



OPEN Stability evaluation of oxyhydrogen and hydrogen nanobubbles under thermal and pH stress conditions

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Nanobubbles (NBs), gas-dispersed systems < 200 nm in size, are increasingly explored for therapeutic and drug delivery applications but are highly sensitive to environmental conditions. This study evaluated the stability of nanobubbles generated from oxyhydrogen (HHO) and pure hydrogen (H₂) gases under thermal and pH stress. HHO nanobubbles were subjected to heating (80 °C and 100 °C) and freezing (-17 °C), while H₂ nanobubbles were stored at pH 4–9 for seven days. Particle size (mode, mean, D50) and concentration were measured using Nanoparticle Tracking Analysis (NTA). HHO nanobubbles showed significant degradation at 100 °C, with reduced concentration and increased particle size, whereas freezing caused moderate instability. H₂ nanobubbles were most stable at neutral pH (6–7), maintaining size and concentration, while highly acidic (pH 4) and alkaline (pH 9) conditions accelerated particle disintegration. Neutral pH (6–7) is optimal for nanobubble stability, while extreme temperatures (100 °C and freezing) markedly reduce stability. These findings provide insights into designing nanobubble formulations with enhanced environmental resilience for clinical applications such as therapeutic gas infusion and advanced drug delivery.

Keywords Nanobubble, Oxyhydrogen, Hydrogen, Stability, pH, Temperature

Nanobubbles (NBs) are ultra-small gas bubbles, typically < 200 nm in diameter, characterized by unique physicochemical properties such as high internal pressure, large interfacial area, and remarkable stability in aqueous media¹. These properties make NBs highly promising for a wide range of biomedical and therapeutic applications, including as carriers for active compounds, medical gases, and infusion media for tissue regeneration². However, nanobubble stability is not absolute; rather, it is strongly influenced by environmental conditions, particularly temperature and solution pH. Temperature changes can increase kinetic energy and reduce surface tension, whereas extreme pH conditions may alter surface charge and interfacial interactions. The combination of these factors can accelerate coalescence, disintegration, or gas release from nanobubbles³. Therefore, understanding the response of NBs to environmental stress is a critical step in optimizing their formulation for clinical and pharmaceutical applications.

Most previous studies have only examined NB stability under a single condition—for example, exposure to extreme temperatures or specific pH ranges—without providing a systematic analysis of how different gas types respond to diverse environmental stresses. This study was designed as an initial step to map the stress-specific degradation profiles of two gases: HHO (hydrogen–oxygen), tested under thermal/freezing treatments, and pure H₂, tested under pH conditions. This design was chosen so that the most relevant environmental variable for each gas could be analyzed separately, thereby allowing clearer identification of degradation mechanisms. The main contribution of this work lies in providing a comprehensive time-series dataset (mode, mean, median/D50, D10, D90, and concentration) that describes nanobubble stability dynamics over time—a feature that remains scarce in the existing literature.

Two main approaches were employed: thermal treatment (heating and freezing) and pH treatment (pH 4–9) to evaluate NB physical stability. HHO was selected as the model for thermal testing due to its high reactivity and propensity to dissociate when exposed to heat. HHO, produced through water electrolysis, contains O₂ and H₂ in an explosive mixture (commonly referred to as Brown's gas), which theoretically generates higher internal

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pressure compared to single-gas nanobubbles. These characteristics make HHO an ideal model system to evaluate interfacial resilience under extreme thermal stress, such as near-boiling heating or prolonged freezing. Under such conditions, pressure differences between the constituent gases in HHO bubbles are expected to induce more dramatic changes, thereby revealing the physical stability limits of NBs⁴.

In contrast, pure H₂ was chosen as the model for pH stability testing because of its inert nature, small molecular size, and lack of reactivity toward H⁺ or OH⁻ ions. This prevents confounding chemical reactions arising from the gas itself, allowing any observed changes in NB size or concentration to be attributed directly to pH effects. Furthermore, molecular hydrogen has long been recognized in the medical literature as a therapeutic gas with antioxidant and anti-inflammatory activities and is widely used in infusion therapies across various preclinical studies^{2,5}. Thus, evaluating the stability of hydrogen nanobubbles across biological to extreme pH ranges is essential to validate their potential as safe and precise medical gas delivery systems.

This study did not assess the combined effects of pH and temperature. This was a methodological decision, as the research was designed as a stepwise investigation, with the current stage focusing on isolating single variables for each gas. Combined pH–temperature evaluations will be performed in future studies, with the present findings serving as the scientific foundation for designing more complex experimental combinations. To evaluate the effects of thermal and pH treatments on NB physical parameters, Nanoparticle Tracking Analysis (NTA) was employed. This technique enables direct monitoring of particle size distribution (mode, mean, median) and particle concentration in solution. Using this approach, the present study aims to establish a solid scientific basis for identifying optimal parameters to preserve NB physical stability. The findings are expected to support the development of NB formulations that are more resilient to environmental variations, while also expanding opportunities for their application in gas therapy, regenerative medicine, and nanobubble-based drug delivery systems.

Methods

Materials

Oxyhydrogen nanobubble (HHO) solution prepared in reverse osmosis (RO) water was used for temperature treatments, while hydrogen nanobubble (H₂) solution prepared in RO water was used for pH treatments. To maintain pH stability during sample preparation and analysis, a buffer consisting of 1% ascorbic acid and 1% sodium bicarbonate was employed.

