(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
01 October 2026 (01.10.2026)

WIPO | PCT

(10) International Publication Number
WO 2026/207299 A1

(51) International Patent Classification:
G01N 33/84 (2006.01) *G01N 33/53* (2006.01)
C07K 14/47 (2006.01)

(21) International Application Number:
PCT/US2026/021042

(22) International Filing Date:
26 March 2026 (26.03.2026)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/777,941 26 March 2025 (26.03.2025) US
63/799,110 02 May 2025 (02.05.2025) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,

(54) Title: SYSTEMS AND METHODS FOR CALCIUM SEQUESTRATION AND PRECIPITATION

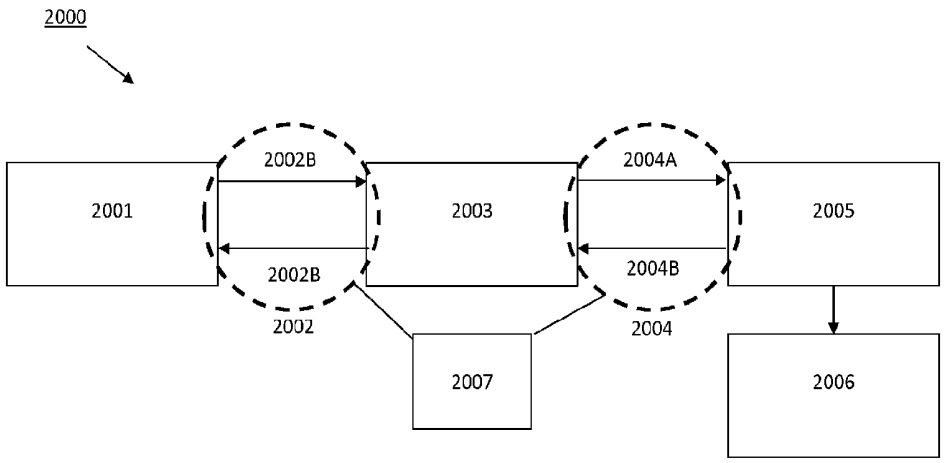


FIG. 1B

(57) Abstract: This disclosure relates generally to methods and devices for capturing calcium. In particular, this disclosure provides a method comprising contacting at least one calcium-sequestering protein to a first solution for a first predetermined amount of time, thereby allowing the at least one calcium-sequestering protein to bind to calcium in the first solution; and contacting the at least one calcium-sequestering protein with a second solution for a second predetermined amount of time, the second predetermined amount of time being until a portion of the calcium is released from the at least one calcium-sequestering protein. This disclosure further provides a device comprising a sample interface configured to receive a sample comprising calcium; and a substrate embedded with a calcium-sequestering protein, thereby allowing the substrate to capture at least a portion of the calcium. The methods and devices herein allow for sequestering large quantities of calcium for downstream applications.

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CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UY, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MU, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

SYSTEMS AND METHODS FOR CALCIUM SEQUESTRATION AND PRECIPITATION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Application No. 63/777,941, filed on March 26, 2025, and U.S. Provisional Application No. 63/799,110, filed on May 2, 2025, the entire contents of each of which is incorporated herein by reference.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in xml format and is hereby incorporated by reference in its entirety. Said xml copy, created on March 25, 2026, is named "110697-016502_US_SL" and is 16,544 bytes in size.

BACKGROUND

[0003] Calcium is an expensive metal and is a component needed in many industrial processes. For example, large quantities of industrial-scale calcium are essential for downstream production of products like concrete. Calcium is traditionally produced by heating limestone with aluminum, a process that requires the quarrying of both materials. Thus, there is a need for renewable techniques for calcium production or reclaiming with minimal maintenance.

BRIEF SUMMARY

[0004] In some aspects, the techniques described herein relate to a method of capturing calcium, the method including: contacting at least one calcium-sequestering protein to a first calcium-containing solution for a first predetermined amount of time, thereby allowing the at least one calcium-sequestering protein to capture calcium in the first calcium-containing solution; contacting the at least one calcium-sequestering protein with a second solution for a second predetermined amount of time, the second predetermined amount of time being until a portion of the captured calcium is released from the at least one calcium-sequestering protein. In some aspects, the second solution further includes one or more counterions to provide for at least a portion of the released calcium to precipitate. In some aspects, the first calcium-containing solution includes calcium at a concentration above 150 mM. In some aspects, the portion of the captured calcium released from the at least one calcium-sequestering protein includes at least 50% of the calcium captured by the at least one calcium-sequestering protein.

In some aspects, the at least one calcium-sequestering protein includes a calsequestrin protein. In some aspects, the at least one calcium-sequestering protein includes a variant of a calsequestrin protein. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 1. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 2. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 7. In some aspects, the calcium-sequestering protein is bound to a sample interface. In some aspects, the sample interface includes a membrane filter. In some aspects, the membrane filter includes a dialysis filter stack.

