Supporting Information

Non-destructive Colorimetric Detection of Milk Freshness by Starch-Alginate Sensor Beads Stabilized by Nanoemulsions

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S.1. Experimental Section

S.1.1. Morphology and Resilience of SAB

Morphology of the dry SAB was assessed by capturing secondary electrons using ZEISS Sigma 300 Field Emission Scanning Electron Microscope at an acceleration voltage of 20 kV, whereas that of the wet SAB were determined using an inverted optical microscope (MX 21i, Olympus). The microemulsions encapsulated in the bead were observed using a phase contrast microscope (ch20i, Olympus).

FTIR-ATR spectra of the beads were obtained using JASCO FT/IR-4700 spectrophotometer equipped with ATR-PRO ONE, utilizing a monolithic diamond crystal for ATR analysis.

The moisture content, solubility, swelling, and leaching of the beads were calculated as follows

$$\text{Moisture content (\%)} = [(W_0 - W_1)/ W_0] \times 100 \quad \text{(S.1)}$$

where W_0 and W_1 are the weight of the beads before and after drying at 105^{o}C for 24 h, respectively.

$$\text{Swelling (\%)} = [(W_s - W_0)/W_0] \times 100 \quad \text{(S.2)}$$

where W_s is the weight of the bead after immersing it in 50 mL distilled water for 24 h.

$$\text{Solubility (\%)} = [(W_0 - W_2)/W_0] \times 100 \quad \text{(S.3)}$$

where W_2 is the dry weight of the insoluble matter after stirring the beads at 150 - 200 rpm at room temperature for 24 h.

S.1.2. Colorimetric Response of SAB

0.1 w/v % solution of BPE and 0.1 M buffer solutions of pH 1–12 were prepared. The color parameters, variation, and absorption spectra of the BPE solution were recorded.

The difference in color of BPE solution for adjacent pH was calculated as:

$$\Delta E = \sqrt{(R_0^* - R^*)^2 + (G_0^* - G^*)^2 + (B_0^* - B^*)^2} \quad \text{(S.4)}$$

where, R_0^*, G_0^*, and B_0^* are the color parameters for BPE solution at pH *x*, and R^*, G^*, and B^* are the color parameters for BPE solution at the following pH in ascending order.

0.1g of beads were immersed in 10mL of fresh milk at 25°C and 70-80% RH for 5 min. Similarly, color parameters of the bead and pH of the milk were recorded at 12 h and 24 h, respectively, and then compared with that of the fresh milk to detect spoilage.

S.1.3. Leaching

Absorbance maxima of 0.1 w/v% BPE solution in distilled water (A_B) were recorded and compared with that of the leachates of 0.1 g of SAB (A_A) immersed in 20 mL distilled water, fresh milk, and spoiled milk for 1 h. The amount of BPE leached out from the bead is calculated as

$$\text{Leached BPE per gram of SAB} = (A_A/A_B) \times 0.2 \text{ g} \quad \text{(S.5)}$$

S.1.4. Cytotoxicity assay

Cytotoxicity was evaluated using L929 mouse fibroblast cells procured from National Centre for Cell Science (Pune, India) cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, including penicillin, streptomycin, and amphotericin (5000 units). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. UV-sterilized beads were placed in 1 cm diameter onto the sub-confluent monolayers in 24-well culture plates and incubated for 1 h (maximum time that the dry SAB comes in contact with the milk sample). Morphological changes in the cells were observed using an inverted phase-contrast microscope (Olympus CKX41) equipped with an Optika Vision-Pro imaging system. Cell viability was quantified using MTT assay as

$$\text{Cell viability (\%)} = \left(\frac{A540\ of\ test}{A540\ of\ control}\right) \times 100 \quad \text{(S.6)}$$

where, A_{540} is the absorbance at 540 nm.