Phosphate-buffered saline (PBS) was used as the diluent prior to analysis using Nanoparticle Tracking Analysis (NTA) to ensure that particle concentrations fell within the instrument's detection range. Additionally, sodium dodecyl sulfate (SDS) and Water One were used as rinsing solutions to clean the NTA instrument before and after each measurement, thereby preventing cross-sample contamination. All materials were prepared under sterile conditions and in accordance with laboratory protocols to ensure the integrity of the research data. No specific microbial tests were performed to confirm sterility. However, all preparations were conducted aseptically to minimize contamination.

Experimental design

Thermal and freezing treatments (HHO Nanobubbles)

To evaluate the effect of thermal stress on the physicochemical characteristics of oxyhydrogen nanobubbles (HHO NBs), several primary treatment groups were designed, accompanied by additional control groups to clarify the specific contributions of the freezing and incubation processes.

RO (Control): Post-production samples without additional treatment (baseline).

RO T80C: HHO NB samples heated at 80 °C for 10 min.

RO T100C: HHO NB samples heated at 100 °C for 10 min.

RO FREEZE: HHO NB samples frozen in a standard freezer (approximately –17 °C) for 192 h (8 days).

RO FREEZE-ONLY (control): HHO NB samples frozen and immediately analyzed after thawing, without incubation, to assess the direct effect of the freezing process.

The heating duration of 10 min was selected based on preliminary testing, which indicated that changes in nanobubble size and concentration were most pronounced within time intervals shorter than 15 min. Although only a single heating duration (10 min) and freezing period (8 days) were employed, preliminary trials using shorter and longer intervals exhibited comparable trends. Therefore, the selected time points were considered representative of short-term stress exposure.

This duration was also chosen to simulate short-term clinical use, as nanobubble suspensions are typically administered within minutes after preparation. The freezing duration of 192 h (8 days) was designed to represent short- to medium-term storage conditions relevant to biomedical applications. It is acknowledged that incorporating additional time points (e.g., 1, 3, 5, and 20 min for heating, and 1, 3, and 5 days for freezing) would provide a more comprehensive understanding of degradation kinetics. This limitation is explicitly noted and will be addressed in future studies. Two control groups were included in this study: (1) RO, consisting of post-production samples without additional treatment (baseline), and (2) FREEZE-ONLY, in which samples were frozen and immediately analyzed after thawing, to distinguish the direct effects of freezing from those related to storage.

pH treatments (Hydrogen Nanobubbles)

Hydrogen nanobubble (H₂ NB) samples were adjusted to target pH values (4–9) using 1% ascorbic acid and 1% sodium bicarbonate solutions. All samples were stored for 0, 1, 3, 5, and 7 days under refrigerated conditions (approximately 4 °C). The observation period was limited to seven days, which was chosen to represent short- to medium term storage conditions most relevant for immediate clinical preparation and use.

Target pH	Ascorbic Acid 1% (μL)	Sodium Bicarbonate 1% (μL)	H ₂ Volume (μL)	Description
4	600	0	19,400	Strongly acidic
5	400	0	19,600	Moderately acidic
6	200	0	19,800	Slightly acidic
7	0	0	20,000	Neutral (control)
8	0	200	19,800	Slightly alkaline
9	0	400	19,600	Moderately alkaline

Table 1. pH buffer composition for hydrogen nanobubble solution Treatments.

Sample	Mode (nm)	Mean (nm)	D10 (nm)	D50 (nm)	D90 (nm)	Concentrations (p/ml)
RO (Control)	85.0	249	74	175	502	7.41×10^9
TO FREEZE-ONLY (Control)	97.5	225	86	173	458	7.29×10^9
RO T80C	82.5	155	56	107	308	7.31×10^9
RO T100C	122.5	262	94	195	473	4.83×10^9
RO FREEZE	112.5	213	86	151	401	7.12×10^9

Table 2. Characteristics of nanobubbles under thermal and freezing treatments using HHO Gas.

Table 1 presents the variation in volumes of 1% ascorbic acid and 1% sodium bicarbonate used to produce solutions with specific target pH values (pH 4 to pH 9). Each solution was prepared to a fixed total volume of 20 mL using hydrogen nanobubble water as the solvent, adjusted to achieve the desired pH value. Descriptive annotations are provided to categorize the level of acidity or alkalinity of each mixture.

Measurement parameters

All nanobubble characteristics, including those subjected to thermal and pH treatments, were measured using the NanoSight Pro system (Malvern Panalytical, UK) based on Nanoparticle Tracking Analysis (NTA) technology. Analyses were conducted at room temperature precisely controlled by a Peltier module, employing a Low Volume Flow Cell (LVFC) to ensure stable flow and accurate detection. The instrument used was the NS XPLOER (Malvern Panalytical), equipped with a 405 nm violet laser for nanoscale particle detection.

Prior to analysis, all samples were diluted 50-fold with phosphate-buffered saline (PBS) to ensure that particle concentrations fell within the optimal detection range. Parameters measured included mode (dominant size), mean (average size), D10, D50, D90 (size distribution), and particle concentration (particles/mL). Camera settings such as gain, shutter speed, and laser intensity were optimized according to the particle type being observed, including adjustments for dilution factors.

