Feature Article

The Winner's Article of the First Dr. Masao Horiba's Award

Development of an Intracellular pH Measurement Method using DNA as a Sensing Material

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While carrying out this research, we studied the relationship between stability of the double helix structure formed by DNA (deoxyribonucleic acid) and pH. Based on obtained thermodynamic data we discovered a DNA structure of a pH sensitivity that was not previously thought to exist in living bodies. Further, we considered the newly discovered pH-sensitive DNA as a new material for pH sensing and have since developed a unique pH sensor that can measure the pH in cells using DNA as detection medium. This was achieved by combining the pH-sensitive DNA to FRET (Fluorescence Resonance Energy Transfer). We have introduced the principles and application of a pH sensor that utilizes nucleic acids.

Introduction

Research into using nucleic acids as sensing material has started to garner attention in the field of nano-technology and bio-technology because nucleic acids can 'recognize' molecules on the nano-scale. The higher-order structures of nucleic acids can work as sensors sensitive to environmental variations of metal ions and pH, and small molecules in solutions. It is expected that a high-sensitivity sensor for environmental variations and small molecules can be developed if nucleic acids can be used as detecting media. However, targeted structures have to be searched for by trial-and-error methods making the research difficult because nucleic acids form varied higher-order structures.

These higher-order structures include not only the double helices formed with Watson-Crick base pairs but also non-Watson-Crick base pair regions (Hoogsteen base pair, etc) and multiple-helix structures. It is expected that a fully understanding of the chemical (thermodynamic) characteristics of higher-order structures of nucleic acids will allow free designs of nucleic acids tailored to the purpose of the sensor and will significantly revive the diminishing use of nucleic acids as sensors.

In this situation, we analyzed the higher-order structures of nucleic acids based on thermodynamics as an example of developing nucleic acid sensors. Then we created a new pH sensitive DNA using thermodynamic data and developed a new pH sensor that uses DNA.

Higher-order Structures of Nucleic Acids

Major higher-order structures of nucleic acids include double helix structures composed of the Watson-Crick base pairs. However, a nucleic-acid molecule has non-Watson-Crick base pair regions or multiple-helices other than the Watson-Crick base pair double helices. These structures have a deep involvement with features of nucleic acids as yet unexplored.

The non-Watson-Crick base pair regions are divided into parts in a state that do and parts that do not form base pairs, and parts that form base pairs with combinations other than the Watson-Crick base pairs. The former includes the bulge, internal loop, terminal mismatch,

hairpin loop, dangling end, etc (Figure 1). The latter includes the mismatch, Hoogsteen base pairs, etc. The non-Watson-Crick base pair regions combined with Watson-Crick base pairs form higher-order structures of nucleic acids. For example, the non-Watson-Crick base pair regions such as dangling end, mismatch and hairpin loop exist in tRNA. These non-Watson-Crick base pair regions determine the formation and stability of the higher-order structures of tRNA. In addition to contributing to the nucleic acid higher-order structural stability, the non-Watson-Crick base pair regions exist in:

(1) Protein recognizing regions,

(2) Metal-ion coordination regions and

(3) Catalytically active regions for cutting and combining nucleic acids.

They assume varied roles within the living body according to their features[1]-[14]. In other words, it is necessary to clarify the higher-structures of nucleic acids and the features accompanying them at the molecular level in order to utilize them as nucleic acid molecular materials or as detecting media in the biotechnology field.

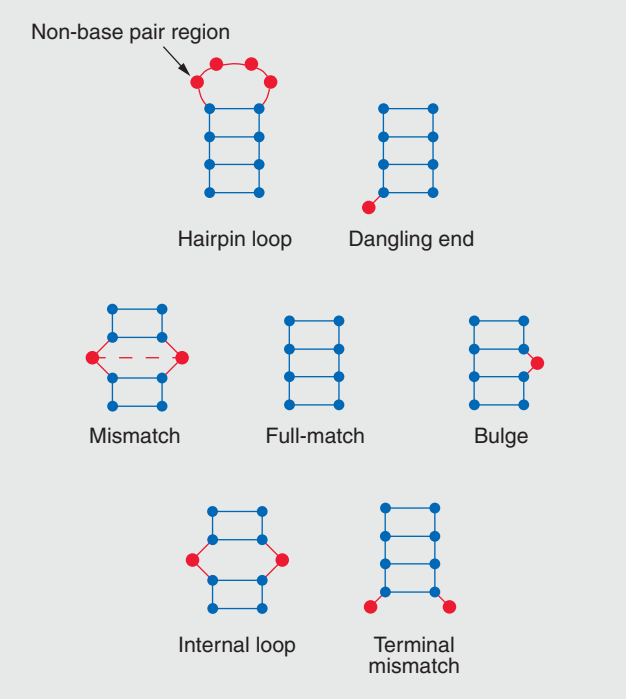

Figure 1 Non Watson-Crick Base Pair Regions and Multiple Chain Structures of Nucleic Acids

