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Thermohypersensitive polydiacetylene vesicles embedded in calcium-alginate hydrogel beads

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Abstract−Polydiacetylenes (PDAs) are widely adapted materials for the development of sensors with liposome-like biomimetic structures, and the sensing results are often detectable with the naked eye. In addition, PDA-based sensors encapsulated within hydrogels have been intensively studied due to their superiority over solution-embedded-type and/or solid-immobilized-type sensors. Hydrogel-type PDA sensors are more stable and equipped with physically controllable high surface areas and are thus potentially utilizable in many applications. However, PDAs have intrinsic color-transitioning properties when exposed to temperatures greater than 50-60 °C, which cannot be used for more practical applications. In this study, we employed a calcium-alginate polymer to maximize the utility of a PDA-based hydrogel-type sensor and discovered that the sensor can be hypersensitive to temperature increases at a lower temperature range. We report the characterization of potential factors, gelation periods, and gelation agents that correlate with the sensitivity of the so-called PDA-alginate hydrogel. We expect that our findings can be applied in future research on industrially applicable developments for the maintenance of cold-chain delivery systems, temperature-sensitive chemicals, or food. Moreover, our materials will also provide a history of temperature changes because the corresponding color will not revert back even after the temperature decreases to the normal range.

Keywords: Polydiacetylene (PDA), Calcium-alginate Hydrogel, Biomimetic Sensors, Hydrogel Beads, Thermohypersensitivity

INTRODUCTION

Polydiacetylenes (PDAs) are highly conjugated molecules that consist of amphiphilic monomers. The monomers of polydiacetylenes are characterized by carboxylate and alkyl chains and thus have a self-assembling nature in an aqueous environment. Upon 254 nm UV light exposure, the assembled monomers undergo photopolymerization. The resulting liposome-like biomimetic vesicles with visible blue color display an irreversible color transition to red when exposed to stimuli such as mechanical stress, pH adjustments, and temperature increases [1]. Due to their distinct colorimetric response to various stresses, PDAs have been easily incorporated into liposomal vesicles for various biological sensing purposes regarding cellular components [2,3] as well as microorganisms [4-6]. Furthermore, PDA vesicles have been embedded in a number of hydrogel materials, including agar [7], polyethylene glycol-diacrylate (PEG-DA) [8], polydimethylsiloxane (PDMS) [9], polyethylene oxide (PEO) [10], polyurethane (PU) [11], and alginate [10,12-14], for advanced detection purposes.

To fabricate a more finely tuned temperature-responsive sensor, PDAs were previously exploited. Although the conventional temperature for the color transition of PDA vesicles is 60 °C [15], which is not beneficial for any temperature sensing applications, much research has been carried out to adjust the starting temperature of the color transition. To increase the sensing temperature by stabilizing the structure of the vesicles, electrophoretic methods and the incorporation of another polymer have been successfully utilized to sense temperature increases to higher than 200 °C [16,17]. However, in these sensor systems, it is difficult to fully utilize the irreversible color transition of PDAs, as the required temperatures are too high. On the other hand, in attempts to decrease the threshold temperature, several temperature sensors with threshold temperatures below the conventional color transition temperature, as low as 5 °C, were fabricated [18,19]. However, these sensors require the complicated synthesis of functional-group-substituted diacetylene monomers for PDA vesicles. Furthermore, the extra steps to prepare the PDAs conjugated with functional groups result in the loss of sensing molecules, potentially decreasing the sensing capacity of the sensors [20]. Therefore, a more comprehensive PDA-based sensor platform with modification-free PDAs and the facile immobilization of vesicles that thoroughly utilize irreversible colorimetric responses is needed.

