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A new glucosamine-containing amphiphilic spin probe

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ABSTRACT

A new, nonionic amphiphilic spin probe for investigating the extracellular matrix close to the cell membrane by EPR spectroscopy has been synthesized and characterized. A pyrrolidine type nitroxide spinlabel has been introduced to the third position of a nonionic sugar polar head (glucosamine) bonded to a lipophilic stearic acid acyl chain anchor. The compound is soluble in polar organic solvents such as ethanol and chloroform, but is sparingly soluble in water.

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The extracellular space in tissues is filled with an intricate network of macromolecules constituting the extracellular matrix. It is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organised meshwork in close association with the surface (glycocalyx) of the cell that produced them. Besides its scaffold-forming function that stabilizes the structure of the tissue, it has a far more active and complex role in regulating the behaviour and function of the cells in contact with it. Despite the rapid progress in characterizing its major components, our understanding of its organization is still incomplete.¹

EPR has proved to be a powerful method for monitoring the biological characteristics of plasma membranes, their interactions with spin-labelled compounds and the dynamics of the latter.² The fine structure of the EPR spectra provides information on the dynamics, polarity, pH, and structural and redox properties of the environment of the spin-labelled molecule.³ The successful application of spin-labelled compounds depends strongly on properly designed spin probes, which are mostly stable free radicals of the nitroxide type.⁴

The ideal spin probe for investigating the extracellular matrix close to the cell surface should be an amphiphilic molecule that can be easily and well anchored into the phospholipid bilayer, with negligible surfactant effects on the integrity of the cell membrane. It should therefore contain a single alkyl chain, but be sufficiently water soluble to pass through the water phase from the film on the test tube wall to the cell surface in the labelling procedure. Charged groups in the polar head should be avoided to reduce their possible haemolytic effects. The nitroxide group-containing moiety should sense the extracellular matrix, therefore it should be positioned as high as possible on the polar head, with a predominantly perpendicular orientation to the membrane surface (along the *z* axis) of the $2p\pi$ orbital containing the unpaired electron, to ensure the highest possible sensitivity of the probe.

The design of the new spin probe **8** reported here was based on an amphiphilic molecule composed of three components: a stearoyl alkyl chain as a flexible lipophilic anchor, a glucosamine sugar moiety as a nonionic polar bulky head and a pyrrolidine type nitroxide free radical as the spin-label. The bulky polar head bearing the nitroxide spin-label is the more rigid part of the molecule and is much larger in cross-section than the flexible alkyl chain.

To synthesize the desired spin probe, we used the synthetic strategy presented in Scheme 1. In the first step, the lipophilic alkyl chain was introduced to the starting sugar, α -D-glucosamine hydrochloride **1**, by selective N-acylation with stearoyl chloride in dioxane/water mixture at ambient temperature.⁵ In the two following steps, 1-O-benzyl and 4',6'-benzylidene protecting groups were introduced according to known procedures, leaving the 3'-hydroxy group of **4** free for a further reaction step.⁶ Fischer glycosylation of **2** with benzyl alcohol over a prolonged reaction time in refluxing benzene gave predominantly the α -anomer of **3**.⁷

The dihydropyrrole-type nitroxide spin-label was introduced by a Williamson reaction, using bromo-derivative **5**.⁸ The reaction was carried out in anhydrous dioxane with sodium hydride as the base and 15-crown-5 as a catalyst to give **6**⁹ in good yield.¹⁰ Next, the benzylidene and benzyl protecting groups were removed by hydrogenolysis in acetic acid/methanol mixture. Also in this reaction step, the nitroxide group was reduced to an amine and, surprisingly, the double bond of the dihydropyrrole ring was reduced, affording a new chiral centre. The resulting secondary



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Scheme 1. Reaction and conditions: (a) H₃C(CH₂)₁₆COCl, NaHCO₃, dioxane/water, rt, 6 h, 94%; (b) BnOH, PTSA, benzene, reflux, 12 h, 65%; (c) PhCHO, (EtO)₃CH, PTSA, DMF, dioxane, rt, 12 h, 74%; (d) NaH, 15-crown-5, dioxane, 50 °C, 40 h, 79%; (e) H₂, Pd/C, AcOH, MeOH, 64%; (f) H₂O₂, Na₂WO₄·2H₂O, Na-EDTA, MeOH, water, rt, 48 h, 56%.

amine **7** was oxidized to nitroxide $\mathbf{8}^{11}$ using hydrogen peroxide in the presence of sodium wolframate as catalyst.¹² HPLC analysis revealed that the final compound **8** was a mixture composed of four diastereoisomers—two pairs of mutarotamers that can interconvert in solution.

The new spin probe **8** was found to be sufficiently soluble in ethanol and only sparingly soluble in water (Fig. 1). Its surfactant properties were estimated from its haemolytic activity.¹¹ In contrast to some other amphipathic spin probes possessing charged groups on their polar heads, compound **8** exerts practically no haemolytic activity, even at the high molar ratio of one molecule of **8** per 100 lipid molecules in membranes of erythrocytes.¹³

In order to test the suitability of the new spin probe for extracellular matrix investigation in cell cultures, it was incorporated into liposomes composed of gangliosides (type III) from bovine brain and lecithins (Emulmetic 320).¹⁴ The X-band spectra of compound **8** recorded at different temperatures (Fig. 2) show significant immobilization of the spin probe, consistent with the position of the spin probe in the sugar chain region of gangliosides close to the lipid-water interface.

In conclusion, we have synthesized a new, nonionic amphiphilic spin probe for investigating the extracellular glycocalyx. Prelimin-



Figure 1. X-band EPR spectra of **8** in (a) phosphate buffer solution (pH 7.4, saturated solution of **8**, estimated solubility, 11 μ M), (b) ethanol (1 mM) in the presence of atmospheric O₂ and (c) de-aerated ethanol solution (1 mM), all at room temperature.



Figure 2. X-band EPR spectra of **8** incorporated into liposomes¹⁵ recorded at different temperatures: (a) 47 °C, (b) 37 °C, (c) 27 °C, and (d) 20 °C.

ary EPR measurements on liposomes have confirmed its potential for characterizing the order and dynamic properties of the extracellular matrix close to the cell surface. The properties of cancer cell surfaces are now under investigation, and EPR studies are expected to provide useful information on the glycocalyx properties of relevant biological samples.

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