Construction of a New Nucleic-acid Structure Transition to pH

Multi-helices composed of Hoogsteen base pairs among varied non-base pair regions can be used not only for understanding and predicting known higher-order structures but also designing nucleic acid molecules with new features. For example, a parallel-type triple chain that forms T × A • T (×: Hoogsteen base pair, •: Watson-Crick base pair) and C^+ × G • C base pair by the Hoogsteen base pair requires a protonation of cytosine (C) base at N3 to form stable base pairs (Figure 2)*1. The p*Ka*2 of the protonation*3 of C base is 5.5. Therefore, parallel-type triple chains are formed only when pH is low. In other words, it is supposed that a new nucleic structure transition that depends on the change in pH as an environmental factor can be constructed by using the Hoogsteen base pair including protonation of the C base.

*1: A: Adenine, T: Thymine, G: Guanine, C: Cytosine.

*2: Dissociation index (The common logarithm of the inverse of the dissociation constant *K*a).

*3: A molecule is added with a proton (H^+) to be converted into an ion.

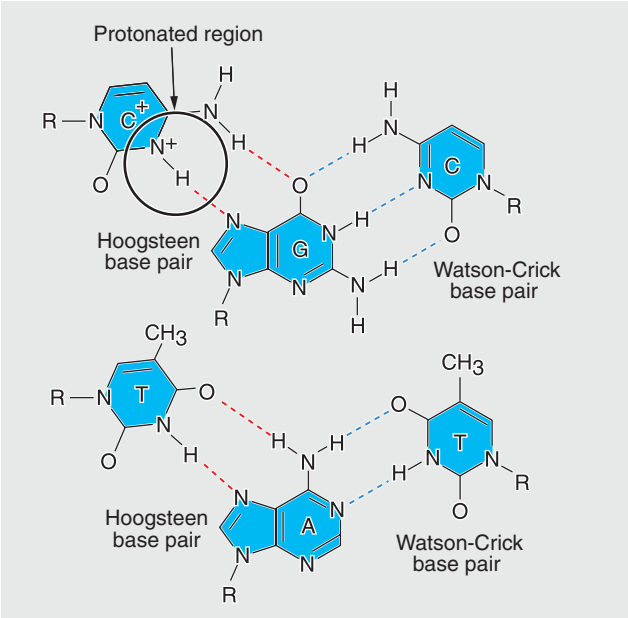

Figure 2 Hoogsteen Base Pairs and Watson-Crick Base Pairs in Parallel-type Triple Chains

Therefore, we studied the relationship between the detailed stability and pH of the Hoogsteen base pair focusing on DNA multi-helices composed of Hoogsteen base pairs towards development of nucleic molecules with new features. As a result, it has become clear that the stability of the anti-parallel-type double helix, composed of the Watson-Crick base pair, and the stability of the triple helix combined with the double helix and the Hoogsteen base pair are approximately the same at pH 5.0[7]. Therefore, we designed a parallel-type double helix (5′-TCTTTCTCTTCT-3′/ 5′-AGAAAGAGAAGA-3′)*4 formed only with the Hoogsteen base pair, based on the thermodynamic data. We studied the structure transition using pH, and found that the two DNA chains are antiparallel-type double helices with bulge bases*5 of pH 7.0. However, they are parallel-type double helices formed only by the Hoogsteen base pairs of pH 5.0 (Figure 3). This pH-dependent structure transition is caused by:

- The protonated cytosine base and the parallel-type double helices of the Hoogsteen base pairs formed at pH 5.0
- The parallel-type double helices destabilized by the lack of protonated bases
- The antiparallel-type double helices with bulge bases formed by the Watson-Crick base pairs at pH 7.0.