Measurements were performed under the following conditions: five recordings per sample, temperature of 25 °C, 3,000 frames per recording, and a flow rate of 30 μL/min. Each data point in the figures represents the mean of five independent recordings, and the corresponding error bars indicate the standard deviation (SD) across these replicates. This approach minimizes instrument variability and improves the reliability of NTA-based size and concentration data.

Results

Thermal and freezing treatments on oxyhydrogen nanobubbles (HHO)

Table 2 presents the analysis results of nanobubble size and concentration formed in reverse osmosis (RO) water following high-temperature treatments (80 °C and 100 °C) and freezing. The measured parameters included mode, mean, size distribution (D10, D50, D90), and particle concentration.

For the RO control, nanobubbles exhibited a mode diameter of 85 nm and a mean size of 249 nm, with D10, D50, and D90 values of 74 nm, 175 nm, and 502 nm, respectively. The particle concentration remained high at 7.41×10^9 particles/mL, indicating stable baseline conditions. In the freeze-only control, the mode diameter was 97.5 nm and the mean size was 225 nm, while D10, D50, and D90 were 86 nm, 173 nm, and 458 nm, respectively. The concentration was measured at 7.29×10^9 particles/mL, which is comparable to the untreated control, suggesting that sample preparation for freezing did not markedly alter nanobubble stability.

For the 80 °C treatment, heating resulted in a reduction in particle dimensions, with the mean size decreasing to 155 nm, the D50 to 107 nm, and the D90 to 308 nm, while the mode diameter was 82.5 nm. Despite these changes in size distribution, the concentration remained stable at 7.31×10^9 particles/mL. These results suggest that moderate heating primarily reduced particle size without significantly affecting the total number of detectable nanobubbles. In contrast, the 100 °C treatment induced pronounced destabilization. The mode diameter increased to 122.5 nm, the mean to 262 nm, and the D50 to 195 nm, with a D90 of 473 nm. Importantly, the concentration decreased substantially to 4.83×10^9 particles/mL. This indicates that exposure to boiling conditions promoted agglomeration, leading to an increase in particle size and a concurrent reduction in particle number.

Finally, freezing treatment produced a mode diameter of 112.5 nm and a mean size of 213 nm, with D50 and D90 values of 151 nm and 401 nm, respectively. The concentration remained relatively stable at 7.12×10^9 particles/mL. This suggests that freezing altered size distribution, particularly by reducing the median and upper percentile diameters, but did not markedly decrease nanobubble concentration.

pH treatments on hydrogen nanobubbles (H₂)

To assess the effect of thermal and freezing treatments on nanobubble size, both mean and mode diameters were compared across all conditions (Fig. 1).

Figure 1 illustrates the effect of thermal and freezing treatments on nanobubble size distribution, as represented by mean and mode diameters. In the control sample, the mean diameter was 249 nm while the mode was 85 nm, indicating a broad distribution with a predominance of smaller particles. The freeze-only control exhibited similar values (mean 225 nm, mode 98 nm), suggesting that preparation for freezing did not markedly alter nanobubble stability. Heating at 80 °C reduced both mean (155 nm) and mode (83 nm) diameters, reflecting a shrinkage of the overall particle population without significant loss of stability. In contrast, heating at 100 °C caused a substantial increase in both mean (262 nm) and mode (123 nm) diameters, consistent with agglomeration and destabilization under boiling conditions. Freezing treatment resulted in intermediate values (mean 213 nm, mode 113 nm), indicating redistribution of particle sizes and partial destabilization, although the extent of enlargement was less pronounced than at 100 °C.

To further characterize changes in size distribution beyond mean and mode values, nanobubble diameters at the 10th, 50th, and 90th percentiles (D10, D50, and D90) were compared across thermal and freezing treatments (Fig. 2).

Figure 2 presents the effect of thermal and freezing treatments on nanobubble size distribution, as represented by the 10th (D10), 50th (D50), and 90th (D90) percentiles. In the control sample, values were 74 nm (D10), 175 nm (D50), and 502 nm (D90), reflecting a wide size distribution dominated by smaller particles but with a significant fraction of larger bubbles. The freeze-only control showed comparable results (D10 86 nm, D50 173 nm, D90 458 nm), suggesting minimal effect of sample preparation for freezing. Heating at 80 °C caused a reduction in all percentiles, with D10 decreasing to 56 nm, D50 to 107 nm, and D90 to 308 nm, indicating shrinkage of both small and large nanobubble populations. In contrast, heating at 100 °C produced enlarged distributions, with D10 94 nm, D50 195 nm, and D90 473 nm, consistent with agglomeration and instability under boiling conditions. Freezing treatment resulted in intermediate values (D10 86 nm, D50 151 nm, D90 401 nm), reflecting redistribution of the size distribution and partial loss of larger bubbles, although the changes were less severe compared to the 100 °C treatment.

In addition to size distribution, nanobubble concentration was evaluated under thermal and freezing treatments to assess the overall stability of particle populations (Fig. 3).