To overcome the intrinsic limitations of solution-embedded PDA vesicles, we developed a PDA-hydrogel sensor platform for the visible detection of temperature increases in this study. In the process of development, the portability of the sensors was greatly enhanced

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by the immobilization of the sensing molecules within a hydrogel bead. Specifically, PDA vesicles were embedded in a calcium-alginate hydrogel matrix. Alginate, a naturally occurring anionic biopolymer, was selected as the hydrogel material for its ease of control of the gelation level as well as the facile integration of the desired materials into the matrix by simply mixing and dipping into Ca^{2+}-containing solutions [12-14,21-23]. Interestingly, PDA vesicles embedded in the calcium-alginate hydrogel matrix displayed further sensitive colorimetric responses, with far lower color transition temperatures than conventional PDA-based temperature sensors, allowing the detection of temperature increases at low-temperature ranges. The concentration dependency of the so-called thermohypersensitivity was also elucidated, establishing a set of references for further sensitivity tuning and utilization. Thus, with the easy fabrication and reference for sensitivity tuning, our PDA-calcium-alginate hydrogel sensor platform could be applied at the required temperatures for specific uses and be utilized in applications such as the maintenance of cold-chain systems or the delivery of temperature-sensitive chemicals, as the color transition is maintained even if the temperature is lowered back.

RESULTS AND DISCUSSION

1. Preparation of PDA-Alginate Hydrogel Beads and Their Thermohypersensitivity

The overall experimental process, from the preparation of the PDA-alginate hydrogel beads to the incubation of the beads, for investigating the thermohypersensitivity of our sensor is illustrated in Fig. 1. The PDA-alginate hydrogel precursor solution was prepared by mixing PDA vesicle solution with alginate solution. Droplets of the precursor solution were introduced to vials containing $CaCl_2$ solutions at various concentrations, and the droplets subsequently settled to the bottom of the vials during the gelation process (Fig. 1(a)). The bead-like PDA-alginate hydrogels were carefully collected and transferred to a well plate for further incubation at various temperatures (Fig. 1(b)). To characterize other potential factors affecting the thermohypersensitivity, PDA-alginate hydrogel beads were prepared with various gelation periods and gelation agents. Additionally, the distinct and superior characteristic of Ca^{2+} as an alginate gelation agent was demonstrated by comparing it to another divalent cation, Ba^{2+}, which has a higher affinity for alginate fibers.

2. Temperature- and Ca^{2+}-concentration-dependent Thermohypersensitivity

Four types of PDA-alginate hydrogel beads gelated in various Ca^{2+} concentrations at 1, 5, 10, and 20% were investigated for their temperature-dependent chromatic behaviors at various temperatures: 5, 15, 30, and 60 °C, respectively. The beads were incubated at 60 °C for 3 hours, and the incubations at other temperatures were performed for 12 hours. PDA-alginate hydrogel beads showed a chromatic shift from blue to red with respect to time and temperature during the incubation process. PDA vesicles became unstable at high temperature due to their intrinsic thermochromism [1] and produced notable chromatic changes (Fig. 2). The images of each type of hydrogel bead in the incubation conditions were captured every 15 minutes until the end of the incubation, and the intensity of the red color in the images was analyzed and calculated