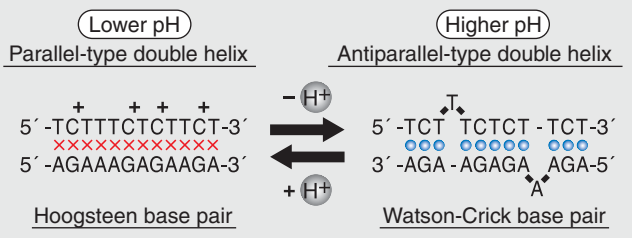

Figure 3 Structure Transition by pH of Parallel-type Double Helix Formed only with Hoogsteen Base Pairs, and Antiparallel-type Double Helix of Watson-Crick Base Pairs with Bulge-type Bases

*4: Nucleotides composed of cyclic five-carbon sugar, phosphoric acid and bases combined are connected to one another with phosphoric acid linking the positions 5′ and 3′ of five-carbon sugar to form a polynucleotide chain. ATGC bases are all heterocyclic including nitrogen. A prime [′] is added to the position of carbon of sugar to avoid ambiguity in the numbering systems of heterocycles and sugar. 5′ and 3′ herein mean the end position of a polynucleotide chain.
ATGC bases are classified into two types including purine (combinations of five-member and six-member rings) and pyrimidine (six-member ring). AG and TC bases are purine and pyrimidine respectively.

*5: A base protruding from a double chain without intruding into a base pair.

Development of a pH Sensor that Operates in a Test Tube

We then developed a pH sensor that can measure pH in a test tube by combining the FRET (Fluorescence Resonance Energy Transfer) and the newly discovered nucleic acid structure whose transition depends on pH changes. At first, we constructed a system (first generation) that can observe changes in colors of fluorescence by two fluorescent molecules approach each other when the structure of DNA is altered by the changes in pH. FRET is an energy transmission by resonances generated among fluorescent molecules. The energy transmission efficiency and the acceptor fluorescence intensity increase as the distance between the energy donor and acceptor is reduced. The fluorescence intensity is in inverse proportion to the sextuplicate intermolecular distance and is used to analyze the higher-order structures of biological molecules or intermolecular interactions. It is predicted that a structure altered by changed pH makes two fluorescent molecules approach each other to change fluorescence intensity.

As shown in Figure 4, we observed that Fluorescein which is a fluorescent molecule functioning as donor added to the end of 5′ of one DNA helix, and TAMRA which is a fluorescent molecule functioning as acceptor added to the end of 5′ of another DNA helix generate green and orange fluorescence at pH 7.0 and pH 5.0 respectively. The color changes into orange and green were not observed when no pH sensitive DNA was used. As expected, the color changes because an antiparallel-type double helix with bulge bases is formed with the Watson-Crick type base pairs at pH7.0, the distance between the fluorescent dyes increases and only green fluorescence of the donor is observed. Meanwhile, orange fluorescence of an acceptor is observed because a parallel-type double helix is formed causing an energy transmission between fluorescent molecules at pH5.0. Thus, we could construct a system that can easily identify pH changes indicated by color changes using DNA as the detecting medium.

