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Investigating BioCaRGOS, a Sol–Gel Matrix for the Stability of Heme Proteins under Enzymatic Degradation and Low pH

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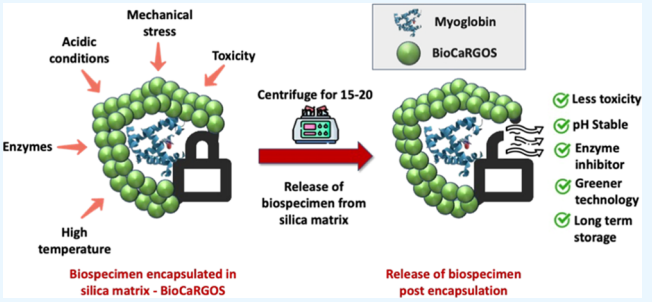
ABSTRACT: There have been significant advances in the development of vaccines for the prevention of various infectious diseases in the last few decades. These vaccines are mainly composed of proteins and nucleic acids. Poor handling and storage, exposure to high temperatures that lead to enzymatic degradation, pH variation, and various other stresses can denature the proteins or nucleic acids present in any vaccine formulation. Therefore, it is necessary to maintain a proper environment to preserve the integrity of biospecimens. To overcome these challenges, we report a practical and user-friendly approach for sol−gels called "BioCaRGOS" that can stabilize heme proteins not only in the presence of degrading enzymes and acidic pH but simultaneously maintain stability at room temperature. Heme proteins, such as myoglobin and cytochrome c, have been used for this study.



INTRODUCTION

Therapeutic proteins make up an important class of biomolecules that are used clinically in a wide range of treatments. The advantages of protein-based drugs include better specificity and fewer side effects relative to other methods. They are also considered safer than gene therapy as they do not alter the genetic structure.[1] Significant challenges to the use of therapeutic proteins include their large molecular size and degradation due to low chemical and physical stability.[2−4] Of these, harsh process conditions such as elevated temperature, pH variation, organic solvents, and deactivation enzymes are the major barriers. Due to this, most proteins are typically stored at low temperatures (≤4 °C) resulting in a dependence on refrigeration for storage and cold-chain for transportation.[5] As an alternative to cold storage, various techniques such as addition of stabilizing agents (sugars, salts, and polyols),[6,7] immobilization of the protein onto surfaces, and encapsulation of proteins into hydrated organic and inorganic matrices have been employed. Two of the most common techniques for protein immobilization are sol−gel encapsulation and adsorption onto inorganic porous materials (e.g., silica, activated carbon, aluminum oxide, iron oxide, and metal−organic frameworks).

Silica sol−gel is a porous and amorphous material that can be synthesized at low temperatures and mild pHs. Over the past three decades, there has been considerable advancement in the sol−gel stabilization of proteins and nucleic acids. However, these techniques have certain limitations, such as the use of a highly concentrated silica precursor (30−50 v/v % TMOS/TEOS), the presence of a methanol byproduct, and lengthy synthetic procedures. Additionally, the glass-like matrix of silica sol−gels requires the use of harsh chemicals to release the biospecimen. To overcome these challenges, we have developed a biocompatible and a greener approach that avoids the use of acids, bases, or alcohols for synthesis while providing facile recovery of the biospecimen. Our BioCaRGOS (Capture and Release Gels for Optimized Storage of biospecimens) system utilizes a short microwave treatment for the hydrolysis of tetramethyl orthosilicate (TMOS). The resulting solution is then subjected to rotary evaporation to remove the methanol byproduct from the desired orthosilicic acid [$Si(OH)_4$]. Previously, we demonstrated excellent stability of various proteins and nucleic acids, such as hemoglobin and miRNA 21, in BioCaRGOS and developed separation strategies to extract biospecimens, such as KRAS ctDNA g12D, post encapsulation for downstream compatibility.[8−11]

