Special Issue Article

Release of calcium ions from particulate monosodium titanates for dental mineralization applications

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Abstract: Purpose: The calcium ion [Ca(II)] release from monosodium titanates (MST) complexed to calcium ions [Ca(II)], referred to as MST-Ca(II), was examined under varying incubation times, pH conditions, and ion equilibrium disruptions. **Methods:** Sample supernatants were analyzed for Ca(II) using the QuantiChrom Calcium Assay Kit. **Results:** No Ca(II) was detected in native MST (control) supernatants but was detected in MST-Ca(II) supernatants. At pH 7, Ca(II) release increased from 0 to 2.5 mg/dL over 3 days ($P < 0.05$ compared to MST control), remaining constant over the completed incubation times. At pH 5, 15 mg/dL of Ca(II) was immediately released with no further release. When the pH was modulated pH 4 to pH 9, Ca(II) concentration dropped from 25 mg/dL to ~0 mg/dL. Finally, when equilibrium was disrupted by partial replacement of the supernatant with sterile water, Ca(II) release was ongoing, reaching a cumulative total of 20 mg/dL over 35 days. (*Am J Dent* 2018;31(Sp Is B):42B-48B).

Clinical significance: The current results suggest that particulate MST-Ca(II) complexes exhibit sustained release of calcium, and that release might be customized by conditions of pH and ionic strength. Thus, these complexes appear promising for biological applications where calcium-mediated mineralization or re-mineralization are desired.

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Introduction

Monosodium titanates (MST) are highly porous inorganic particulate materials with the ability to adsorb and release a variety of metal ions over a wide range of environmental conditions.[1,2] MST were originally developed as a sorbent for radioactive waste[3] but are currently being explored for use in biological contexts. The micron-sized MST particles feature an amorphous inner core and a nano-sized fibrous outer region where ion exchange with metal ions occurs. These properties make them candidates for use as delivery vehicles for therapeutic ions applied as antimicrobials, anti-inflammatories, or chemotherapeutic agents.[4-7] Native MST shows little to no cytotoxicity when in contact with L929 fibroblasts or THP1 monocytes, in vitro[8] and limited toxicity when in contact with oral carcinoma cells and WI-38 lung fibroblasts.[6,7] MST-ion complexes differentially affect the metabolism and cytokine secretion of various cell types depending on the ion delivered and the cell type.[4,6,7] In the current study, we explored MST utility in binding and delivering calcium ions [Ca(II)] for use as remineralization agents in biological contexts.

Calcium ions [Ca(II)] are an essential element of mineralized tissues such as bone, enamel, dentin, and cementum.[9-11] Calcium and inorganic phosphate are tightly regulated within the body to prevent premature calcium phosphate precipitation.[9,12] When small defects in the mineralized tissue occur, cells are stimulated to secrete proteins to concentrate calcium and phosphate at the site of repair.[9,13-15] However, when defects become large, these repair strategies fail. Thus, numerous efforts are ongoing to develop materials and medicaments with the ability to enhance or trigger mineral formation, with limited success.[16-25]

In the current study, as a first step in developing a potential remineralization agent, MST were complexed with Ca(II) and the kinetics of Ca(II) release from MST-Ca(II) complexes under varying conditions of pH, time, and equilibrium disruption, all at biological temperature, were explored. Based on published data with MST and other metal ions cited above, we hypothesized that MST-Ca(II) complexes would bind and release calcium as a function of these conditions.

Materials and Methods

Titanates and titanate-calcium loading - MST was obtained commercially[a] as a 15 wt% slurry (lot #00-QAB-417). The pH of the slurry was adjusted to pH 7 with dilute nitric acid prior to Ca(II) loading. A 0.25 M solution of calcium nitrate[b] was prepared, and the pH of this solution was adjusted to pH 7 with dilute NaOH.[b] The calcium nitrate solution was then added to the MST slurry, and the mixture was stirred for 1 week. The pH dropped to approximately 5.1 after the addition of the Ca solution, and remained at this pH until being adjusted back to ~7 after 24 hours of contact. The final pH after the 1 week contact was 6.2. The loaded solids were then isolated by centrifuging at 3,000 × g for 5 minutes and decanting the supernatant. They were then washed twice with distilled water by dispersing in water and then isolating the solids by centrifugation, followed by decanting the supernatant. A loading of 10 wt% was targeted. Samples of the loaded and washed solids were digested in sulfuric acid and analyzed by ICP-ES to determine the actual loading. Analysis of the material indicated a calcium content of 0.0775 g of Ca(II) per gram of dry solid.