Figure 3 shows that the control sample exhibited a concentration of 7.41×10^9 particles/mL, while the freeze-only control yielded a nearly identical value of 7.29×10^9 particles/mL, indicating that preparation for freezing did not affect particle abundance. Heating at 80 °C maintained a similar concentration of 7.31×10^9 particles/mL, suggesting that moderate thermal exposure altered size distribution but not the number of detectable nanobubbles. In contrast, heating at 100 °C caused a marked decrease to 4.83×10^9 particles/mL, consistent with severe destabilization and collapse of nanobubbles under boiling conditions. Freezing treatment resulted in a concentration of 7.12×10^9 particles/mL, showing that although freezing modified the size profile, the overall particle number remained comparable to the controls.

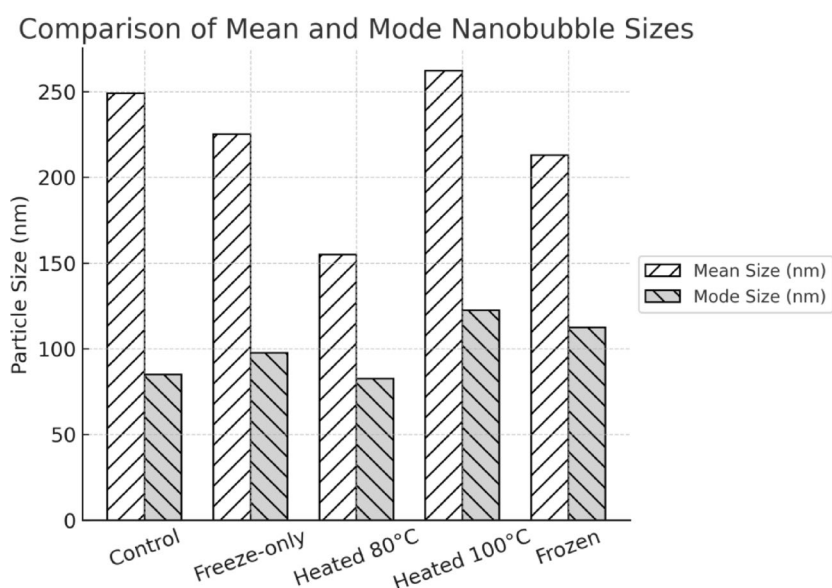


Fig. 1. Comparison of mean and mode for HHO nanobubbles sizes under thermal and freezing treatments.

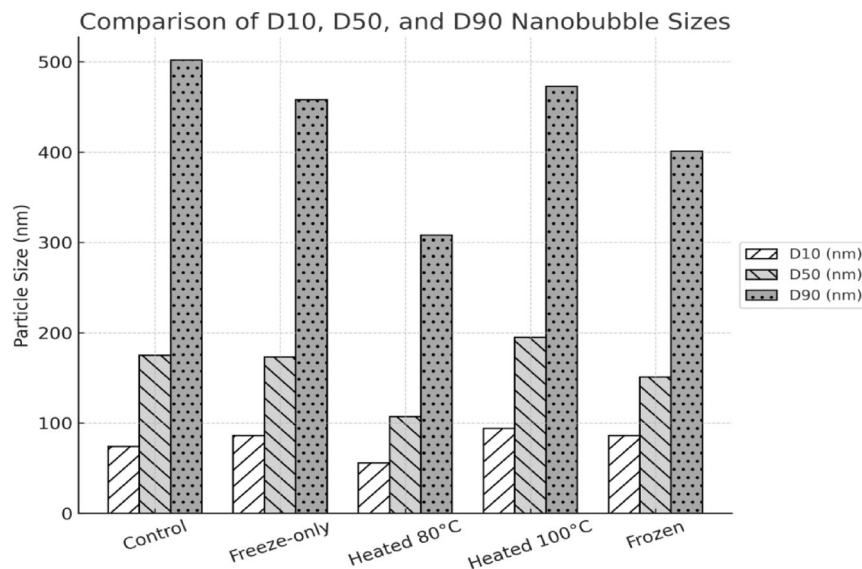


Fig. 2. Comparison of D10, D50, and D90 Nanobubble Sizes under Thermal and Freezing Treatments.

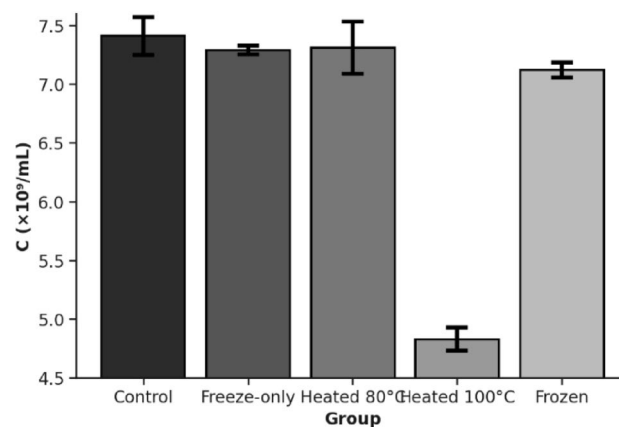


Fig. 3. Comparison of Nanobubble Concentrations under Thermal and Freezing Treatments.

pH treatments on hydrogen nanobubbles (H₂)

Following the evaluation of HHO nanobubbles under thermal treatments, the stability of H₂ nanobubbles was subsequently investigated under pH stress. As an initial parameter, changes in mode diameter were monitored across pH 4–9 during seven days of storage (Fig. 4).

