18. Deines, P., in *Carbonatites: Genesis and Evolution* (ed. Bell, K.), Unwin Hyman, London, 1989, pp. 301–359.
19. Korzhinskiy, A. F. and Mamchur, G. P., *Int. Geol. Rev.*, 1980, **22**, 1390–1396.
20. Reid, D. L. and Cooper, A. F., *Chem. Geol.*, 1992, **94**, 293–305.
21. Simonetti, A. and Bell, K., *J. Petrol.*, 1994, **35**, 1597–1621.
22. Suwa, K., Oana, S., Wada, H. and Osaki, S., *Phys. Chem. Earth*, 1975, **9**, 735–745.
23. Gwalani, L. G., Rock, N. M. S., Chang, W. S., Fernandez, S., Allegre, C. J. and Prinzhofer, A., *Mineral. Petrol.*, 1993, **47**, 219–253.
24. Hoefs, J., in *Stable Isotope Geochemistry*, Springer, Berlin, 1987, p. 241.

ACKNOWLEDGEMENTS. We thank Gilza for sample preparation for isotopic analysis and R. G. Brasilino for helping in petrographic studies. The isotopic analysis was carried out at NEG LABISE during a fellowship to M.K.P. for which he is thankful to TWAS, Italy for travel assistance and to CNPq, Brazil for financial support. Facilities extended by the Director, Rajasthan University Computer Centre are also thankfully acknowledged.

Received 14 July 1997; revised accepted 21 January 1998

Studies on spin-labelled peptide nucleic acid

XiaoXu Li*, ChenGang Huang†, Yanguang Wang†, YaoZu Chen†‡, LiangRen Zhang*, JinFeng Lu*, LiHe Zhang*

*National Laboratory of Natural and Biomimetic Drugs, Beijing Medical University, Beijing 100083, China
†Department of Chemistry, Zhejiang University, Hangzhou 310027, China

Spin-labelled peptide nucleic acid T10 (PNAT10) was synthesized by solid-phase method with Boc strategy. The product was characterized by electron spin resonance (ESR) and time of flight MS (TOF MS) analysis, revealing a facile spin labelling of PNA by *in situ* solid-phase synthesis. The spin-labelled PNA product shows same hybridizing ability as that of native one and may be used in studying the cell membrane permeability of PNA by determination of the ESR signals.

HIGHLY stable analogues of DNA or RNA are of considerable interest in medicinal chemistry and molecular biology due to their possible use as therapeutic agents and as molecular biological tools. Peptide nucleic acid (PNA) is a DNA mimic in which the entire backbone has been replaced by a pseudopeptide backbone composed of *N*-(2-aminoethyl)glycine units[1,2]. PNA can hybridize to the complementary DNA or RNA and exhibits sequence discrimination and thermal stability equal to or better than that of DNA[2–5]. PNA also shows biological effects including *in vitro* transcription and translation modulation and much more biological stability towards proteases and nucleases. These characters make PNA the candidate of choice amongst potential gene-regulating drugs[6–8]. One of the limitations, however, of PNA to be a feasible gene-regulating drug is that PNA cannot enter cell by passive diffusion because of its poor cell membrane permeability[9], and some attempts have been made to improve cell uptake of PNA[10]. The more convenient and versatile method to evaluate cell membrane permeability of PNA is therefore an impetus for studies on its being used as potential gene-regulating drugs.

Conventional nitroxyl spin-label ESR technique has been extensively applied in pharmacology, molecular biology and biophysics[11–13]. We propose here a stable nitroxyl-free radical as a reporter molecule of PNA to investigate the transmembrane behaviour of PNA, and spin-labelled PNA might also be applied for gene probe investigation. In this study we practised a facile *in situ* solid-phase synthesis of spin-labelled PNA by condensation of 3-carboxyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl with the *N*-terminal amino group of PNAT10 fragment in the course of solid-phase synthesis. The assembled structure is shown in Figure 1, the basic character of spin-labelled PNAT10 is presented and a new method for assessment of transmembrane behaviour of PNA is recommended.