Native MST (no calcium) suspensions and MST-Ca(II) pastes were mixed with Millipore water or with calcium-free phosphate buffered saline (Ca-free PBS[c]) to obtain stock suspensions (8,000 mg/L) that were autoclaved (15 minutes, 121ºC) to sterilize prior to further testing.

Scanning electron microscopy (SEM) - SEM characterization of the MST-Ca(II) material was performed using a Sigma VP[d] field emission SEM (FESEM) with secondary electron, back-

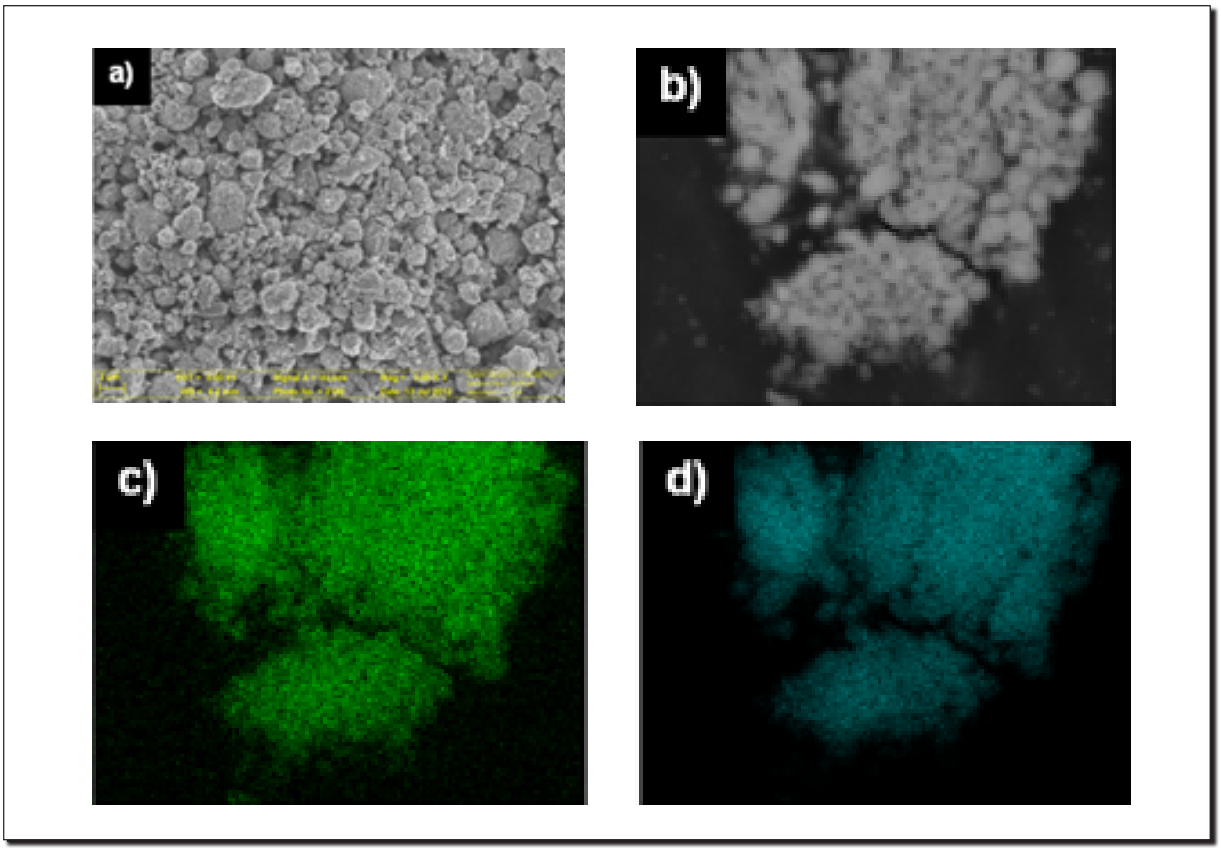

Fig. 1. (**a**) SEM of MST-Ca(II) showing typical particle morphology and size to that of the original MST with no visible evidence of a separate calcium-containing phase precipitated onto the surface of the particles. (**b**) High magnification SEM of MST-Ca(II), (**c**) EDS Elemental Map for calcium, and (**d**) EDS Elemental Map for titanium. Taken together, these provide strong evidence that Ca(II) is exchanged into the crystalline surface of MST, resulting in MST-Ca(II).