Figure 4 presents the changes in nanobubble mode diameter under different pH conditions over seven days of storage. At pH 4, the mode diameter gradually increased from 112.5 nm on day 0 to 127.5 nm on day 7, indicating progressive aggregation and instability in strongly acidic conditions. At pH 5, the mode diameter fluctuated between 82.5 and 97.5 nm, reflecting an intermediate level of stability. In contrast, nanobubbles at pH 6 and 7 maintained relatively constant mode diameters of approximately 82.5–87.5 nm throughout the observation period, suggesting that near-neutral conditions provide the most favorable environment for stability. At pH 8, a slight decrease in mode diameter was observed, from 97.5 nm at day 0 to 82.5 nm at day 7. Nanobubbles at pH 9 demonstrated the highest degree of stability, with mode diameters consistently remaining within the narrow range of 82.5–87.5 nm across all time points. Error bars represent the standard deviation, highlighting the reproducibility and reliability of the measurements. Collectively, these findings demonstrate that nanobubble mode diameter is most stable under near-neutral to alkaline conditions (pH 6–9), whereas acidic environments (pH 4–5) accelerate size changes and reduce stability.

After evaluating changes in mode diameter, mean diameter was also analyzed to provide a broader view of nanobubble size distribution across different pH conditions during seven days of storage (Fig. 5).

Figure 5 shows that nanobubbles at pH 4 exhibited the largest increase in mean diameter, rising from 220 nm on day 0 to 372 nm on day 5, and stabilizing around 365 nm on day 7. At pH 5, mean diameter also increased progressively from 231 nm on day 0 to 311 nm on day 7, indicating gradual aggregation under mildly acidic conditions. In contrast, nanobubbles at pH 6 and 7 remained relatively stable, with mean diameters ranging

Mode dynamics

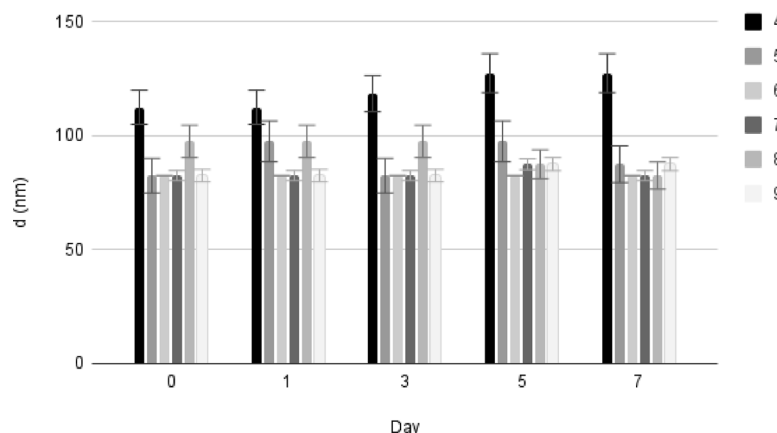


Fig. 4. Changes in nanobubble mode diameter at various pH levels (4–9) during seven days of storage. Each bar represents the mean $\pm$ SD of five independent NTA recordings. Nanobubbles maintained stable mode diameters under neutral to mildly alkaline conditions (pH 6–9), while acidic environments (pH 4–5) induced progressive enlargement, indicating reduced stability.

Mean dynamics

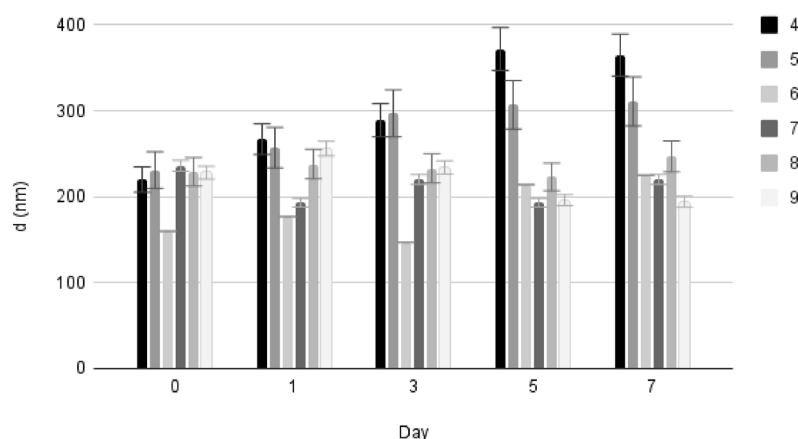


Fig. 5. Changes in nanobubble mean diameter at various pH levels (4–9) during seven days of storage. Each bar represents the mean $\pm$ SD of five independent NTA recordings. Nanobubbles maintained relatively constant mean diameters under neutral to mildly alkaline conditions (pH 6–9), whereas acidic environments (pH 4–5) caused progressive enlargement and reduced stability.

between 160 and 236 nm throughout the observation period. At pH 8 and 9, mean diameters were maintained in a narrower range (approximately 194–247 nm), with only slight variations observed across the seven days. Collectively, these findings demonstrate that neutral to mildly alkaline conditions (pH 6–9) better preserve nanobubble mean size, whereas acidic environments (pH 4–5) promote enlargement and reduced stability.