2. Supplementary Figures and Tables

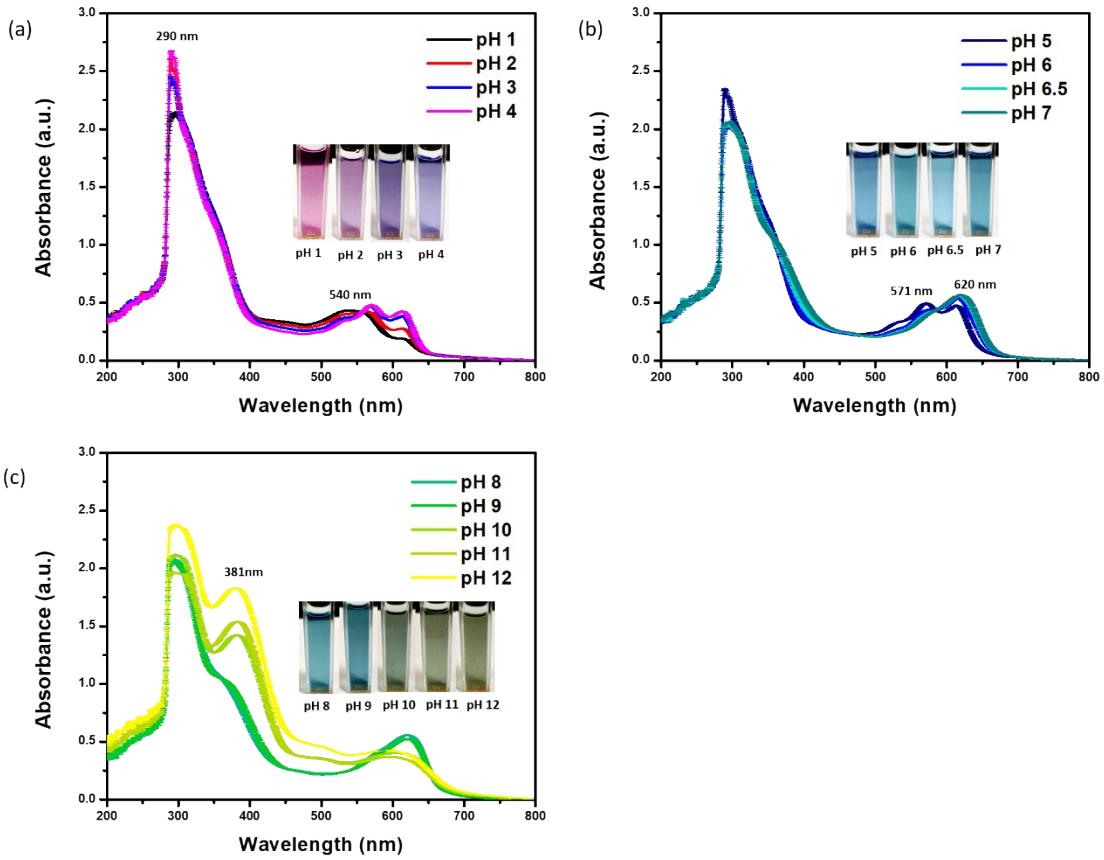

Figure S.1. (a-c) UV-visible spectra and the corresponding color variations of 0.1 w/v% BPE solution with buffer solutions ranging from pH 1-12; Data plotted with *Average ± SD*, n= 3, using Origin Pro 8.1 software.

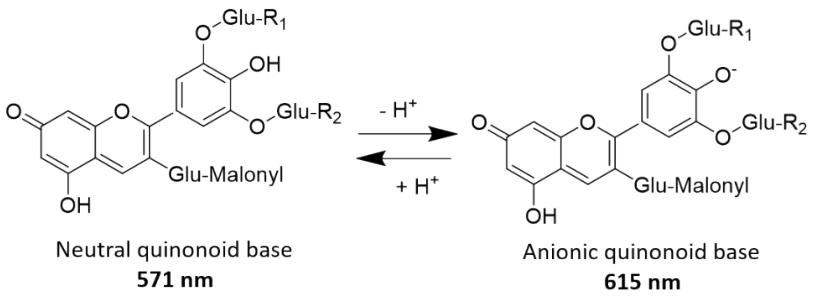

Figure S.2. Possible change in structure of ternatins at pH 5 – 7.

