A novel spin trap for targeting sulfhydryl-containing polypeptides†

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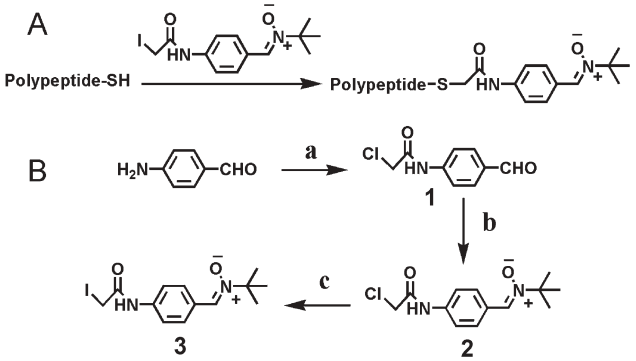
A novel spin trap containing an iodoacetamide group has been synthesized and then used to target polypeptides, *i.e.* glutathione and bovine serum albumin, by which the resulting covalently bonded bioconjugates exhibit great potential for the application of spin trapping of transient radicals in biological systems.

Much of the current interest in spin trapping lies in the detection of biological radicals which are implicated in many physiological and pathological processes.[1] However, these reactive species are unstable and low in concentration, and may site-specifically transform to secondary radical species.[2] Thus, the issue of how to selectively make a spin trap accumulate at the defined site of free radical evolution is very important for the purpose of enhancing spin trapping efficiency and precisely demonstrating the biological significance of the trapped radicals. Recently, a few nitrones derived from α-phenyl-*N*-*tert*-butyl nitrone (PBN) were prepared for targeting mitochondria,[3] cell membranes[4] and lipid membranes[5] *via* noncovalent interactions. A recent study also reported a polyclonal antibody that can recognize adduct species as an antigen which is produced upon spin trapping of suitable protein radicals by 5,5-dimethylpyrroline *N*-oxide (DMPO).[6] Despite the availability of a nitrone bioconjugate as an antigen produced by a covalent conjugation between DMPO derivative and ovalbumin,[6d] little attention has been paid to developing site-specific spin traps for targeting polypeptides *via* covalent connections. With this aim in mind, we report herein the synthesis of *N*-[4-(iodoacetamido)-benzylidene]-*N*-*tert*-butylamine *N*-oxide. This compound has two important properties: while the iodoacetamido moiety can undergo facile reaction with the sulfhydryl group of polypeptides,[7] the nitronyl group can be used to trap free radicals. Thereafter, its covalent targeting for glutathione and bovine serum albumin (BSA) (Scheme 1(A)) as well as enzymatic recognition and proteolysis for the polypeptide-linked spin adducts are also demonstrated.

Functional nitrone **3** was prepared according to a three-step synthesis as indicated in Scheme 1(B). First, 4-(chloroacetamido)benzaldehyde **1** was obtained by acylation of 4-aminobenzaldehyde with chloroacetyl chloride. Then, the condensation of *N*-*tert*-butylhydroxylamine on compound **1** led to *N*-[4-(chloroacetamido)benzylidene]-*N*-*tert*-butylamine *N*-oxide **2**. Finally, the iodination of nitrone **2** afforded a colorless or pale yellow solid.

To evaluate the reactivity of the nitrone **3** with the sulfhydryl group in polypeptides, first, we probed its targeting attack to glutathione (GSH) which is the most abundant non-protein sulfhydryl-containing tripeptide in the cell. The coupling reaction was carried out overnight at room temperature in darkness. The water-soluble bioconjugate (GS-PBN) was subsequently obtained by several extractions and chromatographic purification on Sephadex LH-20. The covalent link between glutathione and the iodoacetamide moiety of the nitrone **3** was characterized by ^{1}H, ^{13}C NMR and MS spectroscopies. To confirm that the covalent connection does not affect the nitronyl group that is the functional site for trapping free radicals, we performed a few spin-trapping EPR experiments in which several oxygen-, carbon- and sulfur-centered radicals were captured by GS-PBN in aqueous or DMSO solution (Table 1). A typical nitroxide signal is demonstrated in Fig. 1(A). The resulting EPR spectrum is unambiguously composed of a triplet of doublets derived from the splitting of nitronyl-N and α-H nuclei, and exhibits the slight anisotropy



Scheme 1 (A) Coupling of the functional nitrone to sulfhydryl-containing biomolecules. (B) The synthesis of the functional nitrone **3**. *Reagents*: (a) chloro acetyl chloride, pyridine, dry ether; (b) *N*-*tert*-butyl hydroxylamine, a small amount of $MgSO_4$, benzene; (c) sodium iodine, acetone.