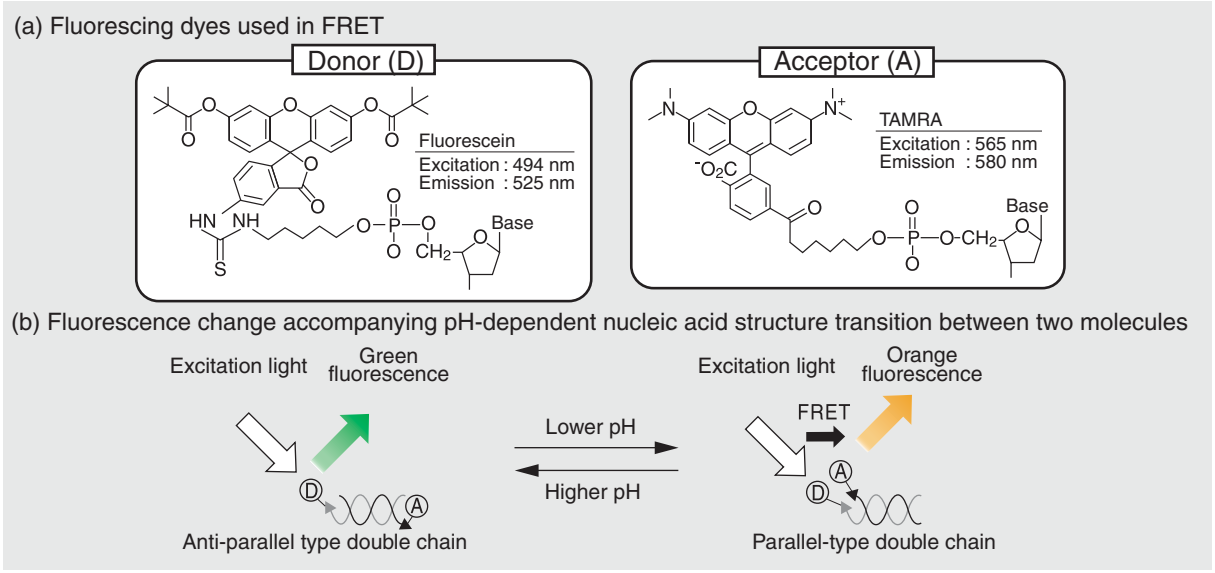

Figure 4 pH Sensor Functioning in Test Tubes

Development of a pH Sensor that Can Measure pH in Cells

Next, we focused on intracellular pH as a target for using nucleic acids as sensors. Changes of pH in cells are related to cancers or cellular deaths. Therefore, the changes can be used as indexes for diagnosing cancers or cellular deaths. Currently, organic compounds are used as reagents for the detection of intracellular pH. However, it is difficult to accurately deliver them into cells and easily measure their pH. In addition, detections with organic compounds are irreversible and not adequate for continuous measurements because they use the intracellular enzyme reactions. Consider then, that it is easier to deliver nucleic acids than organic compounds into cells. The advantages include that changes in the higher-order structures of nucleic acids caused by external factors are reversible (and they are high-speed reactions). Thus, if nucleic acids can be utilized as media to detect pH in cells, they may be used as a better method than organic compounds to measure pH.

The first-generation sensors newly developed for use in cells had drawback that the reactivity and sensitivity in the cells are reduced because bimolecular DNA was used. Therefore, we constructed a hairpin-type sensor as the second-generation sensor where the ends of 3′ of two DNA chains were connected by four T-bases (Figure 5(a)). As in the first-generation sensors fluorescein, which is a fluorescent molecule, was added to one end of 5′ behaving as a donor. TAMRA, which is also a fluorescent molecule, was added to the residual ends of 5′ behaving as an acceptor and providing green fluorescence at pH 7.0 and orange fluorescence, stronger than the first generation, at pH 5.0 (Figure 5(b)). It is supposed that the more significant changes in colors than the first generation were caused by the distance between the two dyes being reduced by the structure of the hairpin-shaped parallel-type double helix in the cells.

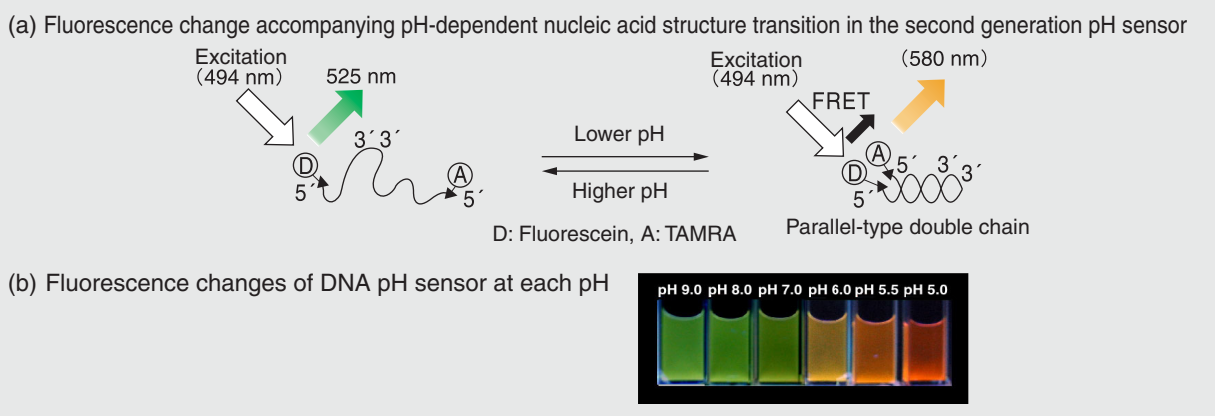

Figure 5 Second Generation pH Sensor Made of two DNA Chains Connected

Further, we introduced the DNA pH sensors into cells and tried to measure pH in cells reduced by apoptosis. The results, as shown in Figure 6, were that the pH sensor emitted green fluorescence in normal cells, and orange fluorescence in abnormal cells where apoptosis was artificially induced by adding apoptosis-inducing peptides. Thus, we found that pH can be detected not only in *in vitro* systems but also in cells by using the newly developed sensor.