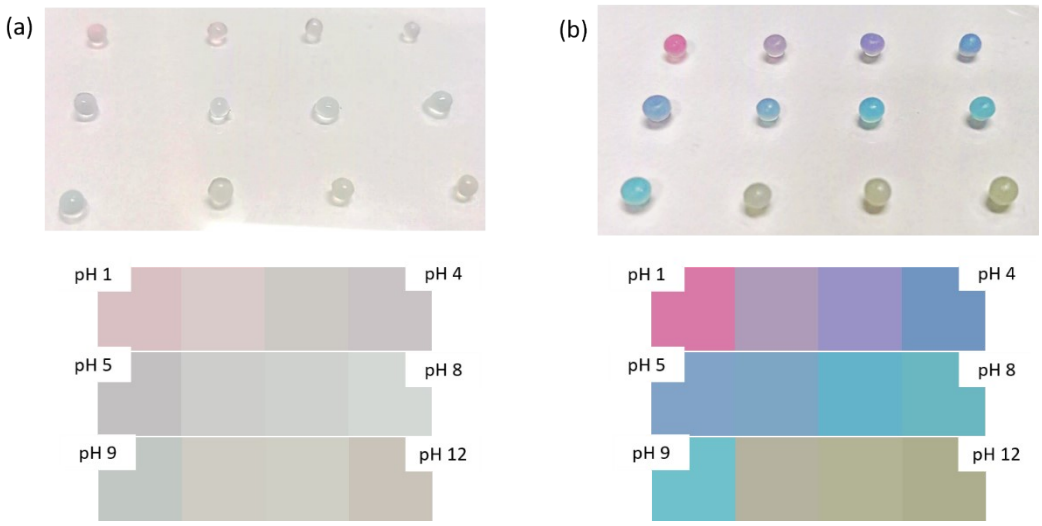

Figure S.3. Colorimetric response of (a) Ca^{2+} crosslinked starch-alginate bead infused with BPE and (b) SAB in 0.1M buffer solutions of pH 1-12.

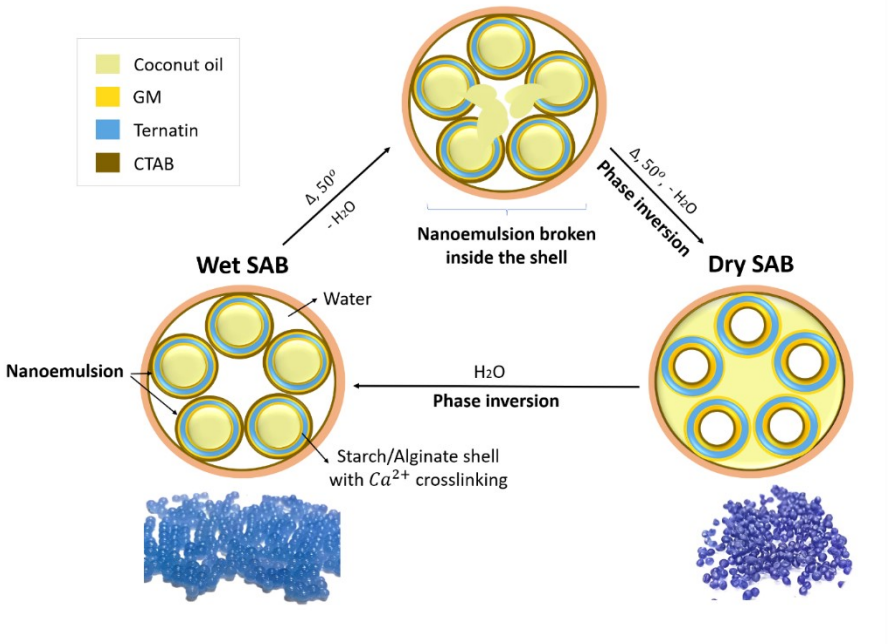

Figure S.4. Structural illustration of the reversible phase inversion in Wet and Dry SAB.