In the current study, we have utilized BioCaRGOS to investigate the stability of the heme proteins myoglobin (Mb) and cytochrome c (Cyt c) and advance BioCaRGOS to be applicable in downstream processes. The two proteins were selected as they have different isoelectric points and are susceptible to denaturation in the presence of proteinase K under low pH conditions. Further, their folding mechanisms in solution are well characterized and easy to assess via

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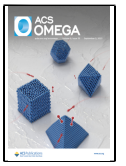

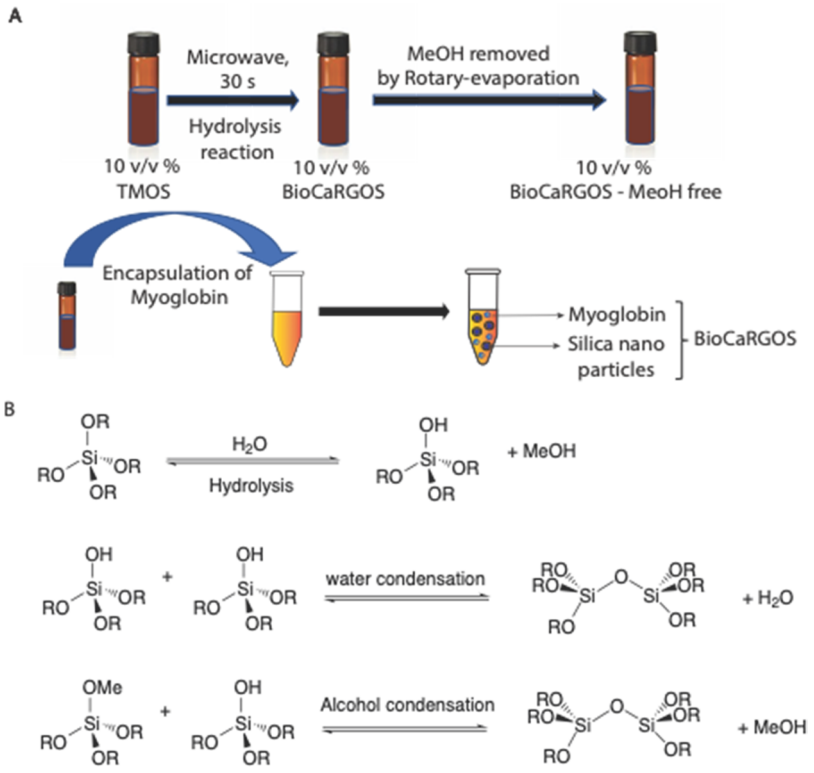

Figure 1. (A) Schematic overview of BioCaRGOS synthesis and Mb encapsulation. (B) Mechanism of BioCaRGOS sol−gel synthesis.

spectroscopic techniques. Mb, a heme-containing protein used for oxygen storage in muscles, has been the subject of examination with regard to protein structure and its stability against protease digestion has been quantified by monitoring change in heme absorption at 409 nm as a function of proteinase K-induced unfolding.[12−14] Cyt c is primarily known for its function in the mitochondria as a key participant in the life-supporting functions of adenosine triphosphate (ATP) synthesis and electron transfer processes in the respiratory system. Due to the presence of the heme prosthetic group, it has gained importance for catalysis in the presence of organic solvents. It can be also used for various other applications such as electron transfer elements between electrodes and surface-bound enzymes.[15] This makes it very crucial to improve the stability of Cyt c under harsh conditions for various applications.

Herein, we report BioCaRGOS as an efficient and greener silica sol−gel approach for the preservation of Mb and Cyt c. The BioCaRGOS strategy includes an efficient release of the protein in its native form from the sol−gel matrix without the use of toxic reagents or harsh conditions. The structural stability of the proteins in BioCaRGOS was established by using various techniques such as ultraviolet−visible (UV−vis) and circular dichroism (CD) spectroscopies. To further confirm the long-term stability in the presence of harsh conditions, we demonstrated excellent stability of Mb in the presence of proteinase K at ambient temperature (25 °C).