In addition to the average particle size represented by the mean diameter, further insight into the size distribution was obtained by analyzing the 10th percentile diameter (D10), which reflects changes in the smallest nanobubble population within the sample (Fig. 6).

Figure 6 illustrates the temporal changes in the 10th-percentile diameter (D10) of nanobubbles under different pH conditions (4–9) during seven days of storage. Overall, D10 values remained within a relatively narrow range of approximately 55–90 nm, indicating limited variation in the smallest fraction of the particle population. At pH 4, D10 increased slightly from 74 nm on day 0 to 82 nm on day 7. At pH 5, values remained essentially stable between 65 and 70 nm throughout the observation period. Nanobubbles at pH 6 exhibited a similarly stable profile, fluctuating modestly between 55 and 68 nm. At pH 7, minor, non-directional fluctuations were observed (62–72 nm), consistent with neutral conditions supporting relative stability. At pH 8, a transient increase to approximately 90 nm on day 5 was followed by a decline to 64 nm on day 7. At pH 9, values varied modestly between 60 and 75 nm without a consistent directional trend. Error bars represent the standard deviation, highlighting the reproducibility of the measurements.

D10 dynamics

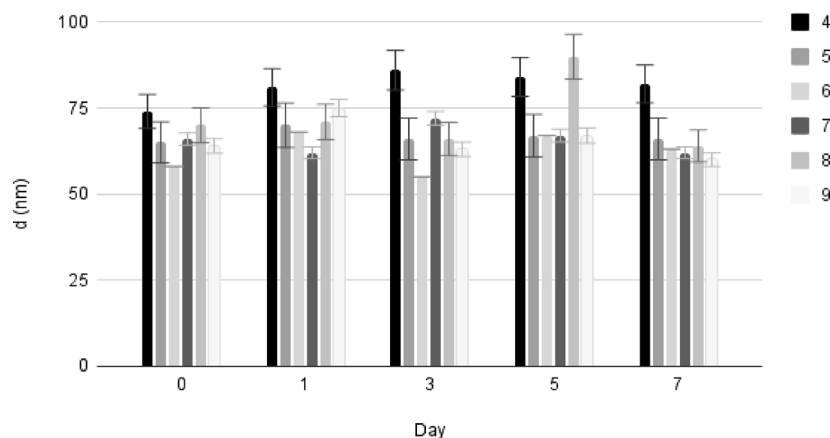


Fig. 6. Changes in nanobubble D10 (10th percentile diameter) at various pH levels (4–9) during seven days of storage. Each bar represents the mean $\pm$ SD of five independent NTA recordings. The smallest particle fraction (D10) remained relatively stable under neutral to alkaline conditions (pH 6–9), whereas acidic conditions (pH 4–5) induced slight fluctuations over time.

D50 dynamics

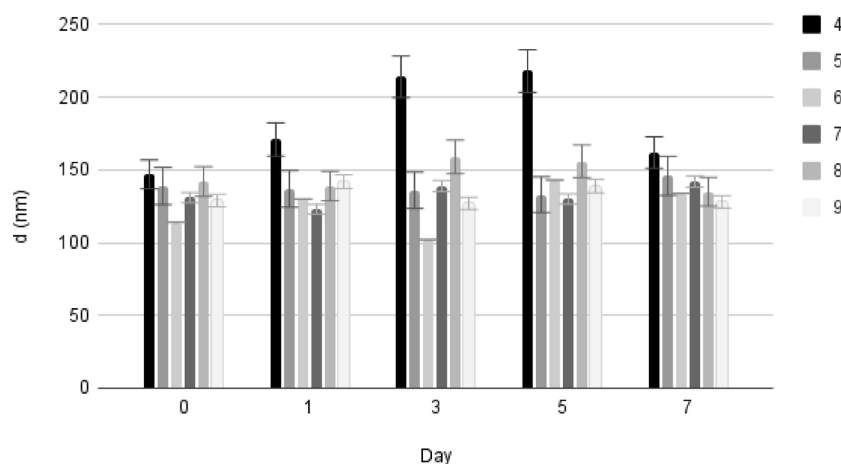


Fig. 7. Changes in nanobubble D50 (median diameter) at various pH levels (4–9) during seven days of storage. Each bar represents the mean $\pm$ SD of five independent NTA recordings. Median particle size (D50) remained consistent under neutral to mildly alkaline conditions (pH 6–9), whereas acidic environments (pH 4–5) led to larger fluctuations, indicating reduced structural stability.

To provide further insight into the size distribution, the results of the 50th percentile diameter (D50), representing the median particle population, are presented next and are illustrated in Fig. 7.

Figure 7 illustrates the temporal changes in the 50th-percentile diameter (D50), representing the median particle size, of nanobubbles stored under different pH conditions (4–9) during seven days of storage. Overall, D50 values ranged between approximately 110–220 nm, with distinct differences observed across pH levels. At pH 4, D50 increased markedly from 147 nm on day 0 to 218 nm on day 5, before declining to 162 nm on day 7, indicating strong instability under acidic stress. At pH 5, values remained relatively stable between 133 and 146 nm, suggesting transitional but preserved stability. Nanobubbles at pH 6 exhibited moderate variability, fluctuating between 102 and 143 nm, reflecting mild instability. At pH 7, D50 remained consistent within 132–142 nm, confirming stability under neutral conditions. At pH 8, values decreased gradually from 118 nm to 110 nm, consistent with moderate alkaline effects. At pH 9, D50 values were maintained within 127–142 nm, indicating stable behavior under strong alkaline conditions. Error bars represent the standard deviation, emphasizing the reproducibility of the measurements.