Boc-protected thyminyl monomer was prepared according to the reported method[14]. The synthesis of spin-labelled PNAT10 was initiated on a Boc-L-Lys(C1Cbz) modified MBHA resin, the PNAT10 strand was elongated based on thyminyl monomer following standard solid-phase peptide synthesis protocols[14,15]. After the 10th thyminyl monomer condensation and Boc-deprotection cycle terminated, the *in situ* spin labelling of PNAT10 was carried out continuously using 3-carboxyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl (ref. 16) as block for solid-phase synthesis. HF cleavage was used to liberate the free product, purification was conducted by C-18 RP-LPLC (0–40% acetonitrile in 0.1% TFA/H_2O). The final product gave the nitroxyl-free radical signals (three peaks, $g = 2.0019$, $a = 15.8$ G,

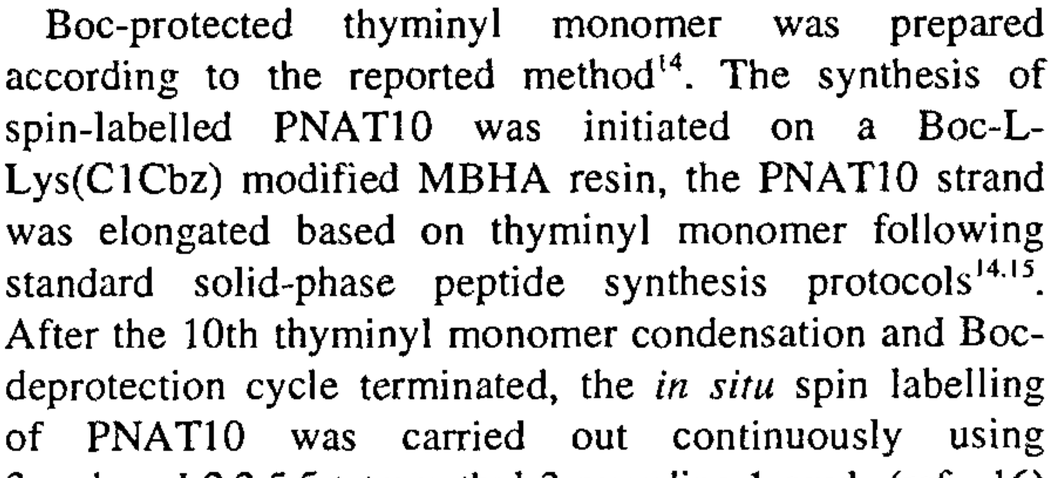

Figure 1. Structure of spin-labelled PNAT10.

‡For correspondence. (e-mail: mengzhou@bltda.com.bta.net.cn)

$\Delta H = 1.8$ G, in water) under ESR spectrum measurement (Figure 2), and revealed successful spin labelling. The molecular weight of the product determined by time of flight MS (TOF MS) was 2973.3, being consistent with the calculated molecular weight. In our works, we designed a spin-labelled PNAT10 and the spin labelling can be achieved by facile *in situ* solid-phase synthesis following the procedure of solid-phase synthesis, the free radical can only be labelled to the *N* terminus of the growing strand, and after coupling of free radical the unreacted free nitroxyl radical was washed off. Since the ESR signal of final product can only be contributed by the covalently linked free radical, the identity of the objective structure of the product can also be confirmed by the ESR signals. The ESR signal product appears to be of a standard nitroxyl-free radical one and shows the convenience and success in solid-phase *in situ* spin labelling. This would facilitate the application of spin-labelled PNA or other PNA analogues.