scattered electron, and in lens secondary electron detectors. It has imaging capability up to ×500,000. Energy dispersive spectroscopy (EDS) (X-Max 20[e]) was performed using an X-Max 20 silicon drift detector (SDD) to detect elements greater than atomic number 3 (Z > 3). EDS data and maps were analyzed using the INCA 4.15[e] data analysis software. Samples of the powdered MST-Ca(II) were mounted in epoxy and either carbon or palladium coated to reduce charging.

MST-Ca(II) release of calcium into water - Under sterile conditions, 100 µL aliquots of Millipore water stock suspension were distributed into sterile microtubes and incubated at 37°C, 100% humidity for time points ranging from 0 hours to 8 weeks depending on the trial (n= 3-6). During incubation, each sample was vortexed once a week to redistribute settled particles. Supernatant and particulates were collected by removing aliquots from incubation and vortexing for 15 seconds. Aliquots were then centrifuged for 30 seconds and the top 50 µL of supernatant were pipetted and transferred to new microtubes. The remaining MST and MST-Ca(II) suspensions (particulates) were also reserved. All samples were stored at 4°C until analysis (section 2.7).

MST-Ca(II) release of calcium into Ca-free PBS - Under sterile conditions, 100 µL aliquots of Ca-free PBS stock suspensions were distributed into sterile microtubes and incubated at 37°C, 100% humidity for time points ranging from 0 hours to 28 days (n= 3). During incubation, each sample was vortexed once a week to redistribute settled particles. Supernatant and particulates were collected by removing aliquots from incubation and vortexing for 15 seconds. Aliquots were then centrifuged for 30 seconds and the top 50 µL of supernatant were pipetted and transferred to new microtubes. The remaining MST and MST-Ca(II) suspensions (particulates) were separately reserved. All samples were stored at 4°C until analysis (section 2.7).

Effect of equilibrium disruption on calcium release - Under sterile conditions, 100 µL aliquots of Millipore water stock suspensions were distributed into sterile microtubes and incubated at 37°C, 100% humidity (n= 3-6). Supernatant samples were collected as described in Section 2.3. Following supernatant collection, new Millipore water (sterile, 50 µL) was added to each aliquot. The aliquots were then vortexed for 15 seconds to redistribute the particles and placed back into incubation. Supernatant collection and water exchange took place every 24 hours (n= 3-6 supernatant samples per day, days 0-35), and particulates were collected every 7 days (n= 3). All supernatant and particulate samples were stored at 4°C for later analysis.

Effect of pH on calcium release - To determine the effect of pH on MST-Ca(II) release of calcium ions, the procedures outlined in Section 2.1 and 2.3 were altered as follows. Prior to the sterilization of the Millipore water stock suspensions, the pH of the suspensions were adjusted using 1.0 N HCl or 1.0 N NaOH (reagents from Sigma Aldrich) until a pH of 4 to 9 was obtained (pH meter, MP220[f]). Supernatant and particulate samples were collected (n=3) for each stock suspension (pH 4,