After evaluating the median particle size through D50, further insight into the upper tail of the size distribution was obtained by analyzing the 90th percentile diameter (D90), which reflects the larger nanobubble population within the samples (Fig. 8).

D90 dynamics

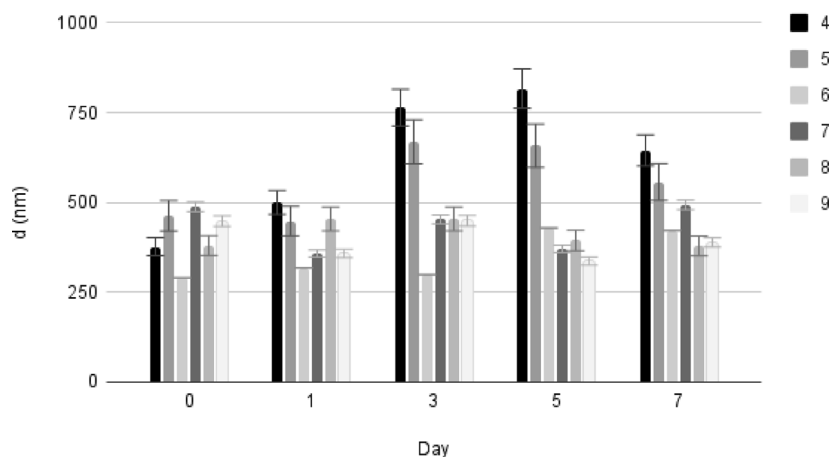


Fig. 8. Changes in nanobubble D90 (90th percentile diameter) at various pH levels (4–9) during seven days of storage. Each bar represents the mean $\pm$ SD of five independent NTA recordings. Larger particle fractions (D90) remained stable under neutral to alkaline conditions (pH 6–9), whereas strongly acidic conditions (pH 4–5) caused pronounced increases in D90, indicating aggregation and reduced stability.

Figure 8 illustrates the temporal changes in the 90th-percentile diameter (D90), representing the larger fraction of nanobubbles, under different pH conditions (4–9) during seven days of storage. At pH 4, D90 increased markedly from 377 nm on day 0 to 817 nm on day 5, before decreasing to 645 nm on day 7, reflecting pronounced aggregation and instability in strongly acidic conditions. At pH 5, values followed a similar trend, rising from 463 nm on day 0 to 669 nm on day 3 and 658 nm on day 5, before decreasing to 557 nm on day 7. At pH 6, D90 values fluctuated moderately between 290 and 429 nm, indicating intermediate stability. At pH 7, values varied between 358 and 493 nm without a consistent directional trend, suggesting partial instability even under neutral conditions. At pH 8, D90 remained relatively stable, ranging between 379 and 454 nm across the storage period. At pH 9, values were also stable within 337–449 nm, suggesting preserved structural integrity under alkaline conditions. Error bars represent the standard deviation, highlighting the reproducibility of the measurements.

Discussion

At baseline, freshly generated nanobubble suspensions exhibited a mode diameter of approximately 80–90 nm and a particle concentration of around 7×10^9 particles/mL, indicating a dense and uniform initial population. These initial characteristics likely contributed to the overall stability observed under moderate stress conditions (e.g., 80 °C heating and freezing), since smaller and more homogeneous nanobubbles are generally more resistant to coalescence and gas diffusion. Conversely, any deviation from this uniformity may have accelerated destabilization when exposed to stronger stress factors, such as high temperature or extreme pH.

The findings of this study indicate that nanobubble (NB) stability is strongly influenced by the type of gas used during formation, as reflected by the distinct responses of HHO and H₂ nanobubbles under thermal and pH stress, respectively. However, a direct comparison of HHO and H₂ nanobubbles under identical stress conditions was not performed in this work. Future studies are therefore required to determine whether the observed differences arise from intrinsic gas properties or from the specific stress environments applied.

Thermal Stress. For nanobubbles generated using HHO gas (oxyhydrogen), exposure to elevated temperatures had a pronounced impact on their physical integrity. Heating to 100 °C resulted in a substantial increase in both mean and median particle size, accompanied by a sharp reduction in particle concentration. This phenomenon can be explained by several physicochemical mechanisms. At high temperatures, increased kinetic energy elevates internal Laplace pressure, leading to gas expansion and accelerated dissolution of nanobubbles into the surrounding medium. Once Laplace pressure exceeds the stabilizing forces of the interfacial layer, nanobubbles tend to coalesce and rupture, releasing gas into the environment. Simultaneously, disruption of the electrical double layer (EDL) at the bubble–water interface weakens the electrostatic repulsion that normally stabilizes nanobubbles, further promoting collapse. These mechanisms are consistent with established theoretical models describing the dynamic balance between Laplace pressure, gas diffusion, and interfacial charge stabilization^{6,10,11}.