EXPERIMENTAL SECTION

Methods. Tetramethyl orthosilicate, sodium chloride, freeze-dried Mb, Cyt C from equine heart with purity >95%, proteinase K, sodium phosphate monobasic, sodium phosphate dibasic, 15.0 mL centrifuge tubes, and UV−vis cuvettes were purchased from Sigma-Aldrich (Saint Louis, MO). Nuclease-free water was purchased from New England BioLabs (MA). The 96-well plates were purchased from Thermo Fisher Scientific (MA). Methanol elimination was performed on Buchi R-124 Rotary Evaporator System (Delaware). A microwave oven (Panasonic NN-SN651B) (400−600 W) was used for 30 s for BioCaRGOS synthesis.

BioCaRGOS Synthesis. A 10.0 v/v % TMOS stock solution was prepared in doubly deionized water and transferred to a 40.0 mL glass vial. The vial was closed with a screw cap and heated via microwave for 30 s to initiate hydrolysis. Post microwave, the solution was then subjected to rotary evaporation for 30 min at a controlled pressure (25 mbar) and temperature (45 °C) to remove the methanol byproduct. This methanol-free TMOS stock solution was cooled to room temperature. After the equilibrium was reached, doubly deionized water was added to formulate the final desired concentration of 0, 1, 2.5, 5, and 7.5 v/v %.

Encapsulation of Protein in BioCaRGOS. Encapsulation of proteins (Mb and Cyt c) was carried out in BioCaRGOS by the addition of 0.06 mL of 10 mg/mL stock to achieve a final concentration of 0.2 mg/mL to which (0−7.5) v/v % BioCaRGOS was added and 0.5 M phosphate buffer pH 7.4 was added to constitute the remainder of the 3.0 mL solution.

Release of Mb Post Encapsulation. Encapsulated Mb was separated from BioCaRGOS matrix using Millipore Ultrafree MC 0.22 μm centrifugal filters (Billerica, MA) spun for 15 min at 14,000*g*. The aqueous filtrate was collected to quantify the amount of Mb released by UV−vis spectroscopy and DLS studies.

Spectroscopic Characterization. Absorbance measurements in the range of 260−700 nm were carried out using a Varian Cary 50 Bio UV−vis spectrometer, Agilent Technologies, Santa Clara, CA. Quartz cuvettes were used for the measurements. Circular dichroism (CD) spectra were

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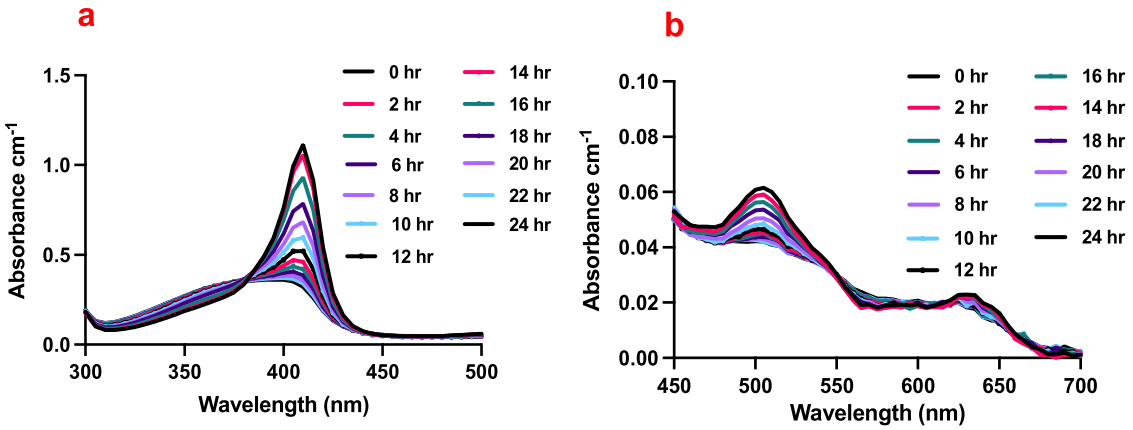