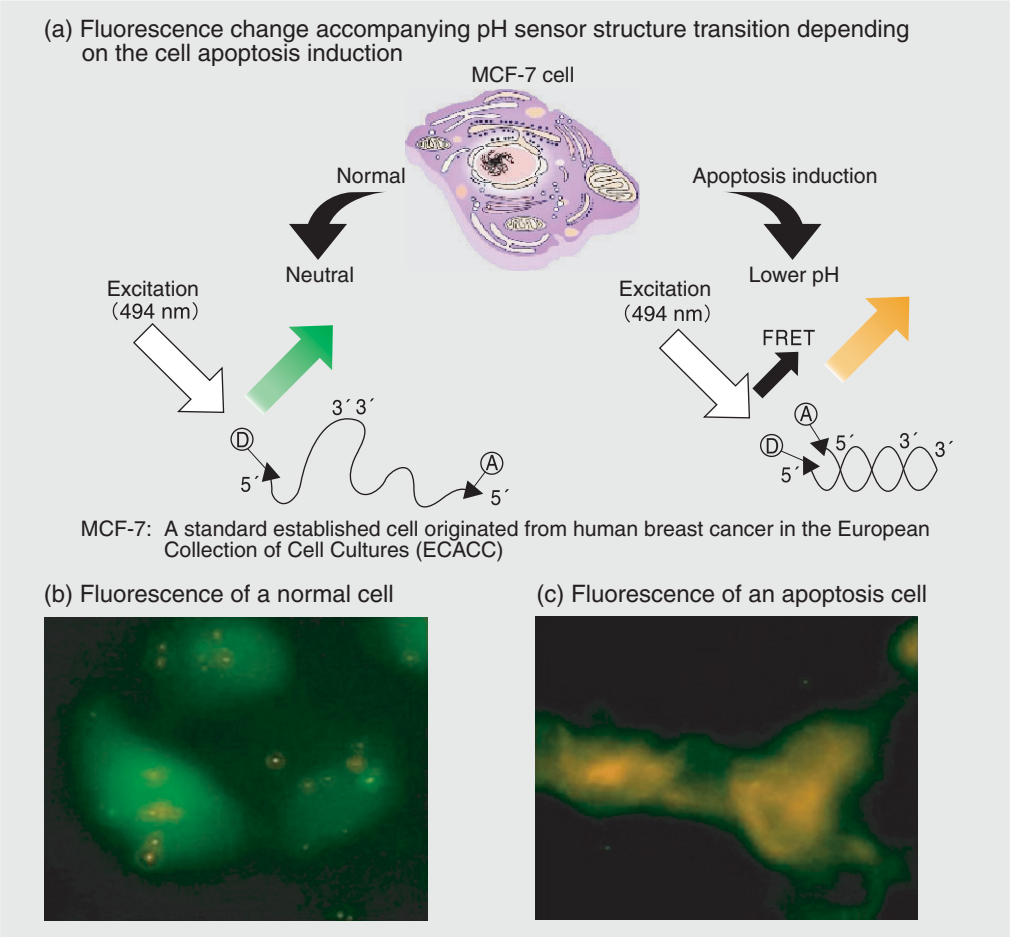

Figure 6 Measurement of Intracellular pH Reduced by Apoptosis

Conclusion

As shown above, we have developed a functional nucleic acid, based on thermodynamic data, which can measure changes in pH by utilizing the unique structure of DNA. Research into nucleic acids utilized as materials have started both at home and abroad. However, there is no example that utilizes DNA structure transitions for pH-detecting media. Therefore, this pH sensor uses an original idea and is unique.

In addition, this pH sensor is superior to the biosensors currently used because it can provide a visual indication of pH. It is expected that new systems for early diagnoses of cancers or detections of apoptosis can be developed using this sensor because it can easily measure pH in cells. Nucleic acid sensors that correspond to every environmental change will be developed through detailed studies of nucleic acid structure transitions based on the changes in environmental factors including not only pH but also metal ions[15],[16] and molecular crowding[17]-[19].

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