[0005] In some aspects, the techniques described herein relate to an extraction device for capturing calcium from a calcium-containing solution, the extraction device including: a sample interface configured to receive a calcium-containing solution; a substrate embedded with a calcium-sequestering protein configured to capture at least a portion of calcium from the calcium-containing solution when contacted with the sample interface containing the calcium-containing solution. In some aspects, the sample interface includes a membrane filter. In some aspects, the membrane filter includes a dialysis filter stack.

[0006] In some aspects, the techniques described herein relate to a method of capturing calcium, the method including: providing an extraction device for capturing calcium from a calcium-containing solution, the extraction device including: a sample interface configured to receive a calcium-containing solution; and a substrate embedded with a calcium-sequestering protein configured to capture at least a portion of calcium from the calcium-containing when contacted with the sample interface containing the calcium-containing solution; contacting the substrate with the sample interface including the calcium-containing solution for a first predetermined amount of time, thereby allowing the calcium-sequestering protein to capture at least a portion of calcium from the calcium-containing solution; and contacting the sample interface with a storage solution for a second predetermined amount of time, the second predetermined amount of time being until a predetermined amount of the calcium is released from the calcium-sequestering protein. In some aspects, the storage solution further includes one or more counterions to provide for at least a portion of the released calcium to precipitate. In some aspects, the calcium-containing solution includes calcium at a concentration above 150 mM. In some aspects, the portion of the captured calcium released from the at least one

calcium-sequestering protein includes at least 50% of the calcium captured by the at least one calcium-sequestering protein. In some aspects, the at least one calcium-sequestering protein includes a calsequestrin protein. In some aspects, the at least one calcium-sequestering protein includes a variant of a calsequestrin protein. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 1. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 2. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 7. In some aspects, the substrate includes a membrane filter. In some aspects, the membrane filter includes a dialysis filter stack.

[0007] In some aspects, the techniques described herein relate to a variant of a calsequestrin protein, wherein the variant exhibits calcium-sequestering activity that is at least 10% greater than the calsequestrin protein. In some aspects, the variant has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 1. In some aspects, the variant has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 2. In some aspects, the variant has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 7.

[0008] In some aspects, the techniques described herein relate to a method for producing a variant of a calsequestrin protein, the method including: cultivating a cell overexpressed with a nucleic acid construct that encodes the variant of the calsequestrin protein; inducing the cell to express the variant of the calsequestrin protein encoded by the nucleic acid construct; lysing the cell; and purifying the variant of the calsequestrin protein from the lysed cell, wherein the variant exhibits calcium-sequestering activity that is at least 10% greater than the calsequestrin protein.

[0009] In some aspects, the techniques described herein relate to a system for producing a variant of a calsequestrin protein, the system including a solution chamber including a calcium-sequestering agent configured to bind to calcium; a first loop in fluid communication with the solution chamber, wherein the first loop is configured to pass a first solution through the solution chamber, the first solution including calcium such that when the first solution is passed through the solution chamber, the calcium-sequestering agent captures the calcium from the first solution; and a second loop in fluid communication with the solution chamber, wherein the second loop is configured to pass a second solution through the solution chamber to extract

the captured calcium from the agent. In some aspects, the first solution is filtered prior to entering the solution chamber. In some aspects, the calcium-sequestering agent includes a calcium-sequestering protein dispersed in solution or a calcium-sequestering protein embedded within a sequestering substrate. In some aspects, the sequestering substrate in calcium-sequestering portion includes one or more membranes, membrane filters, a plurality of beads, is housed within a column, or a combination thereof. In some aspects, a system further includes a reaction portion in fluid communication with the solution chamber, wherein the reaction portion includes a reaction substrate that is configured to allow for precipitation of the extracted calcium. In some aspects, the reaction substrate includes Na2CO3, NaOH, or a combination thereof. In some aspects, the reaction portion includes one or more of sedimentation, a filter, or a combination thereof to precipitate the extracted calcium. In some aspects, the first solution includes calcium at a concentration above 150 mM. In some aspects, the calcium-sequestering agent includes a calsequestrin protein. In some aspects, the calcium-sequestering agent includes a variant of a calsequestrin protein. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 1. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 2. In some aspects, the variant of the calsequestrin protein has an amino acid sequence including at least 90% sequence identity to SEQ ID NO: 7.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The novel features of the present disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure can be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings of which:

[0011] FIG. 1A illustrates a flowchart of a process for capturing calcium, according to an exemplary embodiment of this disclosure; FIG. 1B illustrates a block diagram of a system with which some embodiments may operate for capturing calcium, according to an exemplary embodiment of this disclosure;

[0012] FIGS. 2-3 illustrate purification results of an exemplary variant of a calcium-capturing protein using first and second purification protocols, respectively;