Figure 2. UV−vis spectra of metaquo Mb upon addition of 0.1 wt/v % proteinase K at 25 °C at pH 7.4 over a 24 h period. The Soret bands of metaquo mb (409 nm) and the denatured, free heme are shown in (a), while the Q-band region is shown in (b). The 633 nm band in the Q region is from metaquo Mb.

recorded on a JASCO J-815 spectropolarimeter (JASCO Corporation, Tokyo, Japan) using a cylindrical quartz cell with a path length of 1 mm. Changes in the secondary structure of the protein were monitored in the far-UV region between 190 and 260 nm. Four consecutive scans were averaged and corrected by subtracting the corresponding blanks.

Proteolysis Experiment. For UV−vis absorption measurements, 50 μL of 0.1 wt/v % proteinase K was added to 3 mL of [0−7.5 v/v % BioCaRGOS, 0.2 mg/mL Mb in 0.5 M phosphate buffer pH 7.4]. The samples were incubated for 30 min prior to UV−vis measurements. For CD measurements, the samples were mixed thoroughly and transferred to 1 mm path length quartz cuvettes preequilibrated to the reaction temperature inside the CD sample holder. The CD signal at 222 nm was recorded with an averaging time of 15 s.

RESULTS AND DISCUSSION

BioCaRGOS Synthesis and Encapsulation of Mb. An overall schematic overview of the BioCaRGOS process developed for the encapsulation of biospecimens is shown in Figure 1a. First, the silica precursor TMOS is hydrolyzed in an aqueous solution by heating within a standard benchtop microwave for 30 s. The key hydrolysis reaction is highlighted in Figure 1b. TMOS has four methoxy groups that are hydrolyzed, yielding orthosilicic acid [$Si(OH)_4$] with the release of methanol as a byproduct. The methanol byproduct, which can be deleterious to the biospecimen stability, is removed by rotary evaporation.[11] After the removal of methanol (nearly ∼99%), a known concentration of the BioCaRGOS is added (Mb/Cyt c in 0.5 M phosphate buffer), which results in the condensation of hydrolyzed silica precursor as shown in Figure 1a. This complete self-sterile immobilization of any biospecimen can be achieved in less than 10 min. The process is highly compatible with clinical and downstream processes.

To compare unfolding of Mb within BioCaRGOS to unfolding in controls (i.e., without BioCaRGOS), it is important to establish that the same native structure is the reaction starting point. A previous study encountered difficulties with Mb partially unfolding during encapsulation.[16] Investigation of this protocol identified insufficient water during the microwave hydrolysis step and ineffective removal of the methanol byproduct as likely sources of observed Mb degradation. In this study, we have addressed both these issues by increasing the water volume during the hydrolysis step and subjecting hydrolyzed TMOS to rotary evaporation for 30 min at 45 °C to remove the methanol from BioCaRGOS. With these simple modifications, the UV−vis spectrum of Mb encapsulated in our BioCaRGOS sol−gel matrix is identical to the spectrum of a control sample (w/o BioCaRGOS), indicating that our encapsulation process does not perturb the Mb conformation (Figure S1).

Stability of Mb against Proteinase K. Purified proteins in their native state are known to be slightly disordered with certain sections in their unfolded state.[17] For heme proteins, UV−vis spectroscopy can detect loss or alterations in the heme chromophore and can be an effective indicator of changes in the primary and secondary structure.[18,19] In addition, losses in the heme and the resulting change in the secondary structure are indicative of alteration of tertiary structure conformation as each of the subunits are integral to the tertiary structure of the molecule.[19] Changes within the overall globin structure of Mb subunits are complex and are best described by CD and UV−vis spectroscopy. Figure 2 shows the UV−vis spectra of metaquo Mb in the presence of 0.1 wt/v % proteinase K at pH 7.4 at 25 °C over the period of 24 h in the absence of BioCaRGOS, i.e., control samples. The spectra with the Soret band in the 409 nm region (Figure 2a) and a 633 nm band in the Q-band region (Figure 2b) are indicative of the metaquo state of the protein.[20]