Table 1 EPR hyperfine splitting constants of GS-PBN spin adducts

Adduct	Resource	Solvent	α_N/mT	α_H/mT
$EtO^{\bullet}$	Ethanol/$Pb(OAc)_4$	DMSO[a]	1.37	0.20
$t\text{-}BuO^{\bullet}$	$(t\text{-}BuO)_2$/*hv*	DMSO[a]	1.52	0.32
$CH_3CH^{\bullet}OH$	Fe^{2+}/H_2O_2/ethanol	Water	1.62	0.33
$CH_3^{\bullet}$	Fe^{2+}/H_2O_2/DMSO[a]	Water	1.65	0.35
p-$ClPh^{\bullet}$	*p*-CDT[b]/*hv*	Water	1.60	0.43
$^{\bullet}SO_3^{2-}$	$Na_2SO_3/K_2Cr_2O_7$	Water	1.51	0.19

[a] DMSO, dimethyl sulfoxide. [b] *p*-CDT, *para*-chlorophenyl diazonium tetrafluoraborate.

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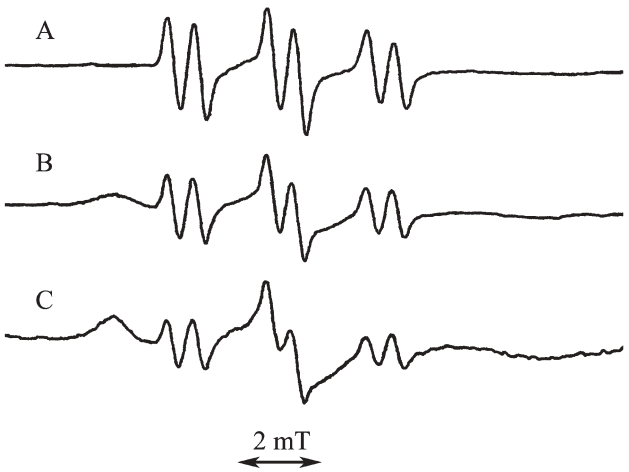

Fig. 1 EPR spectra of free and bound GS-PBN adducts. (A) UV irradiation of the mixture of GS-PBN (3 mM) and *para*-chlorophenyl diazonium tetrafluoraborate (8 mg ml^{-1}). (B) The same as (A), except glutathione s-transferase (10 μM) was added. (C) The same as (A), except glutathione s-transferase (50 μM) was added.

which is characteristic of the incorporation of a bulky glutathionyl group into the nitroxide.

Being an intracellular antioxidant and antitoxicant, GSH takes part in many of the redox and detoxication processes mediated by enzymes such as glutathione peroxidase and glutathione s-transferases (GSTs).[8] Indeed, GSH, and even GSH derivatives may be recognized by these enzymes. In a previous study, a spin-labeled derivative of GSH was recognized by GST and used to characterize the kinetic and binding properties of GST.[9] Thus, it is conceivable that GS-PBN and its spin adducts, as GSH derivatives, may be the substrates for GST. As shown in Fig. 1(B), two EPR spectral components arising from free and bound nitroxides were observed when 10 μM of GST were added to the sample from Fig. 1(A). Increasing the GST concentration to 50 μM resulted in the larger proportion of the bound component (Fig. 1(C)). These results demonstrate that GS-PBN spin adducts can be recognized by GST and probably employed to report the biological environment around the adducts.