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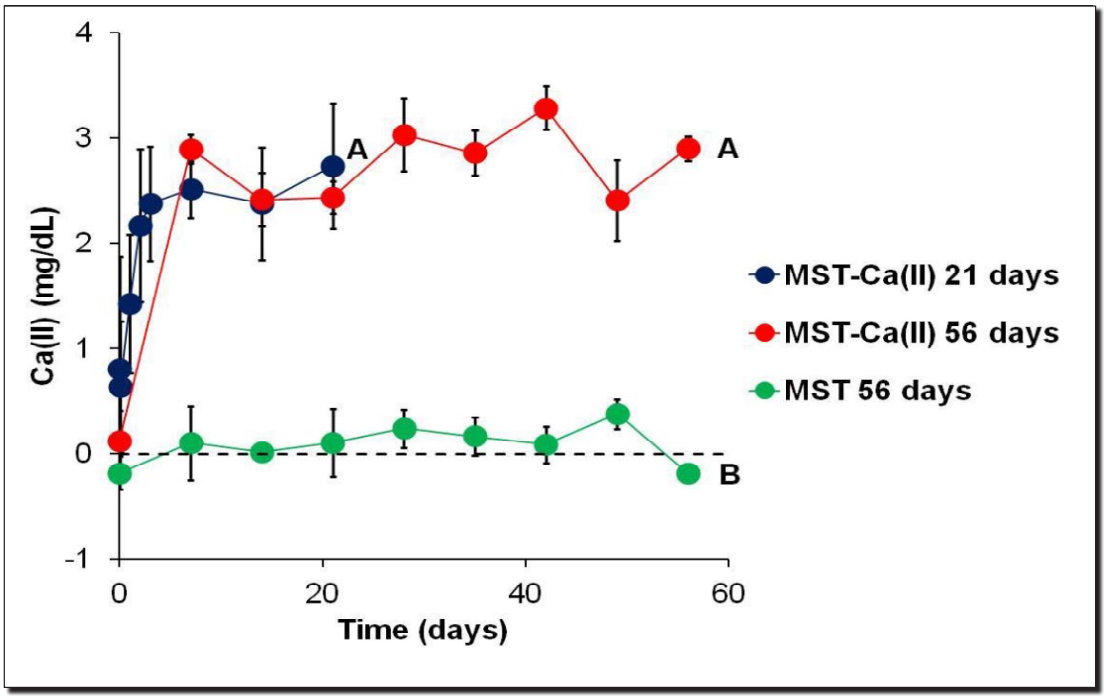

Fig. 2. Ca(II) release into sterile water from MST-Ca(II) during 21 and 56 days of incubation. Ca(II) increased during the initial 3 days of incubation before reaching an equilibrium level of ~ 2.5 mg/dL. No Ca(II) was measured in supernatant samples collected from incubated MST. Ca(II) release from MST-Ca was significantly greater than Ca(II) released from MST (Differences indicated by A, B; P< 0.05, n= 3).

pH 5, pH 6, pH 7, pH 8, and pH 9) and assayed for Ca(II) content (section 2.7). MST and MST-Ca(II) stock suspensions at pH 5 were then sterilized and tested for calcium release following the procedure outlined in Section 2.3, for time points ranging from 0 hours to 7 days (n= 3).

Measurement of calcium - QuantiChrom Calcium Assay Kit[g] (DICA-500) was used according to manufacturer's protocol to measure concentrations of calcium ions. This assay was chosen because it was indicated for detecting calcium in water based and biologically based samples, because of the detection range of the assay, and because of the simplicity of the assay protocol. To assess supernatant samples, 5 µL of each sample or standard were plated in 96-well plates in triplicate. Standards were generated (0-20 mg/dL) by diluting a 20 mg/dL standard solution (provided, BioAssay Systems) with Millipore water. Following plating, 200 µL of the mixed test reagent were added to each well. The plate was incubated at room temperature for 3 minutes and a SpectraMax M2[h] plate reader was used to determine the optical density (OD) of the standards and samples at 612 nm.

The procedure to measure the amount of calcium on the particulates was altered slightly because of known OD interference by MST7. To mitigate particulate interference, particulate samples were reacted with test reagents in a round-bottom 96-well plate. The plates were then centrifuged and 100 µL of developed assay solution, free of particulates (samples and standards), were transferred to new 96-well plate and the OD assessed (612 nm).

All optical densities were converted to calcium concentrations in mg/dL following generation of a standard linear curve to the known standards. Statistical significance and differences were assessed utilizing a Student t-test (α-= 0.05).

Results

MST was reacted with a calcium nitrate solution under conditions such that 100% of the theoretically available sodium was exchanged for calcium ions (equivalent to 10 wt% Ca). Elemental analysis indicated a calcium content of 0.0775 g of Ca(II) per g of dry solid. SEM (Fig. 1a) revealed the calcium-exchanged MST had the same particle size, shape, and morphology of the native MST material and no evidence of precipitated calcium-containing phase on the surface of or otherwise present in the material. Higher magnification in conjunction with elemental mapping (Figs. 1b - d) revealed that both calcium (Fig. 1c) and titanium (Fig. 1d) are evenly distributed over the particles with no regions where calcium is present without titanium. This suggests that the Ca(II) has been incorporated into the MST structural framework by exchange of Ca(II) for sodium ions. If Ca(II) were precipitated, the surface details and spaces between particulates would not have been visible. Following confirmation that calcium precipitation was not occurring, samples were tested for their ability to release the loaded Ca(II) ions.