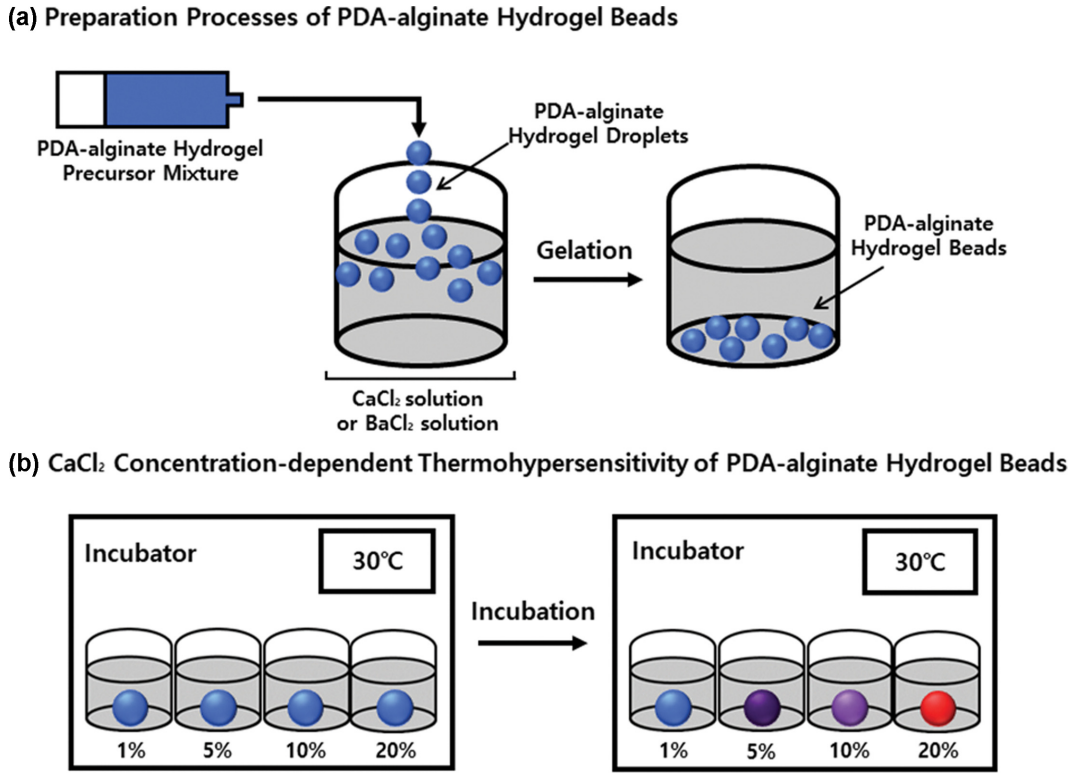

Fig. 1. (a) Overall preparation steps of PDA-alginate hydrogel beads from the injection of the PDA-alginate hydrogel mixture into calcium or barium chloride solutions to the gelation of the PDA-alginate hydrogel beads, (b) Investigation of the thermohypersensitivity at 30 °C.

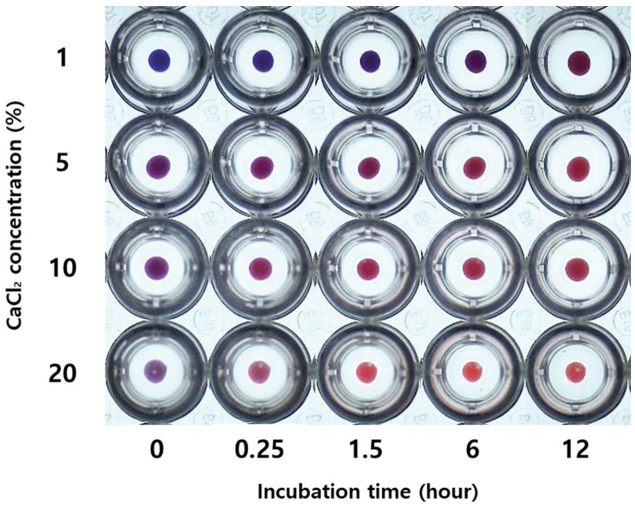

Fig. 2. PDA-alginate hydrogel beads during incubation at 30 °C; Images at 0, 0.25, 1.5, 6, and 12 hours were selected as representatives of the chromatic behaviors recognizable by the naked eye.

for mean R (%) (Fig. 3(a)-(d)).

The mean R (%) of the beads incubated at 60 °C reached a plateau in the first 30 minutes for all bead types gelated in four different Ca^{2+} concentrations, and the mean R (%) of the hydrogel beads incubated at 5 and 15 °C did not show any change throughout the 12 hours of incubation. The chromatic behavior of the hydrogel beads incubated at 30 °C varied depending on the concentration of Ca^{2+}; the maximum mean R (%) of the hydrogel beads gelated in 1 and 5% $CaCl_2$ increased by 20 and 50% compared to the initial value, respectively, for the 12-hour incubation, and that of the beads gelated in 10 and 20% $CaCl_2$ increased by 60 and 80%, respectively, in 2 hours of incubation and remained constant for the rest of the incubation period.

