fig. 2 Mass spectrometry profiles of small molecule reactions of AN-S-BQ (5). A: Reaction of (**5**) with β-mercaptoethanol (**8**); B: (**5**) with sodium azide (**9**); C: (**5**) with 4-aminophenylazide (**10**); D: (**5**) with 1-methoxycyclohexa-1,3-diene (**12**). Raw data were collected using a Finnegan LCQ MS ion trap with electrospray (ESI) ionization. Biomolecule charge deconvolution was accomplished with ProMass (Novatia). See text and ESI† for reaction conditions.



Scheme 3 Crosslinking of AN-SH (**1**) and ANV^{128}-SH by 1,4-benzoquinone (**3**). Note that the regiochemistry of the condensation reactions has not been established.

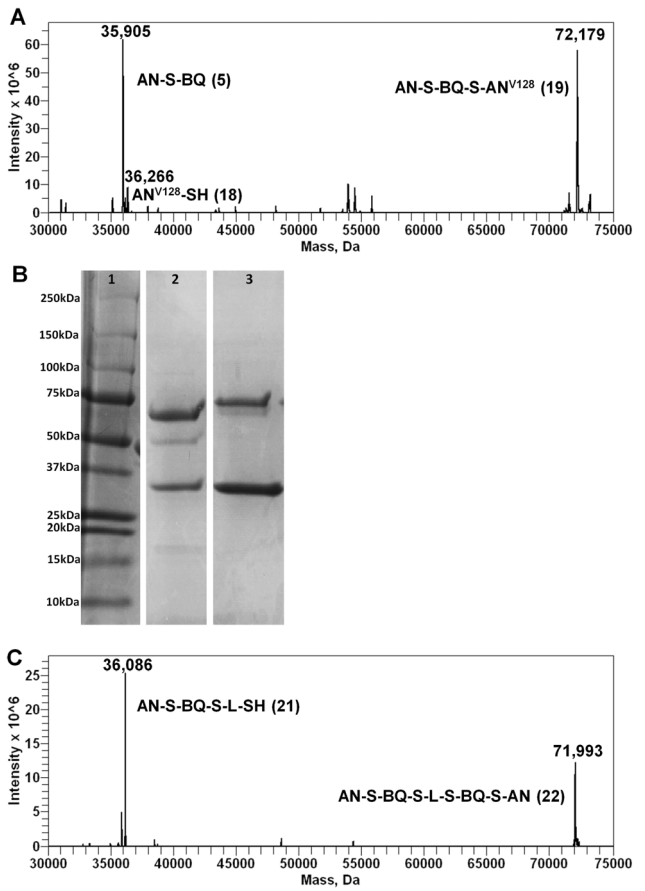
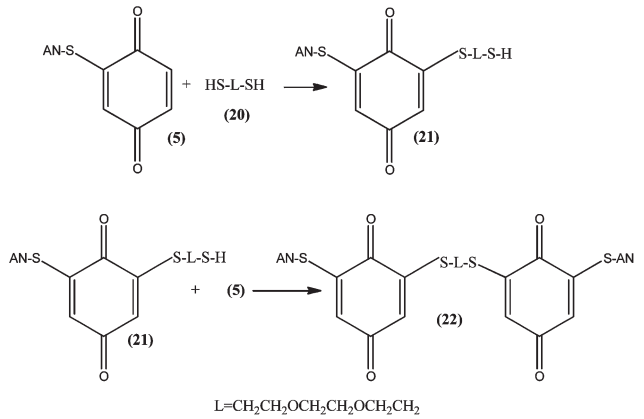


Fig. 3 Mass spectrometry and SDS PAGE profiles of protein crosslinking *via* the agency of AN-S-BQ (**5**). A: Reaction of (**5**) with AN^{V128} (**18**) to give the heterodimer AN-S-BQ-S-AN^{V128} (**19**); B: SDS PAGE analysis of the reaction of (**5**) with annexin proteins. Lane 1, BioRad MW markers. Lane 2, reaction of (**5**) with ANV^{128}-SH (**18**). Lane 3, reaction of (**5**) with tethered (**21**); C: Mass spectrum of reaction of (**5**) with (**21**) to give the homodimer AN-S-BQ-S-L-S-BQ-S-AN (**22**). Raw data were collected using a Finnegan LCQ MS ion trap with electrospray (ESI) ionization. Biomolecule charge deconvolution was accomplished with ProMass (Novatia). See ESI† for experimental conditions.

Following these observations of 1 : 1 small molecule reactions with AN-S-BQ, which under the reaction conditions appear to be free of significant quantities of multiple addition products, we explored the feasibility of linking molecules with potentially high steric demands, such as proteins. The fusion protein diannexin has been shown to suppress apoptosis and protect against reperfusion-ischemia injury in animals[24,25] and is currently in clinical trials for the treatment of ischemia-reperfusion injury following organ transplantation. Accordingly, we initially focused our attention on dimer formation, by incubating (**5**) overnight in 0.1 M phosphate buffer at pH 8, 23 °C, with equimolar amounts of AN-S-BQ (**5**), or with the mutant recombinant form, annexin V-128 (**18**, AN^{V128}-SH). The latter protein possesses a Tc chelation site (Ala-Gly-Gly-Cys-Gly-His) at the N-terminus and is further modified as Cys316Ser to eliminate the cysteine of wild type.[26] Whereas treatment of (**5**) with AN-SH does not produce any practical quantities of the desired homodimer, treatment with equimolar quantities of annexin V-128 (**18**, 10 μM) under the standard conditions yields evidence for the thiol-specific formation of heterodimer (**19**), Scheme 3, molecular weight 72 179 Da (Fig. 3C), in >50% yield as judged from quantitation of SDS gels (Fig. 3B).