MST-Ca(II) and native MST were incubated in water in two separate trials spanning 21 and 56 days. For both trials, the initial Ca(II) concentration was 0 mg/dL for both MST and MST-Ca(II). The MST-Ca(II) supernatant Ca(II) concentration increased over Days 1-3, reaching a steady-state concentration of ~2.5 mg/dL (Fig. 2). Within statistical error, this concentration was maintained over the remaining duration of the 21-day and 56-day time periods of the respective studies. No Ca(II) was detected in native MST supernatants during either trial (Fig. 2).

Under biological conditions, the system would presumably be dynamic in that the biological fluids in contact with the MST-Ca(II) would be changing, thus we sought to determine if

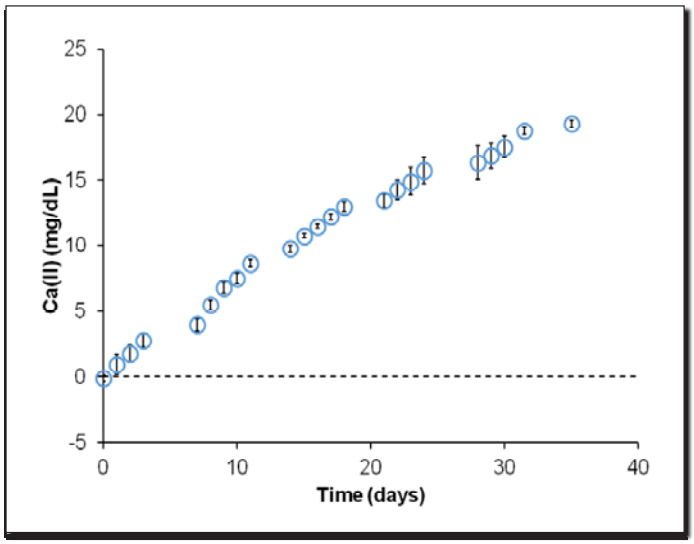

Fig. 3. Daily replacement of 50% of the volume of supernatant over MST-Ca(II) particulates with water. Y-axis indicates the cumulative Ca(II) detected in supernatants. Ca(II) was released by the MST-Ca(II) following each replacement, re-establishing equilibrium. Release continued over 35 days of testing.

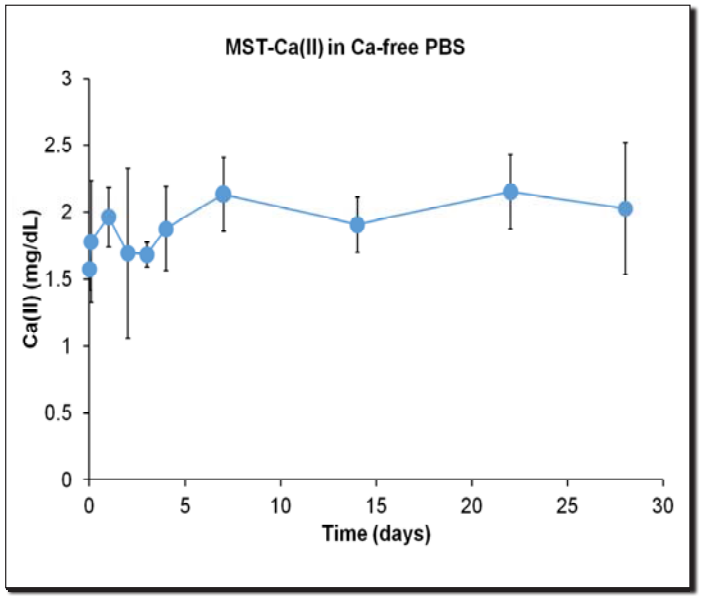

Fig. 4. Ca(II) release into Ca-free PBS from MST-Ca(II) during 28 days of incubation. Ca(II) release was immediate with an average equilibrium of 1.88 mg/dL maintained over the 28 days of incubation.

additional Ca(II) would be released under controlled alterations in conditions. Upon daily disruption of steady-state, Ca(II) was released and measured to range from 0 - 3.0 mg/dL per day. The cumulative Ca(II) released was calculated and plotted against time (Fig. 3). After 35 days, the cumulative Ca(II) released reached 20 mg/dL. At no time was any Ca(II) detected in MST control samples.