In contrast to the thermochromism of PDA vesicles prepared by conventional methods, the chromatic behavior of the PDA-alginate hydrogel beads in this study appeared to change at a lower temperature of 30 °C, while the PDA vesicles suspended in solution changed color at 50-70 °C [24,25]. The distinctive thermohypersensitivity of the PDA-alginate hydrogel beads is mainly attributed to the presence of Ca^{2+} within alginate hydrogels. The higher the con-

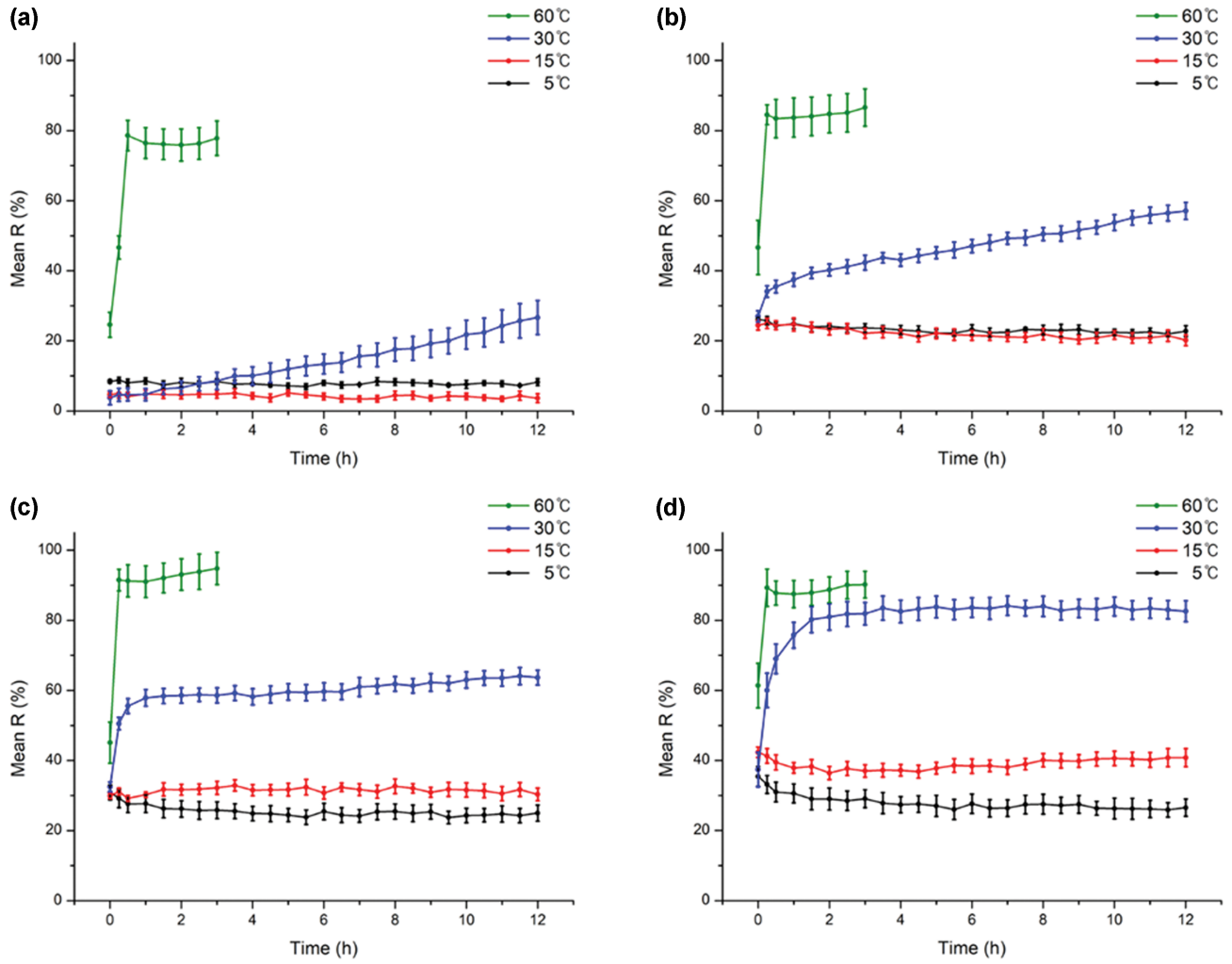

Fig. 3. Temperature-dependent colorimetric behaviors of PDA-alginate hydrogel beads in (a) 1, (b) 5, (c) 10, and (d) 20% (w/v) $CaCl_2$. Error bars represent the standard error of the mean (n=3).

centration of the calcium ions, the more the positive ions are that localize to the carboxylate groups of the PDA molecules, resulting in stronger electrostatic interactions that modify the surface charge distribution. Consequently, structural rearrangements associated with the distortion of the PDA backbone take place [26], making the PDA vesicles exhibit more extreme chromatic behavior with a higher red chromaticity.

3. Gelation-time-dependent Thermohypersensitivity

As the amount of gelation agents correlates with the thermosensitivity of the PDA-alginate hydrogel, further investigation was performed to find the correlation of gelation time with sensitivity because the number of gelation agents permeating into the hydrogel beads is highly dependent on the gelation reaction time. The correlation of the various gelation periods with the thermosensitivity of the PDA-alginate hydrogel was studied using four different gelation periods of 1, 10, 60, and 720 minutes at the same $CaCl_2$ concentration of 20% (w/v). After the assigned gelation periods, the hydrogel beads were transferred to a storage solution NaCl, which maintains a Cl^- molar concentration identical to that of the gelation agent $CaCl_2$. NaCl was chosen as the storage solution in the sense that PDA vesicles are not affected by Na^+ [27], and alginate is generally available in sodium alginate form. The incubation temperature was fixed at 30 °C to ensure that the gelation period was the only variable for this investigation.