Addition of proteinase K to the control Mb samples in the absence of BioCaRGOS results in changes to the spectra in both the UV and visible regions. In the presence of relatively high concentrations of proteinase K, the spectra show a substantial shift in the Soret band from the 409 to 370 nm region (Figure 2a). The broad Soret band in the 370 nm region has been attributed to the absorbance of free heme. Thus, a structural change of the heme pocket upon denaturation results in the release of the heme cofactor.[21−23] The most likely cause of heme loss within the control samples is the unfolding of the helices.

Next, the effect of BioCaRGOS on the stability of Mb in the presence of 0.1 wt/v % proteinase K was evaluated. While maintaining a constant Mb concentration (0.2 mg/mL) and buffer environment (0.5 M phosphate buffer, pH 7.4), samples with a variety of BioCaRGOS concentrations (0−7.5 v/v %) were prepared and spectroscopically monitored over a period of 24 h (Figure 3). Relative to the control Mb samples (i.e., w/

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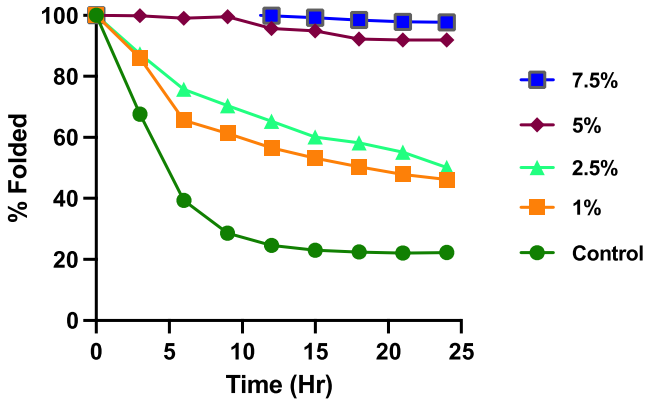

Figure 3. Myoglobin stability with incremental increase in BioCaRGOS concentration (0−7.5 v/v %). An unaltered UV−vis absorbance band (409 nm) of the heme group of Mb was observed in BioCaRGOS formulations (5 and 7.5 v/v %).

o BioCaRGOS), the results show a 2-fold [1.0 v/v % BioCaRGOS] and approx. 3-fold [2.5 v/v % BioCaRGOS] increase in Mb stability in BioCaRGOS matrix over a 24 h period. This demonstrates a BioCaRGOS concentration-dependent trend in determining the physical and chemical stabilities of Mb. Figure 3 shows that 5.0 and 7.5 v/v % BioCaRGOS retained nearly ∼95% stability for 24 h. This suggests that higher BioCaRGOS concentrations were therefore ideal for storing the Mb against proteolytic degradation at room temperature.

Myoglobin contains an abundant amount of a helical structure. A helicity of 82% has been estimated by the simulation of the CD spectrum at 25 °C using the reference spectra of protein secondary structures determined by Chen et al.[24,25] The CD spectra of the Mb in controls and BioCaRGOS (1 and 2.5 v/v %) show prominent bands at 208 nm ($\pi-\pi^*$ transition) and 222 nm ($n-\pi^*$transition). In the presence of proteinase K, the CD spectrum of native Mb, indicative of α-helix, was preserved to a certain extent in 1 v/v % BioCaRGOS, but it was nearly ∼100% preserved in 2.5 v/v % BioCaRGOS formulations as shown in Figure 4, respectively. The results indicate that the secondary structure of Mb was completely unfolded by proteinase K in control samples, whereas in BioCaRGOS, the proteinase K activity was inhibited.