In addition to being dynamic, biological conditions are also ionically complex, thus Ca(II) release from MST-Ca(II) was studied with Ca-free PBS as the bathing solution. Ca(II) release into Ca-free PBS was immediate and attained an average steady-state of 1.88 mg/dL (Fig. 4). This steady-state was maintained over 28 days in incubation.

Ca(II) release from MST-Ca(II) was found to be highly dependent on sample pH. Adjustments to pH resulted in immediate (less than 5 minutes) release of Ca(II) into the supernatant. The quantity of Ca(II) released decreased from 25.7 mg/dL at pH 4 to 0.41 mg/dL at pH 9 (Fig. 5a). The pH versus Ca(II) concentration data had an exponential association: [Ca] = 81.5*exp(-0.988*pH). Despite the pH-dependent calcium concentration in the supernatant, the Ca(II) measured on the particulates was statistically constant and in excess of the standards (Fig. 5b), suggesting a large remaining reservoir of Ca(II) on the MST-Ca(II) particles with saturation of the assay. When samples of pH = 5 were incubated for a period of 7 days (Fig. 6), an initial, steady-state Ca(II) concentration of 15 mg/dL was attained and remained statistically constant over the 7-day trial. Ca(II) steady-state at pH 5 was significantly greater

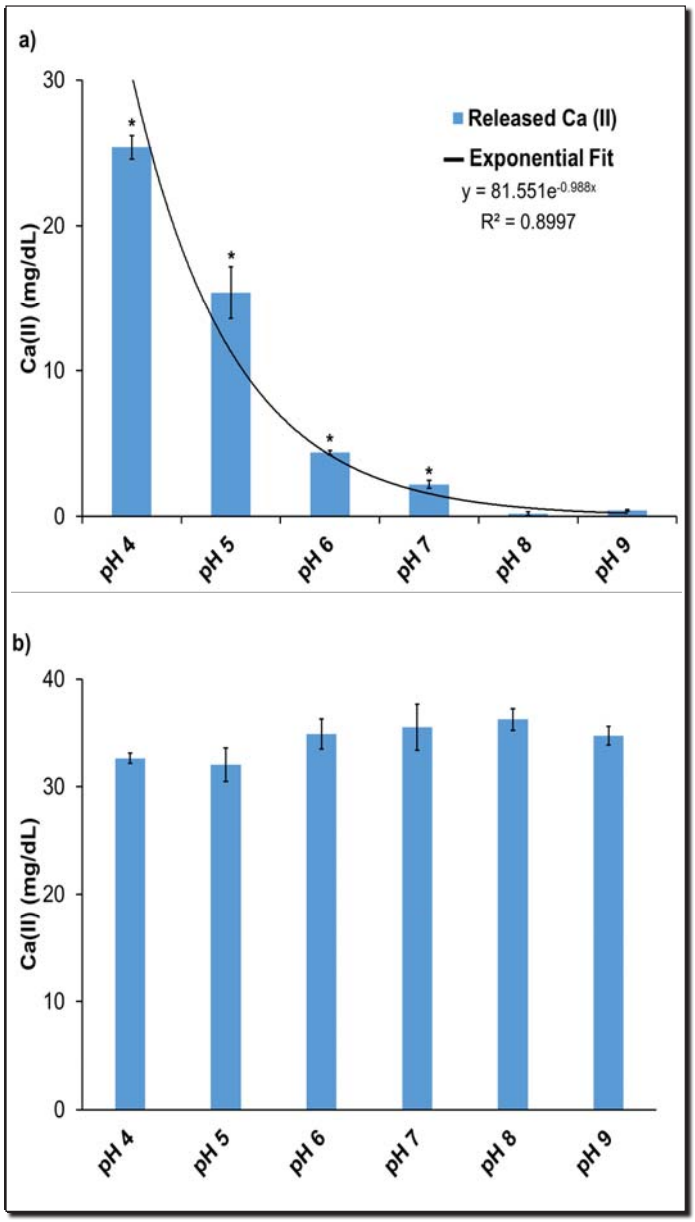

Fig. 5. (**a**) Short-term (< 5 minutes) Ca(II) release from MST-Ca(II): dependence on pH (pH 4 to 9). The amount of Ca(II) detected ranged from 25.4 mg/dL at pH 4 to ~0 mg/dL at pH 9. There was a nearly exponential relationship between pH and calcium concentration (black fitted line). * denotes statistically significant release (P< 0.001). (**b**) Ca(II) measured on MST-Ca(II) particulates (34.4 mg/dL) following release. There was no significant difference in Ca(II) detected on the particulates, suggesting a large reservoir of Ca(II) available for release that exceeded the ability of the assay to detect all the calcium on these particles.