The gelation-time-dependent thermosensitivity is shown in Fig. 4. The beads over the longer gelation periods of 60 and 720 minutes provided a larger mean R (%) difference between the initial state value and the maximum value, while those over the shorter gelation periods of 1 and 10 minutes showed smaller increases in mean R (%). The maximum mean R (%) of the beads over longer gelation periods reached nearly 80%, while those over shorter gelation periods reached approximately 40 and 45% for the 1- and 10-minute gelation periods, respectively. The difference in the mean R (%) at the initial states between the beads over the gelation periods of 60 and 720 minutes was not considered in the determination of thermohypersensitivity because the mean R (%) values of both beads saturated and plateaued at a nearly constant value.

The gelation-time-dependent sensitivity could be elaborated with the concept of captured Ca^{2+} required as a crosslinking agent for alginate fibers during gelation. Considering that the PDA-alginate mixture was dropped into the gelation solution and that alginate does not fully gelate within a 10-minute gelation period [28,29], it is reasonable to assume that the amount of Ca^{2+} delivered to alginate fibers was insufficient. In contrast, alginate fully gelates into hydrogel beads over longer gelation periods. These results support the idea that the amount of Ca^{2+} accumulated in alginate fibers is dependent on the gelation period and, thus, a larger Ca^{2+} amount is delivered to alginate fibers at an extended gelation period. Therefore, the greater portion of the PDA vesicles can be affected by the nearby captured Ca^{2+} during a longer gelation period, resulting in more structural distortion that contributes to the increase in sensitivity [26,30].