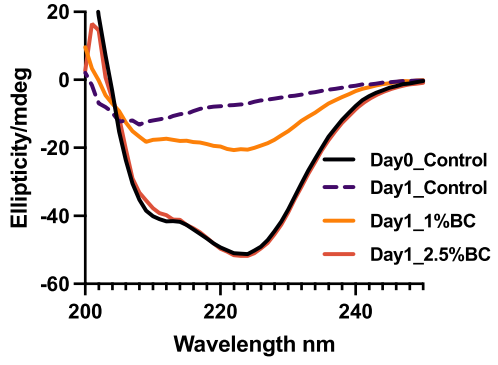

Figure 4. CD spectra of Mb encapsulated in 1 and 2.5 v/v % BioCaRGOS vs controls. Note: Day 0_Control was similar to Day 1_2.5 v/v % BC. Denatured Mb conformation is indicated by the dotted line (purple). CD spectra of Mb encapsulated in 2.5 v/v % BioCaRGOS vs controls demonstrating intact 2° structure of Mb compared to Mb in controls, which was denatured over 24 h.

The unfolding of Mb is observed in the presence of 1% (v/v) BioCaRGOS, albeit more slowly than in control formulations. In the controls, complete unfolding occurs within 24 h. Peterson et al. suggested that heme loss from ferric Mb in their gels and controls occurs only after sufficient unfolding to allow water to enter the pocket, which weakens the Fe-His bond and disrupts hydrophobic interactions.[26] Sol−gels, such as BioCaRGOS, restrict the motions that open the hydrophobic, folded heme pocket, making it difficult for water molecules to encounter the heme group. As a result, we see a slow disruption of mb helicity.

The superior protein storage demonstrated by higher (2.5 v/v %) BioCaRGOS formulations highlights another key feature of the system. The conditions mimicked in BioCaRGOS formulations are very similar to the biological pH and ionic strength. Under these conditions, the Mb and the silica will exhibit negative charges as the pH of the formulations is greater than their isoelectric points of Mb ∼ 6.5−6.7 and silica nanoparticles ca. 1.5−3.6, respectively. Therefore, electrostatic repulsive forces dominate and the Mb is stabilized. On the other hand, proteinase K has an isoelectric point (pI = ∼10) greater than its near-physiological pH environment (pH 7−8). As such, it will electrostatically adsorb on the negatively charged silica of BioCaRGOS resulting in deactivation of the proteinase K. Therefore, BioCaRGOS not only provides stabilization to biospecimen encapsulated in it but also denatures enzymes that can degrade them, and thus they prove to be an efficient storage solution.

Long-Term Storage of Mb in the Presence of Proteinase K. It is well known that the structural integrity of proteins is disturbed when degrading enzymes, such as proteinase K, are present. To investigate the long-term stability of Mb in the presence of proteinase K in BioCaRGOS, we evaluated the UV−vis absorbance of the heme group of Mb over 15 days. The data demonstrated that Mb encapsulated in BioCaRGOS (5 and 7.5 v/v %) in the presence of proteinase K was not perturbed by the addition of proteinase K indicating a robust silica network that preserved the Mb structure. On the other hand, Mb in control samples (without BioCaRGOS) showed a steep decrease in terms of stability of Mb upon the addition of proteinase K in just 24 h (Figure 5). As a result, >90% of the Mb structure is intact over 15 days in BioCaRGOS, whereas Mb in controls is deactivated within the first week of storage.

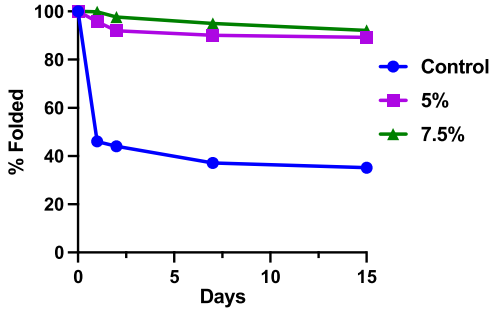

Figure 5. Long-term storage in the presence of proteinase K of Mb encapsulated in 5 and 7.5 v/v % BioCaRGOS demonstrating excellent stability compared to Mb in control samples.