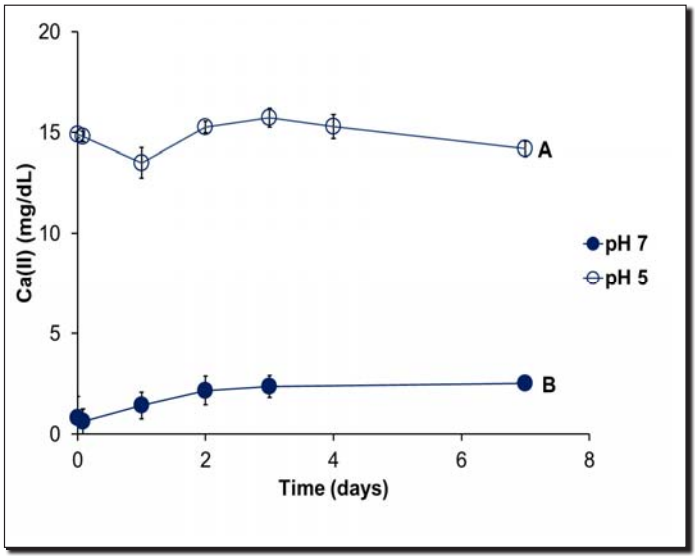

Fig. 6. 7-day release of Ca(II) from MST-Ca(II) particulates at pH 5 versus pH 7. At pH 5, an average of ~15 mg/dL Ca(II) was detected at each time point. This is in contrast to pH 7 where an equilibrium of ~2.5 mg/dL Ca(II) was not attained until Day 3. Statistically significant differences denoted by A,B (P< 0.05).

(P< 0.05) than the steady-state concentration attained for samples at pH 7.

Assessing the amount of Ca(II) remaining on MST-Ca(II) under the various release conditions, it was found that with the exception of Ca-free PBS release, all MST-Ca(II) samples had greater than 20 mg/dL of Ca(II) remaining on them (Table), exceeding the maximum assay standard. For Ca-free PBS release, an average of 14.5 mg/dL of Ca(II) were measured on the MST-Ca(II) particulates (P< 0.05), independent of the amount of time the particulates were incubated.

Discussion

In the current study, calcium ions were successfully loaded and subsequently released from MST in a sustained and controlled manner. Previous testing demonstrated that contact of MST with a solution containing calcium ions would result in the exchange of Ca(II) for sodium ions in the MST;[2] however, subsequent release was unknown. Release into water over both 3 and 8 weeks at physiological pH resulted in a steady-state Ca(II) concentration of ~2.5 mg/dL. This value is similar to the concentration of Ca(II) measured in saliva, which ranges from 2.07 to 4.16 mg/dL depending on salivary flow rate.[26] Further tests demonstrated that interrupting this equilibrium resulted in additional Ca(II) release from the MST-Ca(II) until equilibrium was reestablished. Additionally, a large quantity of Ca(II) was measured as remaining on the particles, suggesting a large reservoir. Thus, MST-Ca(II) has conceivable utility as an ongoing ion source of calcium in applications where Ca(II) is removed from the surrounding microfluidic environment and incorporated into mineralized matrices such as bone and dental defects or dental restoration margins.

MST-Ca(II) was extremely pH responsive with an exponential and rapid relationship between pH and measured released Ca(II). This attribute has potential therapeutic advantages. At pHs lower than physiological pH, such as may occur during infection or under overgrown biofilms;[27-29] a greater concentration of Ca(II) would be released to establish equilibrium, resulting in a large localized available calcium ion concentration for remineralization.

Table. Average Ca(II) measured on MST-Ca(II) particles following various release conditions (see description). * indicates statistically difference from other samples tested (P< 0.05). Note: precision is low for calcium concentrations >20 mg/dL because these values are extrapolated from a standard curve in which the highest standard is 20 mg/dL.