4. Gelation-agent-dependent Thermohypersensitivity

As demonstrated, the thermohypersensitivity is highly dependent on Ca^{2+}. We therefore further investigated whether the application of other divalent cations as gelation agents would yield another degree of thermohypersensitivity. Among the most prevalent cations, Mg^{2+} was excluded because gelation requires much higher concentration of gelation agents and longer time as long as several hours as compared to other divalent cations [31]. $BaCl_2$ was utilized for this investigation and prepared at 0.45 M, an equivalent molar concentration to that of Ca^{2+} used in this study, for a correlative comparison. The PDA-barium-alginate hydrogels were prepared using the same conditions that Ca^{2+} affected the thermosensitivity, a 720-minute gelation period, and 3 hours of incubation at 30 °C. However, the sensitivity of the PDA-barium-alginate hydrogel beads was very different from that of the PDA-calcium-alginate beads. A comparison of the sensitivities between those two types of beads is shown in Fig. 5. Compared to the gradual increase

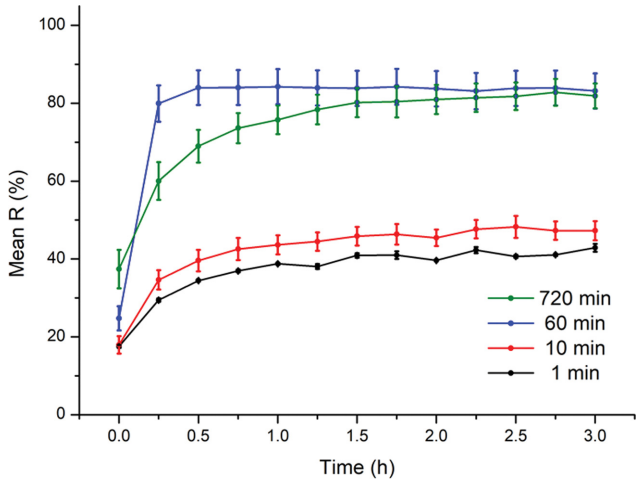

Fig. 4. Gelation-period-derived differences in the sensitivity of PDA-alginate hydrogels using 20% (w/v) $CaCl_2$ as a gelation agent at 30 °C. The gelation periods were 1, 10, 60, and 720 minutes, respectively. Error bars represent the standard error of the mean (n=3).

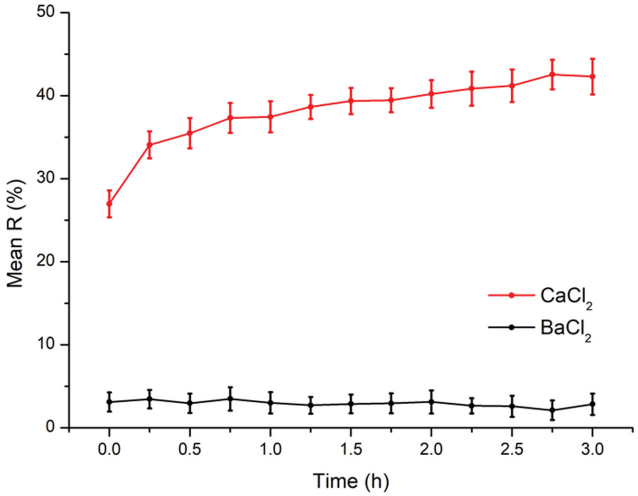

Fig. 5. Gelation-agent-derived differences in the sensitivity of PDA-alginate hydrogels at 30 °C using 0.45 M $CaCl_2$ and 0.45 M $BaCl_2$ as the gelation agents. Error bars represent the standard error of the mean (n=3).

in mean R (%) for the PDA-calcium-alginate beads, the sensitivity of the PDA-barium-alginate did not change at all throughout the 3 hours of incubation.

The zero sensitivity of the PDA-barium-alginate hydrogels is attributed to the distinct affinity of the alginate polymers to barium. Since alginate fibers have a higher affinity for Ba^{2+} than Ca^{2+} because Ba^{2+} binds to both the G- and M-blocks of alginate, whereas Ca^{2+} binds to the G- and MG-blocks that generally exist in smaller portions than the former [28,32], it is assumed that an identical amount of Ba^{2+} holds more alginate polymers compared to Ca^{2+}. Consequently, a greater portion of captured cations interacts with the carboxylate of the PDA [26,30] in the calcium-alginate than with the barium-alginate, causing more structural distortion and thus increasing the overall sensitivity of the PDA-calcium-alginate hydrogels.