The difficulties encountered in crosslinking two units of AN-SH (**1**) above, can be circumvented by crosslinking pairs of AN-S-BQ (**5**) substrates as shown in Scheme 4. Thus **5** (10 μM) is first treated with 1 mM bis-thiol (**20**) for 1 h and the isolated



Scheme 4 Crosslinking of pairs of AN-S-BQ adducts (**5**) mediated by the dithiol linker (**21**). Note that the regiochemistry of the condensation reactions has not been established.

tethered entity (**21**) is then condensed with an equimolar quantity of (**5**), which yields the homodimer (**22**), of mass 71 993 Da (Fig. 3C). Confirmation of the formation of dimers is obtained from SDS PAGE. Fig. 3B shows bands corresponding to ~72 kDa products from the reaction of (**5**), with annexin V-128 (**18**), lane 2, and the reaction mediated by the tethered entity (**21**) to give (**22**), lane 3, respectively. Under the same conditions, (**5**) itself fails to dimerize after more than two days, clearly implicating the thiol tether in the dimerization process leading to (**22**).

Conjugations of protein thiols to 1,4-benzoquinone (**3**) generally occur rapidly and can be conveniently carried out in minutes with BQ at 1 mM and micromolar concentrations of protein. The second addition of protein to quinone is generally more difficult to effect, since high concentrations of the protein factors are not as readily achieved and the reactivity of the benzoquinone moiety, as in (**5**), may be attenuated by the presence of the globular protein substituent. Consequently, (**5**) may discriminate amongst various thiol-containing proteins on the basis of their thiol nucleophilicities,[27] as well as the need to sterically accommodate their overall structures. It is noteworthy that the thiol reactivity of annexin V-128 (**18**) with 1,4-benzoquinone (**3**) is far greater than that of annexin V (**1**). For example, at one extreme, when annexin V (**1**), 3 μM at 23 °C is reacted with 1 mM BQ at pH 5.5 for 2 h, only ~5–10% of (**5**) is formed. By contrast, annexin V-128 (**18**) at 3 μM reacts completely with as little as 4 μM of BQ in 1 minute. The relatively low thiol reactivity of annexin V (**1**) may account for the failure to observe the formation of AN-S-BQ-S-AN homodimer formation, analogous to (**19**). The alternative strategy of dimer formation by crosslinking a pair of AN-S-BQ conjugates (Scheme 4) obviates the need for robust protein thiol nucleophiles such as annexin V-128 in the rate-limiting coupling step; the entity (**20**), bearing unencumbered nucleophilic thiols, can be exploited for both the initial linkage to (**5**) as well as in the ultimate crosslinking step.

Significantly, the calcium-dependent binding properties of the annexin components of the conjugates, (**5**), (**19**), and (**22**), are not compromised by benzoquinone modification. In a sedimentation assay testing the ability of annexin V to associate with acidic phospholipid liposomes in a calcium-dependent manner[28] the conjugated annexins behaved similarly to the unmodified protein. Annexins incubated with acidic phospholipid liposomes in the presence of $CaCl_2$ or EGTA were co-sedimented by centrifugation, and pellets and supernatants were subjected to SDS-PAGE. Modified and unmodified annexins co-migrated with the pelleted liposomes rather than supernatant in the presence of $CaCl_2$ but not EGTA (ESI†). It is noteworthy that these thiol-linked conjugates are intact after being subjected to boiling and reducing conditions while being processed on SDS gels.

Nevertheless the formation of conjugates herein presents two levels of complexity of mechanistic and synthetic interest. Their redox properties provide pathways to potentially reactive quinone forms. The quinones may be regarded as multivalent scaffolds having several sites at which attachments may occur. Consequently, their redox properties, in conjunction with their substitution pattern, can generate multiple and diverse adducts through cycles of oxidation-reduction. The ability to fully exploit BQ-mediated approaches to crosslink proteins and give homogeneous products, will undoubtedly, be dependent upon the steric demands of protein substrates. As well, potential intramolecular reactions within a given protein-quinone conjugate could conceivably block further adduct formation. The latter can, in principle, be controlled by pH, redox conditions and medium effects.

Conclusions

In summary, we have demonstrated that the annexin conjugate, AN-S-BQ (**5**) can serve as a versatile substrate for a variety of functionalities (thiols, amines, and dienes). The conjugate (**5**) can be regarded as a prototype of a hybrid molecule consisting of a holoprotein fused to an auxiliary organic chemical entity capable of orthogonal[29] reactivity to that of the protein. In this instance the auxiliary actually serves in the dual capacity of thiol-specifically crosslinking proteins or incorporating onto the hybrid framework an assortment of organic chemical functionalities that are normally unreactive with proteins. Such functionalities have the potential for connecting various biological and spectroscopic entities to the protein *via* the quinone bridge.

Indeed, the scope of reactivity of quinones as carriers of biologically active entities, including the influence of pH, substituent effects, the regiochemistry of adducts, their affinity for oxygen and nitrogen nucleophiles, as well as the pharmacological activity of various conjugates, are currently under investigation.

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