An *in vitro* targeting attack to the sulfhydryl-containing protein was performed with a mixture of the nitrone **3** and BSA. The mixture was then incubated overnight at 30 °C in darkness. Excess nitrone **3** was subsequently removed by Sephadex G-25. UV-VIS spectra indicated that besides the protein-specific absorbance at 280 nm the BSA linked PBN (BSA-PBN) exhibited another absorbance shoulder at 316 nm (Fig. 2). The appearance of the new absorbance implies the existence of a chromophoric group (PBN moiety) in the modified protein. To estimate the spin trapping properties of the nitrone-labeled BSA, *para*-chlorophenyl radicals were generated by UV photolysis of *para*-chlorophenyl diazonium tetrafluoraborate in the presence of BSA-PBN. After the irradiation, a spectrum characteristic of an immobilized nitroxide was observable, as illustrated in Fig. 3(A). Under the same experimental conditions, UV-irradiation of BSA-PBN or diazonium alone did not produce any detectable EPR signal (data not shown). Although the broad line in protein-linked spin adduct hampers the expedient recognition of the adduct, we can obtain some additional information concerning protein dynamic effects,

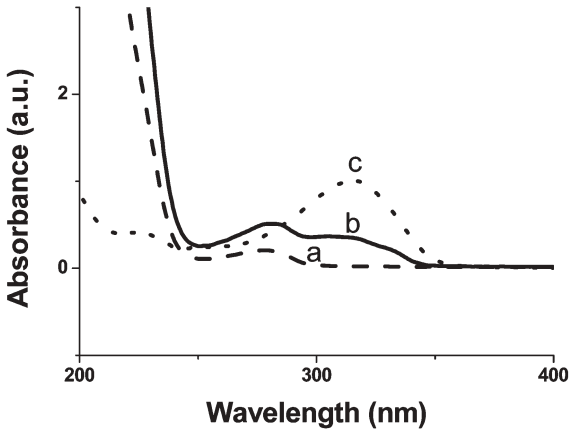

Fig. 2 UV-VIS spectra (H_2O) of (a) bovine serum albumin (BSA) (dashed line), (b) the BSA linked PBN (solid line) and (c) the nitrone **3** (dotted line).

such as rotational-hindrance or binding to immobilized environments. Assignment of the broad signal can be performed by analysing a well-resolved spectrum after proteolysis of the protein-linked spin adduct. As shown in Fig. 3(B), the broad signal became well-resolved after the sample was incubated with pronase, a nonspecific protease. Comparing the hyperfine splitting constants (hfsc) for the EPR signal from Fig. 3(B) (α_N = 1.60 mT; α_H = 0.42 mT) and that from spin adduct of GS-PBN and *para*-chlorophenyl radicals (α_N = 1.60 mT; α_H = 0.43 mT), we find that both are dramatically concordant, by which the trapped radical can be confidently determined to be the *para*-chlorophenyl radical.

In summary, the newly synthesized nitrone **3** can efficiently target some sulfhydryl-containing biomolecules, such as glutathione and BSA. The resulting bioconjugates, GS-PBN and BSA-PBN, not only can efficiently trap reactive radicals, but more interestingly, their spin adducts further exhibit the characteristics of immobilization, like spin labelling (either directly by a protein-linked spin adduct or indirectly through an enzyme-recognized immobile nitroxide). The present study demonstrates that with the help of the covalent link or molecular recognition, the targeting for polypeptides makes possible the transportation of a spin trap to the area(s) of interest in which the monitored radical species is site-specifically generated. In addition, characterization of EPR spectra for immobile adducts can be achieved through proteolysis.

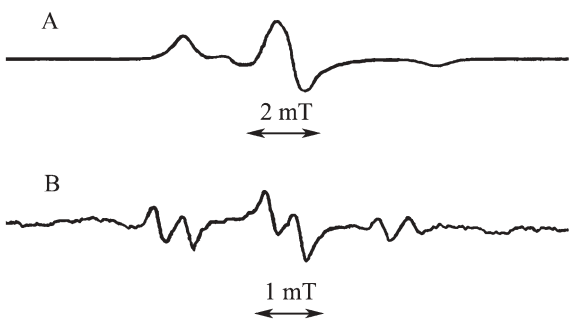

Fig. 3 (A) UV irradiation of the aqueous solution of a small amount of *para*-chlorophenyl diazonium tetrafluoraborate and the attached protein (0.5 mM). (B) The sample from spectrum (A) was treated with pronase (20 mg ml^{-1}) for 24 h at room temperature.

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