Temperature-Controlled Conversion of Boc-Protected Methylene Blue: Advancing Solid-State Time-Temperature Indicators

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Cold-chain management is of high importance in preserving perishable products and in retaining quality. A visible marker on packages indicating complete maintenance of the cold chain assures safe consumption of products by end-users and assists in reducing waste. Time-temperature indicators (TTIs) are integrated markers that provide information about exposure of packages to adverse temperature and have been gaining increased attention by consumers. Here we present a methylene-blue-based derivative, N,N,N′,N′-tetramethyl-N,N′-Boc-phe-nothiazine-3,7-diamine (BocPTDA), that can be used as a solid-state organic TTI dye, exhibiting an irreversible change from colorless to blue green upon heating. The conversion properties, studied using a silicagel-coated plate, confirmed that BocPTDA undergoes a color change above 20°C. At temperatures of 4°C and below, no visible changes are exhibited, making BocPTDA a well-suited marker for monitoring abrupt temperature deviations indicating improper cold-chain management. Thus, application of BocPTDA-based TTI systems on packages could inform consumers about the cold-chain maintenance, assuring quality and safe consumption.

In the modern world, transport of a variety of perishable products around the globe is inevitable. Cold-chain management is therefore highly important in preserving these products’ quality. With a growing public awareness[1] and evolving legislative requirements,[2] there is a high need to track and record the cold-chain. Electronic log devices (eTLog) are prevalent in monitoring temperature during the transportation of high value sensitive products[3] such as pharmaceuticals,[4] vaccines,[5] like mRNA-based Covid-19 vaccines,[6] and refrigerated and frozen consumer food packages. But the transport data of eTLog devices is not commonly available to end-consumers, which is important if any intermittent increase in temperature (so called temperature abuse) had occurred.[7] Since eTLog devices are too expensive (~100 USD/piece) to use on food packages like those for chilled seafood and meat products, alternative time-temperature indicators are developed.[7] These TTIs exhibit a change in color indicating temperature abuse, assuring consumer about proper cold-chain management and any potential loss of quality. Several TTI systems have been designed so far, including, viscosity-based,[8] enzymatic-reaction based,[9] microbial-based systems,[10] temperature-sensitive organic dyes,[11] inorganic nanomaterials,[12] and others.[13] These TTIs have the advantage of being cheap and easy to use, as they require no additional software or hardware.

Viscosity-based TTI sensors are commercially available and broadly applied (e.g. Monitor Mark™,[14] WarmMark™,[15] and Timestrip®[16]). Here, a pigment is mixed in a viscous fluid and placed in a reservoir bulb (some sensors need to be activated at the beginning of temperature monitoring by pressing the bulb) that is connected to a flow-enabled porous wick capillary. As the viscosity of fluid changes with temperature, the flow rate in the capillary varies and the fluid travels different lengths. Upon reaching a certain point on a pre-calibrated scale, which can be defined for any particular product’s expiration in optimal storage, the product is deemed expired. However, a sharp rise in temperature for a short time (temperature abuse) followed by cooling back to acceptable temperatures is not detectable by these devices. Further, non-linear flow of fluid in the wick over large time scale scan create problems with accuracy.[17] In addition to capillary TTIs, the viscosity principle was also applied to blur a printed barcode as temperatures increase.[18]

Alternatively, enzymatic reactions have been used in design of TTIs. In these, lipases and pH indicators with esters are separated in two compartments in a bulb which are mixed by pressing the bulb at the beginning of package monitoring.[19] As the lipase hydrolyzes the ester – given that the reaction rate is dependent on the temperature – a change in pH occurs and upon reaching a certain pH-value, a change in indicator color is exhibited. While this method is precise in monitoring temperature changes, an aqueous matrix is needed for the reaction, limiting possible broad applications.

Organic molecules with inherent color affected by temperature are cheap alternatives to viscosity- and enzyme-based systems. Currently, a commercially available solid-state TTI label, OnVu®, uses a light-temperature interconvertible dye, mercocya-
nine (blue) to spiropyran (beige) (Figure 1A).[20] The printed spiropyran label is activated at the beginning of temperature monitoring. However, the blue pigment is sensitive to light exposure and needs special lamination for protection. To overcome this, a non-interconverting system was conceived by Beverina et al., based on a tert-butyloxy carbonyl (Boc-) and DMAP protected squaraine (Figure 1B).[21] This dye showed TTI behavior by undergoing a temperature-controlled removal of the Boc group and simultaneous liberation of the DMAP. Thus, on silica plates, a color change from colorless to deep blue for formation of squaraine is observed. This color change is initiated within 5 min at 25°C and fully developed in 3 h. These findings encouraged us to apply the Boc-protection strategy to other dyes for temperature sensing. Here we focused on the broadly available dye methylene blue (MB) and report the synthesis and TTI applications of a Boc-protected MB derivative (N,N,N',N'-tetramethyl-N10-Boc-phenothiazine-3,7-diamine; BocPTDA, 2), its stability on solid matrices, and detection of temperature abuse (Figure 1C).