Freezing Stress. Freezing treatment for eight days at –17 °C also altered nanobubble characteristics, as reflected by decreased particle concentration and changes in size distribution. These effects are most likely caused by ice crystallization, which can disrupt the interfacial structure of nanobubbles, damage their protective surface layer, and compromise their ability to maintain internal pressure. Nevertheless, compared with thermal stress, freezing primarily affected nanobubble morphology rather than inducing complete disintegration.

pH Stress. In contrast, nanobubbles generated using pure hydrogen gas (H₂) exhibited greater resistance to pH variation, particularly within the neutral range (pH 6–7). Under these conditions, physical parameters such as mode, mean, and median diameters remained stable throughout the seven-day storage period, and particle

concentration also remained relatively high. This suggests that near-neutral pH provides an optimal balance between interfacial tension and electrostatic repulsion. At physiological pH, the concentration of H^+ and OH^- ions is balanced, helping stabilize the nanobubble–water interface by maintaining surface charge distribution and suppressing coalescence. This balance minimizes fluctuations in Laplace pressure, thereby contributing to the preservation of nanobubble stability^{7,8}.

Under strongly acidic conditions (pH 4), particle size increased sharply while concentration declined drastically from the first day, reflecting rapid disintegration triggered by the high concentration of H^+ ions. These ions interact with the nanobubble interface, disrupting surface stability and promoting gas release and coalescence⁹. At pH 5, degradation occurred more gradually, representing a transitional state between unstable and stable conditions. Conversely, under alkaline environments (pH 8–9), nanobubbles showed moderate stability, with slight fluctuations in size and a gradual decrease in concentration between days 3 and 7. The reduction in stability under alkaline conditions may be attributed to OH^- ions, which alter interfacial tension and surface charge distribution, weakening the EDL and promoting gas diffusion into the surrounding medium. Previous reports have also shown that OH^- ions can modify interfacial water structure and destabilize colloidal systems^{9,11}. Moreover, because ascorbate and bicarbonate ions were used as buffering agents in this study, their potential effects on ionic strength and redox balance at the bubble interface cannot be excluded and warrant further investigation.

Initial pH Differences. At day 0, slight variations were already evident among the initial hydrogen nanobubble samples prepared at different pH levels. Samples generated under acidic conditions (pH 4–5) exhibited slightly larger mean diameters and lower concentrations compared with those prepared at neutral or alkaline pH. These initial differences suggest that proton enrichment at low pH may immediately weaken the interfacial charge balance, even before prolonged storage, thereby predisposing nanobubbles to faster disintegration. In contrast, samples prepared at neutral to mildly alkaline pH (6–9) displayed smaller and more uniform size distributions at baseline, which likely contributed to their greater stability during subsequent storage.

Mechanistic Interpretation. The overall findings align with fundamental theories of nanobubble behavior. Nanobubbles are generated through cavitation, electrolysis, or gas supersaturation, forming nanoscale gas domains stabilized by high internal Laplace pressure. Their persistence in liquid is attributed to the presence of surface charge and the EDL, which suppress coalescence via electrostatic repulsion. Over time, nanobubbles gradually decay through gas diffusion, coalescence, or interfacial collapse processes that are accelerated under thermal or pH stress. These mechanisms explain the vulnerability of HHO nanobubbles to high-temperature disruption and the relative stability of H_2 nanobubbles at neutral pH^{10,11}.

Supporting these findings, internal monitoring at the Indonesian Molecule Institute (IMI) has demonstrated that HHO-based nanobubbles can reach concentrations as high as 2.99×10^{10} particles/mL¹². Although not directly part of this study, these internal data provide additional context and highlight the potential of local technology to generate high-density nanobubble formulations, which may improve efficiency in therapeutic gas delivery and regenerative applications.

Limitations and Future Implications. Several limitations should be acknowledged. First, only single-variable stress conditions were examined thermal and freezing for HHO nanobubbles, and pH variation for H_2 nanobubbles while combined stress evaluations were not performed. Second, the observation period was limited to seven days, representing short- to medium-term storage; longer-term assessments are necessary to evaluate true stability for pharmaceutical use. Third, characterization relied solely on NTA without complementary analyses such as zeta potential or interfacial chemistry, which would provide a deeper mechanistic understanding. Fourth, microbial contamination assays were not conducted, even though all preparations were performed aseptically; thus, biological factors potentially affecting stability cannot be completely ruled out. Addressing these limitations in future studies will help establish a more comprehensive understanding of nanobubble resilience and support their development for clinical and regenerative applications.

Conclusion

This study systematically evaluated the stability of HHO and H_2 nanobubbles under thermal, freezing, and pH stress by tracking multiple parameters, including mode, mean, D10, D50, D90, and particle concentration. Distinct degradation patterns were observed: HHO nanobubbles were highly sensitive to boiling and freezing, whereas H_2 nanobubbles maintained maximal stability at neutral pH. These findings provide new insights into gas specific stability mechanisms and underscore the value of multi-parameter analysis for nanobubble characterization. The results establish a foundation for developing more robust nanobubble formulations, and future work should include cross-condition comparisons, zeta potential analysis, and extended storage studies to advance clinical and regenerative applications.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

V.A. performed the experimental work, including sample preparation, nanobubble characterization, and data analysis. She also drafted the initial manuscript and contributed to subsequent revisions. A.H. conceptualized and designed the study, provided supervision and technical guidance, and contributed to data interpretation and manuscript revision. Both authors read and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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