The melting temperature of hybrid between spin-labelled PNAT10 and its complementary DNA strand dA10 was determined at 1 : 1 PNA : DNA stoichiometry as described[2,3], using the extinction coefficients A 15.4, T 8.8 for both PNAT10 and DNA by heating to 90°C for 5 min, cooling to room temperature, and storing for 30 min followed by storage at 5°C for at least 30 min. The melting curves were recorded in steps of 0.5°C/min. The T_m was determined from the maximum of the first derivative of the plot of A_{260} versus temperature. The buffer used for T_m measurement contained 100 mM NaCl, 10 mM phosphate, 0.1 mM EDTA (pH 7.4). The resultant T_m 78°C, for hybrid of spin-labelled PNAT10 and dA10, is same as that for native PNAT10 and its complementary ssDNA dA10. This result shows that PNA's hybridization ability is not influenced upon being adducted to a spin labelling molecule at its *N*-terminal. This is desired because the result meets the basic requirement for a suitable spin labelling molecule that will not interfere with the system to be labelled[11–13]. Further, the result is also as expected because it has been reported that modification of PNA with small molecules or even protein has little influence on its hybridizing behaviour[10]. The ESR signal was also measured when spin-labelled PNAT10 hybridize to dA10 and dA14, the spectrum obtained is nearly the same as that of spin-labelled PNAT10 only (Figure 2); the isotropic peaks show that no strain is imposed on the spin labelling molecule when spin-labelled PNAT10 hybridize to DNA, this might be due to the labelling molecule's stretching out of the hybrid strand. It appears that the spin labelling on the *N*-terminal of PNA fails to reflect the binding situation of hybrid of PNA and DNA, however, the unchanged ESR signal could easily be quantified and therefore might be used as an indicator of the amount or location of spin-labelled PNA.

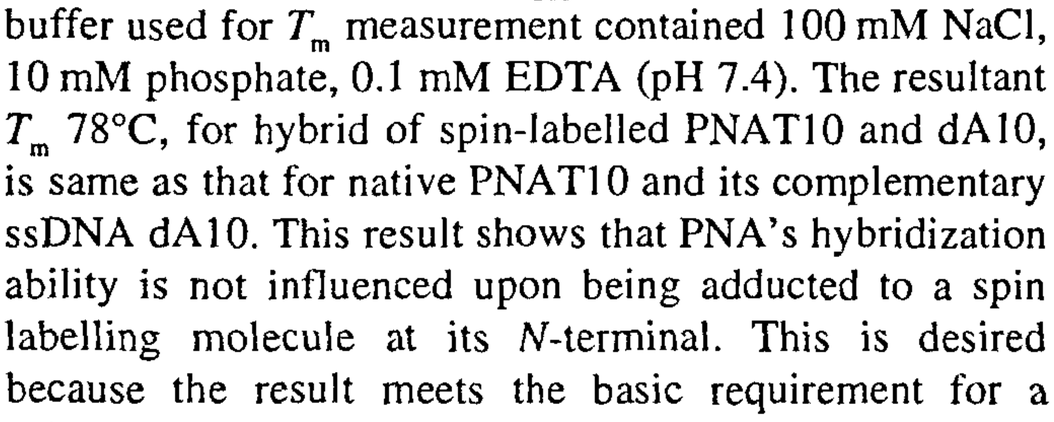

Figure 2. ESR spectrum of spin-labelled PNAT10.

Stable nitroxyl-free radical can be reduced to hydroxyl amine by reducing agent. In erythrocyte the concentration of reducing agent may reach 2–3 mM, which can reduce the free nitroxyl radical that enters into the cell and, hence dramatically quench the ESR signals inside the cell. Based on this principle, experiments were carried out to study cell membrane permeability of kinds of nitroxyl-free radicals and spin-labelled phosphate by monitoring the decrease of ESR signals of spin label when incubated with erythrocyte[17,18]. To assess the membrane permeability of PNA, we designed nitroxyl-free radical as reporting molecule so that the permeability of spin-labelled PNA could be detected by measurement of the ESR signal changes after its incubation with erythrocyte.