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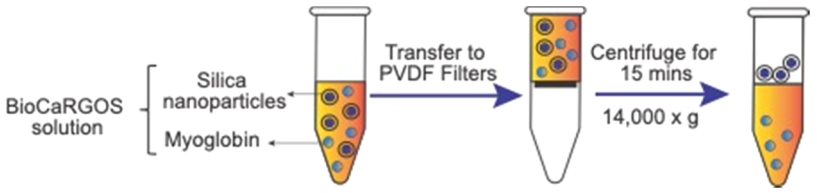

Figure 6. Schematic representation of release of Mb from 5 v/v BioCaRGOS solution.

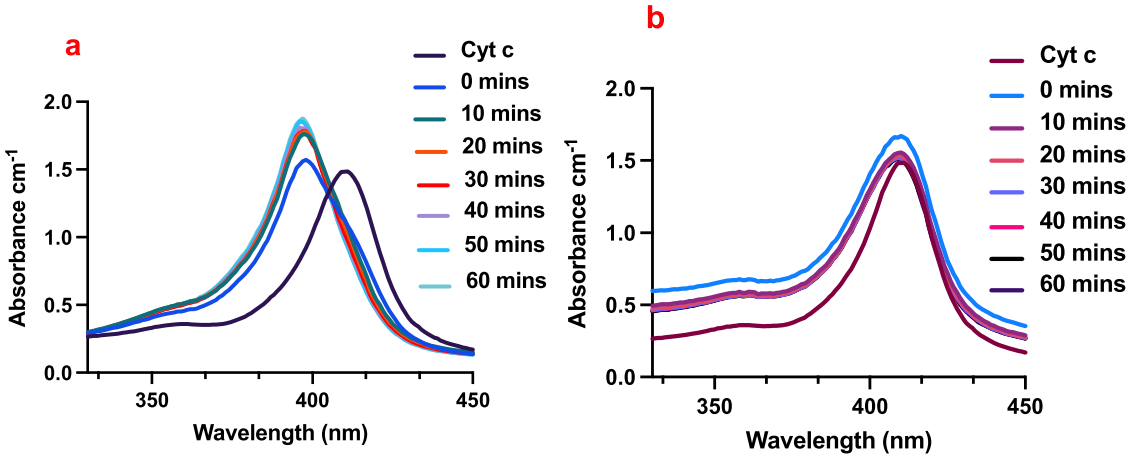

Figure 7. UV−vis absorption spectrum of Cyt c encapsulated at pH 2.5 over the period of 1 h (a) in control samples (w/o BioCaRGOS) and (b) in 7.5 v/v % BioCaRGOS.

Release of Encapsulated Mb from BioCaRGOS Matrix. An important aspect of any stabilizing agent is the ability to release encapsulated biospecimen. A major advantage of the BioCaRGOS system is the ability to release biospecies in an efficient and facile manner. The encapsulated Mb can be released from the BioCaRGOS formulation [5 v/v % TMOS; 0.01 w/v % 0.2 mg/mL Mb; 0.5 M PB, pH 7.4; 3.0 mL] by centrifugation in a 0.22 μm centrifugal filter for 15 min at 14,000g (Figure 6). The recovered Mb in the filtrate displays an intact Mb heme group compared to its native form. We attribute this excellent release phenomenon to the formulation pH of 7.4, which is higher than the isoelectric points of both silica nanoparticles and Mb. Thus, electrostatic repulsion between Mb and the BioCaRGOS prevents binding of Mb onto the silica nanoparticles allowing for ∼98% heme recovery, based on absorbance, post encapsulation (Figure S2).

Dynamic light scattering (DLS) was used to further confirm the removal of silica nanoparticles from the protein solution. No colloidal particles were detected in the filtrate, indicating that silica particles from BioCaRGOS formulation had been separated completely. This was further confirmed with a negative control where the sample [5 v/v % TMOS; 0.01 w/v % Mb; 0.5 M PB, pH 7.4; 3.0 mL] was tested before filtration. Upon measurement of the DLS, the nanoparticles exhibited a size of 180 ± 3.29. This DLS study confirmed the presence of the removal of silica from the protein solution.