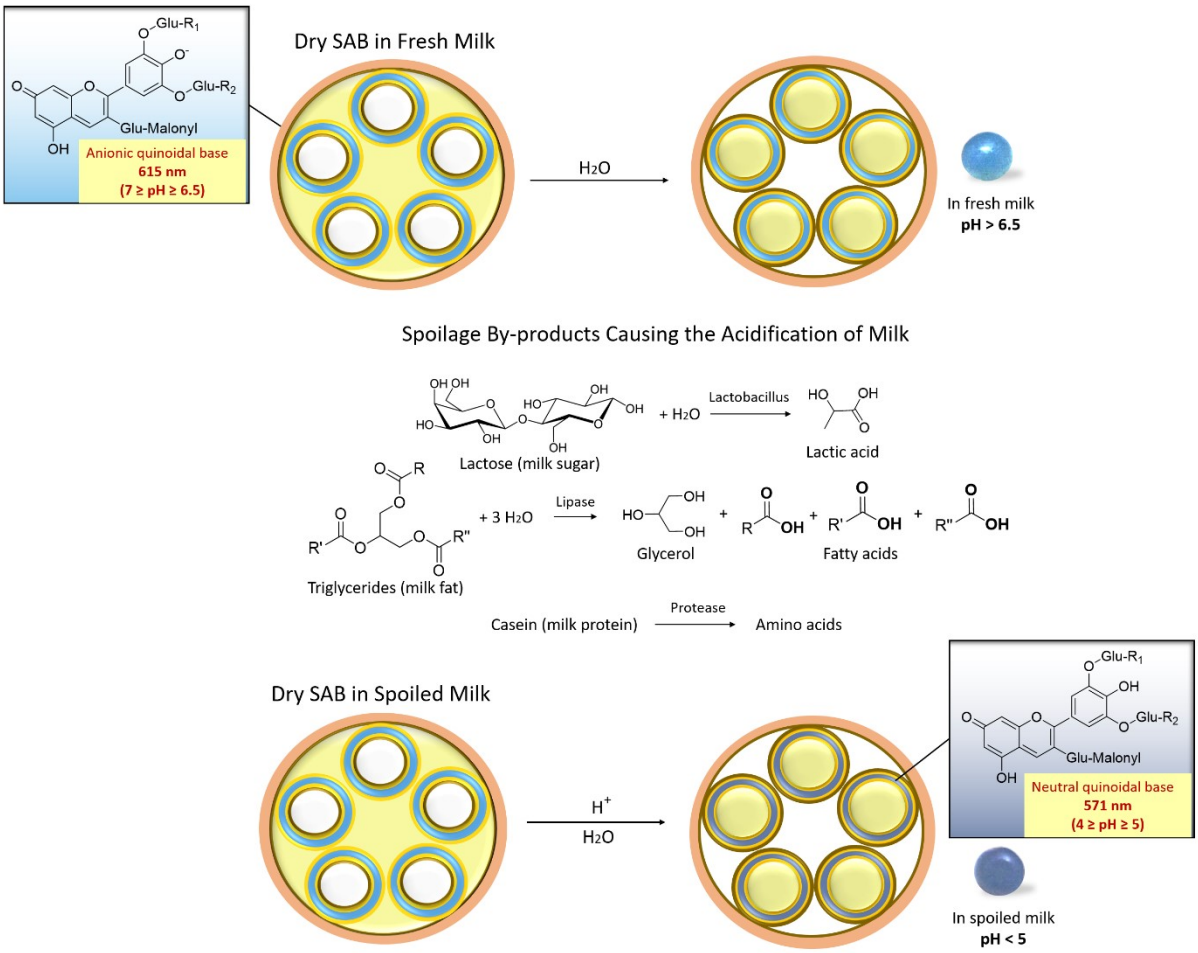

Figure S.5. Mechanistic illustration of color response of Dry SAB in both fresh and spoiled cow milk.

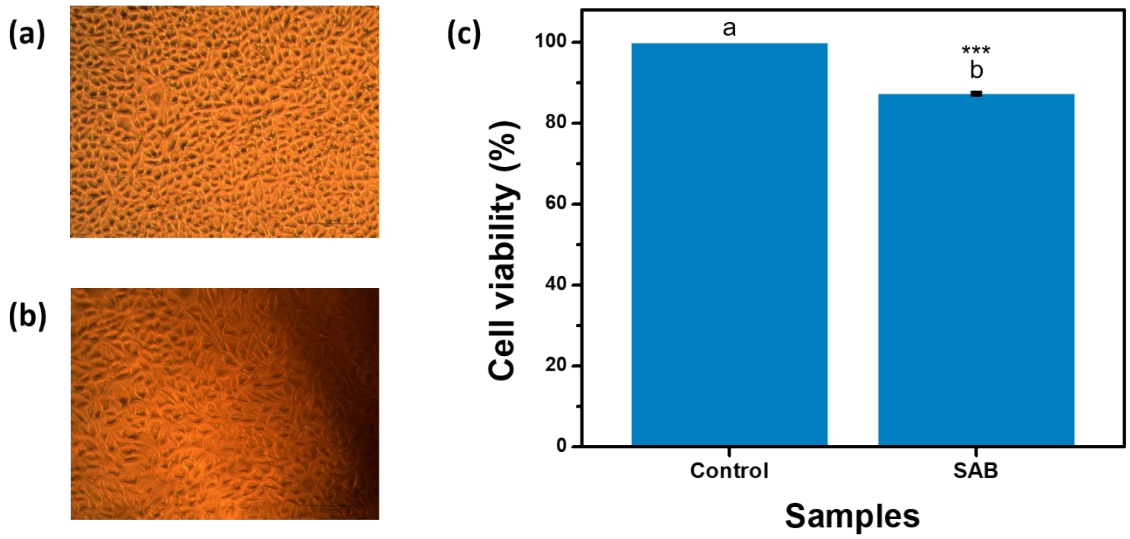

Figure S.6. Microscopic images of L-929 (Mouse fibroblast) cells in (a) control and (b) dry SAB beads during cytotoxicity assay for an incubation time of 1 h; (c) Graphical representation of L-929 cell viability obtained from MTT assay. Data plotted with *Average ± SD*, n = 3, using Origin Pro 8.1 software. Statistical analysis: *Shapiro-Wilk test*: $1 > p > 0.1$; *Tukey's HSD test:* $p < 0.05$, effect size > 1 (for alphabetical notation); and *One sample t test*: $p < 0.05$, effect size > 1 (for '*' notation).

