Development of novel whole-mount \textit{in situ} hybridization (WISH)

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\textbf{ABSTRACT}

We have designed a novel fluorescent PNA probe which can be rapidly introduced into the living cell without any complicated pretreatment. We could post-synthetically incorporate amino acid derivatives having membrane permeability function into the PNA probe by utilizing key compound 1 which we have already developed. We show that the PNA probe will be a candidate for a rapidly penetrating and non-degrading probe for WISH.

\textbf{INTRODUCTION}

WISH (Whole-mount \textit{in situ} Hybridization) is a convenient method for detecting the localization of target mRNA in tissue or embryo by fluorescence.\(^{(1)}\) However, the enzyme resistance of WISH probes is poor because DNA/RNA is used as a medium for the target mRNA binding domain of the WISH probe. Therefore, the necessity for precise sampling and the problem of the false-positive have been identified. Also, since the conventional probe cannot be said to have excellent membrane permeability, it was necessary to use transfection reagents such as DOTAP/DOPE and fixation of the cell. This poor membrane permeability has become a serious defect for the WISH probe, because rapid intracellular introduction is required when a probe is applied to a living cell, especially tissue and embryo.

A peptide nucleic acid (PNA) is a DNA analogue in which the phosphate backbone is replaced by \(N\)-(2-aminoethyl)glycine units (Fig. 1).\(^{(2)}\) The electrostatically neutral peptide backbone prevents inter-strand repulsion of the complementary strand, allowing sequences to be recognized with high affinity and selectivity with respect to hybridization with DNA or RNA. The use of PNAs is also advantageous because they are extremely stable to cellular nucleases and proteases. Unfortunately, the membrane permeability of PNA is also poor. The development of a novel fluorescent PNA probe, which can be introduced into tissue and embryo, is the key to enhancing the application range of the WISH technique. We have already established the protocol for incorporating post-synthetically functionalized molecules in a PNA oligomer by utilizing key compound 1.\(^{(3)}\) We herein report that a novel fluorescent PNA probe having high membrane permeability has been designed and this probe could be rapidly introduced into the living cell without any complicated pretreatment.

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Figure 1}
\end{figure}
RESULTS AND DISCUSSION

Recently, we have reported a strategic synthetic method for functionalized PNA monomers, in which nucleobases were replaced with functional molecules, and application of the functional PNA oligomer to antisense therapy. At that time, we succeeded in introducing post-synthetically a number of functional molecules into the same PNA oligomer using key compound 1, because it has the two different protecting groups of Fmoc and rBoc, which can be selectively deprotected. Designed probe 2 combines the three functional domains of "fluorescence", "membrane permeability", and "sequence recognition" (Fig. 2). For the "sequence recognition" domain the antisense sequence of the exon region of a target mRNA was selected. In the case of the "membrane permeability" domain, 1 was successively introduced several times into the PNA oligomer, deprotected to amino groups by piperidine treatment, and condensed simultaneously with amino acid derivatives having a membrane permeability function. Furthermore, the terminal amino group of the linker was treated with FITC in order to make the "fluorescence" domain. Probe 2 was purified by HPLC, and characterized by MALDI-TOF MS.

The pretreatment is generally carried out by fixation of the tissue and embryo and the usage of transfection reagents because it is most important in WISH that the fluorescence probe is introduced into cells efficiently. Therefore, it is difficult to say that the nature of the tissue or embryo is accurately monitored. Probe 2 was directly added to the living cell culture solution without any pretreatment, this solution was incubated for 12 h, and excess probe was washed out. Observation by fluorescence microscope showed that all the living cells emitted fluorescence, although probe 3 having no "membrane permeability" domain was seldom fluorescent. Judging from the immunostaining procedure, there was no expression of the protein which was correlated with the target mRNA. These results suggest that the designed WISH probe rapidly penetrated the living cell, hybridized with the target mRNA, and inhibited the specific protein biosynthesis derived from the target mRNA. Attempts to optimize the membrane permeability using an amino acid derivative are currently in progress in order to design an efficient introduction pattern.

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REFERENCES