Healthy adults' blood was washed three times with isoosmotic PBS (pH 7.0) by centrifugation (10 min × 2000 rpm)[17]. The RBS suspension was adjusted to 50 million/ml with PBS and was incubated at 37°C for 15 min prior to use. The spin-labelled PNAT10 (2 mM) 200 µl was mixed with 200 µl RBC suspension. The mixture was incubated at 37°C. The same volume of sample was picked out from the mixture during incubation periodically and submitted to ESR measurement on a Bruker ESP 300 spectrometer under the same conditions. Since the erythrocyte suspension is free of plasma via washing procedure, ESR signal changes could only be due to intracellular chemical reactions. In our study the spin-labelled PNAT10 showed no significant decrease of ESR signal strength at the same field without widening of peaks even after 5 h incubation with erythrocyte suspension. This result indicates that since the free radical was not reduced by reducing agents inside the cell, the spin-labelled PNA could not or poorly penetrate the cell membrane passively within the observing time. In another experiment, when spin-labelled PNAT10 was incubated with cell lysate, the ESR spectrum as recorded above it showed that the ESR signals decreased significantly, showing nearly 90% decrease in strength at the same field after 15 min (Figure 3). Thus, in this model the decrease of ESR signal during incubation with erythrocyte lysate is due to the release of reducing agents from cell into the mixture, in intact cell suspension

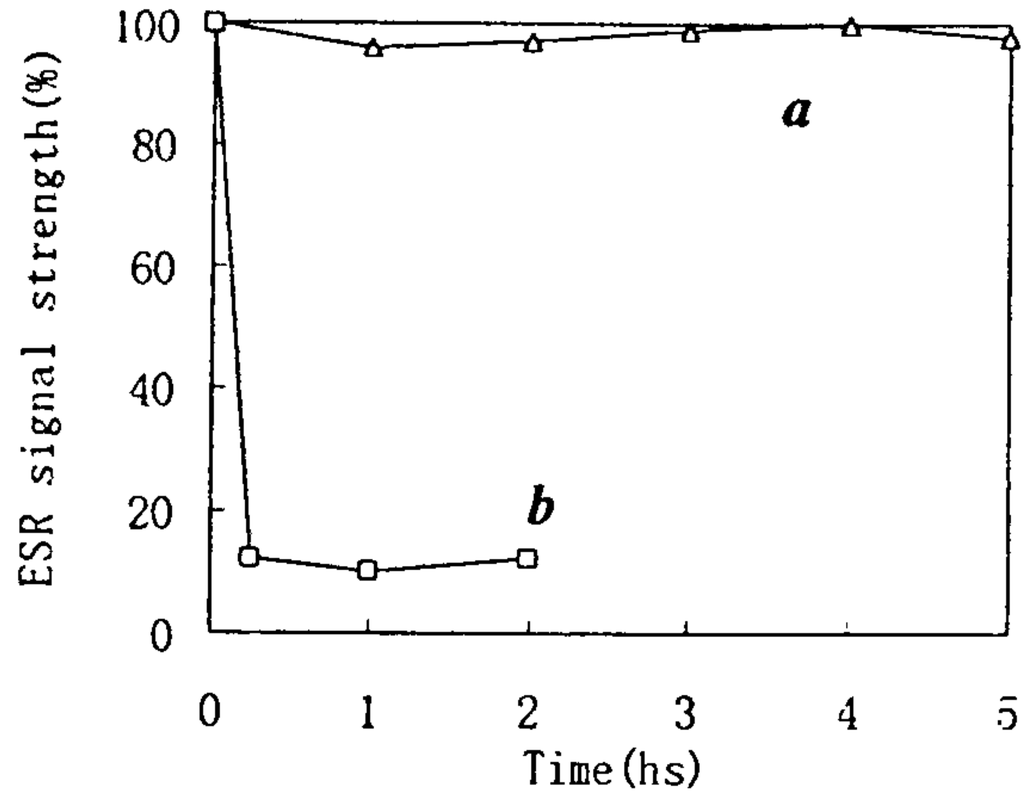

Figure 3. ESR signal strength of spin-labelled PNAT10 incubated with (*a*), erthyrocytes suspension and (*b*), erythrocyte lysate. The strength is presented as the percentage of the initial peak height of low field.

the changes in signal strength can be used as a parameter for demonstrating cell membrane penetrability. The above results support the reported poor cell permeability nature of PNA[10].