The Boc-protected MB derivative 2 was prepared in a two-step reaction, reduction and protection, starting from commercially available methylene blue hydrochloride (see Supporting Information). A reduction with sodium borohydride, followed by treating the product with di-tert-butyl dicarbonate in basic solution and chromatographic purification yielded a pale blue solid 2 in 48% yield. Having confirmed the identity of 2, TTI experiments were carried by staining silica gel thin layer chromatography (TLC) plates with 2. TLC plates of 3 × 2 cm² size were dipped in the staining solution containing 2 at 0.2 mg mL⁻¹ in diethyl ether and dried under airflow at room temperature. The TLC plates stained with 2 were then stored at different temperatures, −20°C to 100°C, and reflectance spectra were measured at various time points to determine their temperature sensitivity and conversion, as well as their capability to sense a short time sharp rise in temperature.

Reflectance measurements of the TLC plates stained with 2 for temperatures at −20°C and 4°C showed no measurable change in reflectance for over 5 days (Figures S7, S8) and also no change in color. It is thus safe to conclude that 2 is stable at low temperatures on a TLC plate. Following this, reflectance spectra were recorded after storing the corresponding TLC plates at 20°C, 40°C, 60°C, 80°C, and 100°C for 120 minutes (Figures 2, S1 to S5). Due to the removal of the Boc group in 2 and following air oxidation, forming 1 (Scheme 1), a clear effect of temperature on reflectance values as well as a visible color change were observed. At 20°C, the 2-stained silica gel plates showed neither a detectable color change nor a decrease in reflectance for the conversion of 2 to MB in 120 minutes, indicating very slow conversion at ambient temperature. At 40°C and higher, the blue-green color formation and corresponding change in reflectance could be clearly seen (Figures 2A, 2C), accelerating with increasing temperature.
At 100 °C, the conversion was rapid (< 3 minutes) and completed in < 15 minutes, with a strong blue-green color formation due to accumulation of MB on the silica plate (Figures S5, S13). To quantify the conversion, the reflectance minimum at 660 nm was plotted against time (Figure 2C).

Analysis of the experiments indicated an exponential decrease, presumably going through a two-step reaction sequence (Scheme 1). In the first step, the Boc protection group was removed by the weak acidic nature of silica gel, resulting in N,N,N',N'-tetramethyl-10H-phenothiazine-3,7-diamine, which is then oxidized to methylene blue in atmospheric oxygen (Scheme 1).

The long term stability of 2 was evaluated at temperatures of −20 °C, 4 °C, and 20 °C. At −20 °C and 4 °C, the conversion of 2 to 1 was very slow, showing only 5% and 10% reduction in reflectance, respectively, after 136 h, without yielding a clear visible color change (Figures S7, S8). At 20 °C, a 30% decrease in reflectance and the formation of a blue green color were observed after 136 h, indicating the suitability of 2 for TTI and for long term storage (Figure 3).

We then investigated the suitability of 2 in temperature abuse conditions by varying the ideal storage temperatures for a short duration (so-called challenging experiments) (Figure 4). For this, the 2-stained silica gel TLC plates were first stored at −20 °C and 4 °C for 16 h and then were heated to 20 °C and 40 °C for 2.5 h, respectively, to mimic temperature abuse. After measuring the reflectance spectra, the samples were cooled to the original starting temperatures and stored for 24 h. Following this, a second heating to 20 °C and 40 °C was applied for 2.5 h, and the plates were cooled and stored further at the original temperatures. Before and after each temperature abuse event, the reflectance spectra were measured. As depicted in Figures 4A and 4B, heating both cold plates to 20 °C showed two significant points of decreased reflectance at 660 nm, as well as the appearance of a visible pale blue green, which agrees with continuous measurement. After heating to 40 °C, the decrease in reflectance at 660 nm was more
intense and nearly complete conversion of 2 to MB was found in first heating cycle only. The color change was strongly visible and persisted during the storage and second abuse phase. As a control experiment, TLC plates stored at a constant temperature of −20 °C and 4 °C for over 3 days showed no significant change in reflectance values.

To evaluate the suitability of 2 in temperature abuse detection after long-term storage, the TTI samples were stored at −20 °C and 4 °C for 136 h first and then heated to 40 °C for 90 min. This heating showed a significant decrease (> 30%) in reflectance (Figure 4C) and appearance of a blue-green color. This indicates that 2 is stable for long time storage and is suitable for monitoring temperature abuse events of perishable products.

In conclusion, we presented N19-Boc-protected N,N,N′,N′-tetramethylphenothiazine-3,7-diamine (BocPTDA), a derivative of methylene blue, as suitable organic time-temperature indicator (TTI) dye. The colorless dye molecule was coated on silica gel plates with ease, which, upon heating, exhibited a blue-green color through a two-step reaction sequence yielding methylene blue. The long term stability of the dye on silica plates was proved by storing the plates at −20 °C and 4 °C, respectively, for over 136 h. Different temperature abuse conditions were employed to record abrupt temperature rises and color changes, and detected the increase to 20 °C to 40 °C for 90 minutes. As no pre-activation is required for BocPTDA and the formed color is stable over a long time, BocPTDA is suitable for TTI applications to ensure the end-user about proper cold-chain management and product quality. We are currently working on modified solid matrices to detect smaller temperature rises, that is, 5 to 10 °C, for short durations from ideal cold-storage, which could be suitable for application in temperature fluctuations of poultry and meat products.

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Conflict of Interest

The authors declare no conflict of interest.

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