Table S.1. Advantages and limitations of the existing techniques for monitoring milk freshness[36-38].

Type of Method/Sensor	Advantages	Limitations
Conventional microbiological methods (plate count, culturing, ELISA, PCR)	Highly accurate, standardized protocols, widely accepted for safety compliance and can identify pathogens.	Laborious and time-consuming (24–72 h or longer), require lab, equipment, and skilled personnel, unsuitable for real-time analysis and on-site monitoring, PCR cannot distinguish between live and dead cells.
pH-based sensors (glass electrodes, and synthetic pH indicators)	pH changes correlate with spoilage. Miniaturized electrodes and colorimetric pH indicators enable intelligent packaging.	Electrodes are fragile, costly, and prone to fouling. Synthetic pH indicators like bromothymol blue and methyl red are non-biodegradable, toxic if ingested in large quantities, and possess risk of leaching, thus contaminating the product.
ATP Bioluminescence	Very fast (minutes to hours), easy-to-handle, and established in food industry (HACCP).	Non-specific (detects both microbial and non-microbial ATP), and requires pretreatment for accuracy.
Impedance / Oxygen sensors (DOX, microfluidics, amperometric O_2 sensors)	Rapid quantitative detection (3–6 h). Miniaturized portable systems greatly benefit the monitoring process.	Non-specific, cause electrode fouling and drift problems, and are more expensive than simple colorimetric methods.
Commercial colorimetric respiration systems (MicroFoss, BacT/ALERT, Soleris, BioLumix,	Automated, faster than conventional methods (6–24 h) and can detect specific groups of	Require lab and equipment, cost-intensive, and unsuitable for real-time analysis.

GreenLight)	microorganisms.	
Anthocyanin-based colorimetric papers and films	Easy-to-prepare biocompatible eco-friendly indicators showing significant color changes over a wider pH range than synthetic indicators. Real time and on-site monitoring possible.	Like most synthetic indicators, color change indicating milk spoilage is often weak, unstable, and often influenced by humidity, light, and temperature.
Fluorescence-based (Riboflavin) sensor	Real time and on-site monitoring possible. Sensitive to microbial activity over time.	Requires fluorimeter. Fluorescence intensity can be influenced by light and turbidity of sample.
Electronic Nose / Tongue	Non-destructive, rapid detection of spoilage indicating VOCs and metabolites.	Require high cost and calibration, sensitive to temperature and humidity changes, can cause sensor drift and reproducibility issues.
Wireless RFID sensors & smart caps	Real time and on-site monitoring possible. Can be integrated directly into packaging. No need for battery.	Limited detection for metal-lined cartons, and inaccurate readings from milk protein adsorption.

Table S.2. Classification of ternatins based on the R_1 and R_2 groups in Figure S.2. showing the delphinidin-3,3′,5′-triglucoside core structure; C, p-coumaroyl; G, D-glucosyl.

Ternatins	**R_1**	**R_2**

Ternatin A1	CGCG	CGCG
Ternatin A2	CGCG	CG
Ternatin A3	CG	CG
Ternatin B1	CGCG	CGC
Ternatin B2	CGC	CG
Ternatin B3	CGCG	C
Ternatin B4	CG	C
Ternatin C1	CGC	None
Ternatin C2	CGCG	None
Ternatin C3	C	None
Ternatin C4	CG	None
Ternatin C5	None	None
Ternatin D1	CGC	CGC
Ternatin D2	CGC	C
Ternatin D3	C	C

Table S.3. Hydrophilicity of the beads; Data: *Average ± SD*, n ≥ 3, using Microsoft Excel 2019; Statistical analysis using Jamovi software: *Shapiro-Wilk test*: 1 > p > 0.1; *Tuskey HSD test:* ($p < 0.05$), effect size > 1 (for alphabetical notation).

Bead	**Moisture content (%)**	**Swelling (%)**	**Solubility (%)**
Wet SAB	84.42 ± 0.21^{a}	97.24 ± 1.73^{a}	87.02 ± 0.69^{a}
Dry SAB	5.46 ± 0.30^{b}	450.68 ± 30.91^{b}	29.14 ± 2.89^{b}