Condition	Ca(II) (mg/dL) ± SD	Description
Water release (56 days)	44.0 ± 2.2	Average Ca(II) on MST-Ca(II) particulates reserved after extended release into water (21 days and 56 days)
PBS release (28 days)	14.5 ± 0.8 *	Average Ca(II) on MST-Ca(II) particulates reserved after extended release into Ca-free PBS (28 days)
pH 5 (7 days)	36.1 ± 1.0	Average Ca(II) on MST-Ca(II) particulate reserved after extended release in water at pH 5 (7 days)
Disrupted equilibrium	29.1 ± 0.8	Average Ca(II) on MST-Ca(II) particulates reserved after 35 days release, daily equilibrium disruption
Immediate pH release	34.4 ± 1.7	Average Ca(II) on MST-Ca(II) particulates reserved after immediate release into water when pH was varied from 4 to 9 (see Fig. 5b for individual values).

Even at pH 4, when greater than 20 mg/dL of Ca(II) was released from MST particles, no significant difference in the amount of Ca(II) was detected on the particulates. This observation suggests that a large reservoir of Ca(II) existed on the MST-Ca(II) particles which could not be fully measured due to limitations of the assay. Comparing theoretical amounts of Ca(II) loaded on MST (utilizing a solution difference method) to measured calcium amounts, only 1/3 of the loaded calcium amount was measured in our release experiments (results not shown). These results suggest either a limitation in the ability to release all Ca(II) from the particulates once loaded or a limitation in the ability of the assay to detect all Ca(II) on the particulates. Regardless, the MST was loaded with a reservoir of calcium ions available for delivery.

In addition, Ca(II) release from MST-Ca(II) was altered by a complex ionic environment, which reduced the steady-state concentration of Ca(II) compared to water release. At the same time, the amount of Ca(II) measured on the materials was greatly reduced compared to materials releasing Ca(II) into water. We hypothesize that calcium ions were interacting with the phosphate reservoir resulting in precipitated calcium phosphate that cannot be separated from white MST-Ca(II) particulates or measured. Problems with measuring Ca(II) in other buffers have been documented previously[30] and more investigation into the effects of other ions on Ca(II) release is needed.

Titanium alloys, which are generally alloys of titanium and oxygen, are extensively used for orthopedic and dental implants and need to integrate with bone to function adequately. In attempts to improve this integration and interface, numerous titanium alloy surface treatments have been developed, including chemical, heat, and micro-arc treatments which oxidize the

titanium, resulting in a titanate surface.[31-33] In this context, titanate is a general term used to describe oxides of titanium on the alloy surfaces. Calcium also has been integrated into these treated surfaces resulting in a layer commonly referred to as calcium titanate.[32-34] However, studies suggest that little calcium is released from the calcium-titanate alloy surfaces.[35] Treated titanium alloy surfaces are fundamentally different than the MST and MST-Ca(II) particulates used in the current study. MST particulates have, by design and synthesis, highly crystalline surfaces with substantial surface area designed for ion exchange. Unlike titanium alloy surfaces treated with calcium, we have shown in the current work that MST-Ca(II) releases Ca(II); this result is consistent with previous studies that have reported release of other ions under physiological conditions.[4-7] The release of calcium ions from MST-Ca(II) complexes offers several potential therapeutic applications.

MST particulates also have similarities to titanium dioxide nanoparticles (anatase). Both MST and titanium dioxide nanoparticles are oxides of titanium, however MST is larger than anatase (on the order of microns versus nanometers, respectively). By definition anatase is crystalline whereas MST has an amorphous core with a well-defined crystalline surface.[4] Both materials have been shown to successfully deliver metals and chemotherapeutic agents in biological settings.[36,37] However, no literature has reported that calcium ions have been delivered from titanium dioxide nanoparticles.

Overall, we demonstrated that Ca(II) can be exchanged onto MST (denoted as MST-Ca(II)) and subsequently released into solution in a controlled and sustained manner. In the future, we intend to investigate the biocompatibility and mineralization capabilities of this material in biological contexts as a prelude to therapeutic applications.

a. Optima Chemical Group, LLC, Douglas, GA, USA.
b. Sigma-Aldrich, St. Louis, MO, USA.
c. Life Technologies, Carlsbad, CA, USA.
d. Carl Zeiss Microscopy LLC, Thornwood, NY, USA.
e. Oxford Instruments, Abingdon, Oxfordshire, UK.
f. Mettler-Toledo, Columbus, OH, USA.
g. BioAssay Systems, Hayward, CA, USA.
h. Molecular Devices, Sunnyvale, CA, USA.

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