Thus, effort should be made to endow PNA with higher cell membrane permeability because a low cell membrane permeability is one of main limitations that hinder many DNA analogues from being used as practical gene targeting drugs, and the chemical modification of PNA might be a promising way to surmount this problem. In these studies, spin labelling of PNAs or their derivatives would be an effective route to assess their cell membrane permeability using the above model, and the spin-labelled PNAT10 could find its application by serving as a negative control. The relative works are in progress.

1. Nielsen, P. E., Egholm, M., Berg, R. H. and Buchardt, O., *Science*, 1991, **254**, 1497–1500.
2. Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Frieier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B. and Nielsen, P. E., *Nature*, 1993, **365**, 566–568.
3. Leijon, M., Graslund, A., Nielsen, P. E., Buchardt, O., Norden, B., Kristensen, S. M. and Ericksson, M., *Biochemistry*, 1994, **33**, 9820–9825.
4. Brown, S. C., Thomson, S. A., Veal, J. M. and Davis, D. G., *Science*, 1994, **265**, 777–780.
5. Cherny, D. Y., Belotserkovskii, B. P., Frank-Kamenetskii, M. D., Egholm, M., Buchardt, O., Berg, R. H. and Nielsen, P. E., *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 1667–1670.
6. Hanvey, J. C., Peffer, N. J., Bisi, J. E., Thomson, S. A., Cadilla, R., Josey, J. A., Ricca, D. J., Hassman, C. F., Bonham, M. A., Au, K. G., Carter, S. G., Bruckenstein, D. A., Boyd, A. L., Noble, S. A. and Babiss, L. E., *Science*, 1992, **258**, 1481–1485.
7. Nielsen, P. E., Egholm, M. and Buchardt, O., *Gene*, 1994, **149**, 139–145.
8. Demidov, V. V., Potaman, V. N., Frank-Kamenetskii, M. D., Egholm, M., Buchardt, O., Sonnichesen, S. H. and Nielsen, P. E., *Biochem. Pharmacol.*, 1994, **48**, 1310–1313.
9. Witlung, P., Kajanus, J., Edwards, K., Nielsen, P. E., Norden, B. and Malstrom, B. G., *FEBS Lett.*, 1995, **365**, 27–29.
10. Pardridge, M., Boado, R. J. and Kang, Y. S., *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 5592–5596.
11. Simeonova, M., Ivanova, T., Raikov, A. and Konstantinov, H., *Acta Physiol. Pharmacol. Bulg.*, 1994, **20**, 77–82.
12. Gutierrez, P. L., Cohen, B. E., Sosnovsky, G., Davis, T. A. and Egorin, M. J., *Cancer Chemother. Pharmacol.*, 1985, **15**, 185–195.
13. Gnewuch, T. G. and Sosnovsky, G., *Chem. Rev.*, 1986, **86**, 203–238.
14. XiaoXu. Li, YanGuang, Wang and YaoZu, Chen, *Chinese Chem. Lett.*, 1997, **8**, 385–386.
15. Dueholm, K. L., Egholm, M., Behrens, C., Christensen, L., Hansen, H. F., Vulpius, T., Petersen, K. H., Berg, R. H., Nielsen, P. E. and Buchardt, O., *J. Org. Chem.*, 1994, **59**, 5767–5773.
16. Couet, W. R., Brasch, R. C., Sosnovsky, G., *Tetrahedron*, 1985, **41**, 1165–1172.
17. Ross, A. H. and McConnel, H. M., *Biochemistry*, 1975, **14**, 2793–2798.
18. Eriksson, U. G., Tozer, T. N., Sosnovsky, G., Lukszo, J. and Brasch, R. C., *J. Pharm. Sci.*, 1986, **75**, 334–337.

ACKNOWLEDGEMENT. This work is financially supported by National Laboratory of Natural and Biomimetic Drugs and the Cao Guang-Biao Foundation of Zhejiang University.

Received 30 May 1997; revised accepted 13 January 1998