Effect of BioCaRGOS and Their Concentration on the Stability of Cyt c at a Low pH. To extend the applicability of BioCaRGOS technology to preserve heme proteins with a higher pI, we investigated Cyt c (pI = ∼9.6) as a model protein. Cytochrome c is not susceptible to unfolding by proteinase K under neutral pH conditions due to the charge repulsion of the positively charged species. Hence, we investigated the scope of BioCaRGOS as an effective stabilizer of Cyt c against harsh acidic conditions (pH = 2.5). UV−vis absorption allows us to infer the conformational changes of proteins in solvent media. The acid-induced denaturation of Cyt c in controls (without BioCaRGOS) and encapsulated in BioCaRGOS was investigated following the shift of the Soret absorption band as a function of time. Acidification affects the absorption spectrum of Cyt c in controls as shown in Figure 7a, the Soret band (which is sensitive to the spin state of the heme-iron and to the nature of the axial ligands) blue-shifts from 410 to ∼394 nm, within 10 min of encapsulation indicating full protein unfolding.[27] Due to the presence of the heme prosthetic group, Cyt c shows some characteristic absorption bands.

As shown in Figure 7b, it is clearly evident that there is no significant shift in the wavelength maxima of Cyt c in the presence of 7.5 v/v % BioCaRGOS even within 1 h of encapsulation and did not affect the polypeptide environment of Cyt c around the heme group. However, it was observed that with low concentrations of BioCaRGOS, there was a blue shift observed albeit more slowly. We further evaluated the stability of Cyt c, over 6 h in control and BioCaRGOS formulations (Figure 8). We attribute this increased stability in 7.5 v/v % BioCaRGOS to increased immobilization, which hampers the biospecimen motions.

CONCLUSIONS

In summary, we have developed not only a biocompatible approach but also a greener and less toxic synthetic approach for the synthesis of sol−gels. This approach was used to demonstrate the effective protein storage against multiple stresses, such as protease degradation and low pH conditions. The model protein used to examine the stability against proteinase K was Mb, and Cyt c was used as a model protein to examine the stability against pH stress. Both the proteins showed exceptional stability in 5 and 7.5 v/v % BioCaRGOS, and it was observed that Mb was preserved efficiently for over

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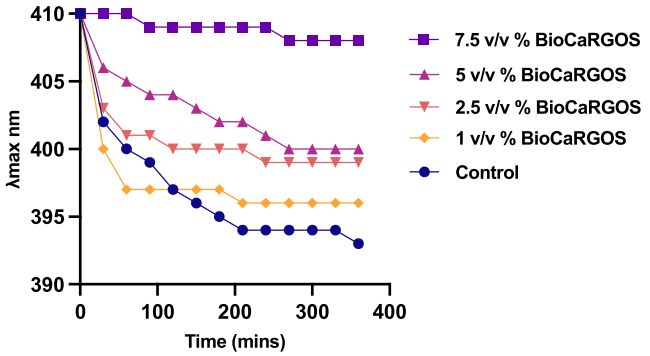

Figure 8. Cyt c stability with incremental increase in BioCaRGOS concentration (0−7.5 v/v %). An unaltered UV−vis absorbance band (409 nm) of the heme group of Cyt c was observed in BioCaRGOS formulations (7.5 v/v %).

25 days even in the presence of proteinase K. The mode of stabilization is attributed to the proteinase K inhibition by BioCaRGOS formulations caused by electrostatic attractions. Furthermore, a greener, practical, and downstream-compatible approach was used to release the biospecimen/protein post encapsulation from the BioCaRGOS matrix. Thus, BioCaRGOS technology can be used not only for long-term storage but also to preserve biospecimens from getting denatured due to multiple stresses over a short-term period.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c04012.

UV−vis absorbance of myoglobin with different concentrations of BioCaRGOS (PDF)

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Notes

The authors declare no competing financial interest.

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