5. Applications as a Temperature History Indicator

A temperature-detecting sensor was developed based on the data generated and the method introduced in this study (Fig. 3) using the optimal concentration of a gelation agent with 20% (w/v) $CaCl_2$. The developed sensor was immediately transferred and kept in a refrigerator at 4 °C before use, and no color transition of the sensor was observed while stored in the refrigerator. However, when the sensor was brought out and exposed to an environment with a temperature of 20 °C, the change in the temperature was successfully detected with an immediate color transition to red. The mean R (%) value of the sensor after the successful detection of temperature reached above 60%, and the intensity of the red color was sufficient to be recognized with the naked eye (Fig. 6). This sensor can be applied for the development of an exposure indicator to room temperature. As the sensor successfully detects a temperature increase to 20 °C, a change to a higher temperature can be detected with more prominent intensity (Fig. 3). For irreversible PDA color transitions, the utilization of the sensor in monitoring fresh food, vaccines, or other temperature-delicate product deliveries represents a great application, regardless of whether the products have been exposed to a higher temperature during delivery.

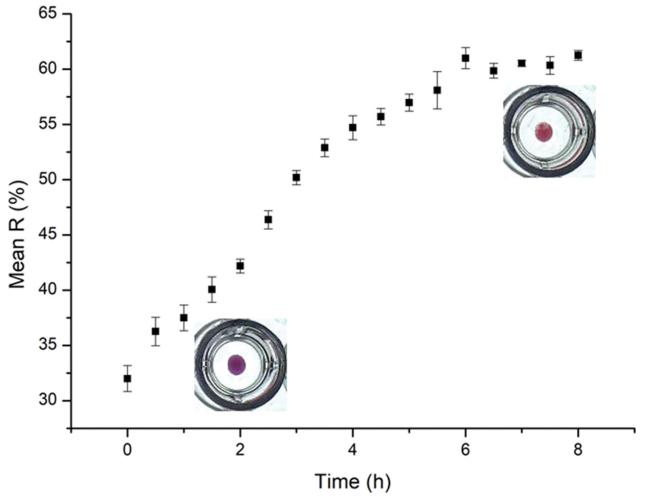

Fig. 6. Color transition of a PDA-alginate hydrogel (gelation with 20% (w/v) $CaCl_2$) at an ambient temperature of 20 °C. Figures at the bottom left and top right represent the initial and final colors of the sensor, respectively. Error bars represent the standard error of the mean (n=3).

EXPERIMENTAL SECTION

1. Preparation of PDA-alginate Hydrogel Beads

PDA vesicles were obtained using the solvent injection method [36] with minor modifications. A 2 mM PCDA (Sigma-Aldrich, Saint Louis, MO) solution was prepared by dissolving 7.492 mg PCDA in 500 μL absolute ethanol (Mallinckrodt Baker Inc., Phillipsburg, NJ) mixed with 10 mL distilled water followed by vigorous stirring for 30 minutes. The solution was incubated for 90 minutes at 80 °C in a water bath, and then large aggregates in the solution were removed using a 0.45 μm pore-sized regenerated cellulose (RC) membrane syringe filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and stored at 4 °C for more than 12 hours. Finally, the 2 mM PDA solution was exposed to 254 nm UV light for 5 minutes using handle UV exposure (VL-4. LC, VILBER LOURMAT, Torcy, France) to be prepared in the blue phase and mixed with 5% (w/v) alginate (Sigma-Aldrich, Saint Louis, MO) solution at a 1 : 1 ratio. The mixture was then dropped into $CaCl_2$ (Sigma-Aldrich, Saint Louis, MO) solution to form bead-like hydrogels.

2. Investigation of the Thermohypersensitivity

PDA-alginate hydrogel beads were obtained through a gelation process in 1, 5, 10, and 20% (w/v) $CaCl_2$ solutions. The hydrogels were kept at 4 °C for longer than 12 hours in the same solutions used for gelation. Three hydrogels from each $CaCl_2$ solution were collected and individually placed in a 96-well plate containing 300 μL of the same $CaCl_2$ solution. The 96-well plate was placed in an incubator (MIR-154-PK, Panasonic Health care Co., Ltd., Japan) and photographed every 15 minutes using a USB microscope (MSP-8000PRO, Freezone, Seoul, Korea) for 12 hours at 5, 15, and 30 °C, and 3 hours at 60 °C. The experiments were repeated for each temperature.

3. Investigation of Gelation-time-dependent Thermohypersensitivity

PDA-alginate hydrogel beads were prepared in 20% (w/v) $CaCl_2$ solution through gelation periods of 1, 10, 60, and 720 minutes. The hydrogels obtained over 1, 10, and 60 minutes were separately stored at 4 °C in a 3.6 M NaCl (Sigma-Aldrich, Saint Louis, MO) solution for longer than 12 hours before use, but the hydrogels with a 720-minute gelation period were used immediately. Three hydrogels of each gelation period were individually placed in a 96-well plate containing 300 μL of 3.6 M NaCl. The plate was placed in the incubator at 30 °C and photographed at an interval of 15 minutes for 3 hours.

4. Investigation of the Gelation-agent-dependent Thermohypersensitivity

The aforementioned method was used to prepare PDA-alginate hydrogel beads using Ca^{2+} and Ba^{2+}. The identical 0.45 M concentration of both $CaCl_2$ (equivalent to 5% (w/v) $CaCl_2$) and 0.45 M $BaCl_2$ (Junsei Chemical Co., Ltd., Japan) was utilized for gelation. The hydrogels were kept at 4 °C in each solution for longer than 12 hours. Three hydrogels obtained from each $CaCl_2$ and $BaCl_2$ solution were collected and separately placed in a 96-well plate containing 300 μL of the same solution used for gelation. The plate

was incubated at 30 °C for 3 hours while taking photographs at 15-minute intervals.

5. Chromatic Analysis of the PDA-alginate Hydrogel Beads

The thermosensitivity of the PDA-alginate hydrogel beads was shown by the blue-to-red color transition and was determined by the intensity of the red color. ImageJ was utilized to analyze photographed images to quantify the chromatic change of the PDA-alginate hydrogel beads. The images of hydrogels were analyzed in RGB mode, and the mean R (%) was calculated with the value of the red color.

$$\text{Mean R (\%)} = \frac{R_{sample} - R_0}{R_{max} - R_0} \times 100$$

Note that

R=red value among the RGB-analyzed values

R_{sample}, R_0, R_{max}=red value of the sample, minimum red value among all samples, and maximum red value among all samples, respectively.

CONCLUSION

Their significant characteristics of innate self-assembly and chromatic transitions have allowed PDAs to be widely adopted as biomimetic sensing materials. Although the physicochemical properties of PDAs have been demonstrated in previous research [1,33-35], to the best of our knowledge, an investigation of the enhanced sensitivity of PDAs embedded in calcium-alginate hydrogels is reported herein for the first time. Our findings demonstrate that the thermohypersensitivity is attributed to the presence of Ca^{2+} encapsulated in the hydrogel matrix and can be characterized by the amount of Ca^{2+} in the gelation solution and the gelation duration. We also demonstrate that a divalent cation's affinity to alginate fibers is another crucial factor determining thermohypersensitivity. The affinity of Ca^{2+} contributes significantly to the sensitivity, while Ba^{2+} has a null contribution. In conclusion, the method developed in this study is applicable to the development of a high temperature-exposure indicator that can be used in monitoring the temperature during the delivery of fresh foods or medicines, such as vaccines, that are required to be stored at a low temperature. The thermosensitivity of an indicator can be further sophisticatedly adjusted to a specific temperature to be used for the delivery of a temperature-sensitive item. We hope that our findings can be used as a means for overcoming the barriers in PDA-related research and extending corresponding applications.

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