[0013] FIG. 4 illustrates purification results of an exemplary variant of a calcium-capturing protein using a purification protocol;

[0014] FIG. 5 illustrates calcium concentration results of a storage buffer before and after exposure to an exemplary extraction device;

[0015] FIGS. 6A-6B illustrate dynamic light scattering (DLS) data of an exemplary variant of a calcium-capturing protein, the DLS data being with or without chelation, respectively;

[0016] FIG. 7 illustrates DLS correlation data of an exemplary variant of a calcium-capturing protein during exposure to buffer at different pH levels;

[0017] FIG. 8 illustrates DLS data of an exemplary variant of a calcium-capturing protein during exposure to different buffers at pH 8;

[0018] FIG. 9 illustrates calcium concentration results of an exemplary variant of a calcium-capturing protein after exposure to a 250-ppm calcium concentration buffer;

[0019] FIG. 10 illustrates calcium concentration results of an exemplary variant of a calcium-capturing protein after exposure to ocean water;

[0020] FIG. 11A illustrates an extraction system according to an exemplary embodiment of this disclosure; FIG. 11B illustrates an example of a filter, water chamber and a pump for driving flow from a calcium-containing solution through the filter; FIG. 11C illustrates nonlimiting examples of a dialysis chamber; FIG. 11D illustrates a schematic view of a dialysis chamber; and FIG. 11E illustrates a schematic view of a dialysis cassette;

[0021] FIGS. 12A-12B illustrate a side by side of an AlphaFold predicted structure of CASQ1 with and without GFP;

[0022] FIGS. 13A-13B illustrate an Alpha fold 3 structure of CASQ1GFP with 54 calcium ions bound (FIG. 13A), and the CD spectrum for CASQ1GFP in the presence of 25 mM EDTA are consistent with partially unfolded proteins (FIG. 13B);

[0023] FIGS. 14A-14B illustrate calcium selective electrode readings before and after dialysis into a calcium containing buffer (FIG. 14A) and a calcium release buffer (FIG. 14B);

[0024] FIGS. 15A-15C illustrate (left) normalized DLS autocorrelation curves of CASQ1GFP, and (right) the corresponding fitted hydrodynamic size distributions for the indicated samples;

[0025] FIGS. 16A-16B illustrate the zeta potential and electrophoretic mobility of CASQ1GFP in response to increasing $CaCl_2$ concentrations (FIG. 16A) and the corresponding data curves (FIG. 16B);

[0026] FIG. 17 illustrates ppm values determined by ICP-MS the calcium capture and release.

[0027] The following description and examples illustrate in detail some embodiments of techniques and technologies described herein. It is to be understood that embodiments are not limited to acting in accordance with the specific examples provided herein, as other approaches are possible. Those of skill in the art will recognize that there may be variations and modifications from the specific examples below that are within the scope of this disclosure.

DETAILED DESCRIPTION

[0028] This disclosure relates generally to methods and systems for calcium sequestration and precipitation. In some embodiments, the present disclosure provides a method of capturing calcium, the method including contacting at least one calcium-sequestering protein to a first calcium-containing solution for a first predetermined amount of time, thereby allowing the at least one calcium-sequestering protein to capture calcium in the first calcium-containing solution. In some embodiments, a method includes contacting the at least one calcium-sequestering protein with a second solution for a second predetermined amount of time, the second predetermined amount of time being until a portion of the captured calcium is released from the at least one calcium-sequestering protein.

[0029] The methods and systems herein allow for such calcium production. In particular, the techniques provided herein allow for renewable methods of calcium capture and release that require minimal maintenance due to the nature of the techniques. Further, the techniques provided herein are scalable for other applications, including industrial, chemical, and structural engineering.

[0030] The following description and examples illustrate in detail some embodiments of techniques and technologies described herein. It is to be understood that embodiments are not limited to acting in accordance with the specific examples provided herein, as other approaches are possible. Those of skill in the art will recognize that there may be variations and modifications from the specific examples below that are within the scope of this disclosure.

[0031] FIG. 1A illustrates a flowchart of an exemplary process 1000 for capturing calcium from a calcium-containing solution. At step 1001, at least one calcium-sequestering protein is allowed to contact a first solution for a first predetermined amount of time, the first solution containing calcium. By contacting calcium in the form of calcium ions or a calcium-based composition present in a first solution, calcium-sequestering proteins are allowed to sequester, bind, or otherwise capture the calcium present in the solution. In some embodiments, the at least one calcium-sequestering protein is allowed to entrap calcium ions present in the first solution via a binding mechanism so as to sequester large quantities of calcium. Once the calcium is captured by the calcium-sequestering protein, the calcium may be stored as bound or stored calcium for downstream release into a second solution.

[0032] In some embodiments, a first solution comprises a calcium solution. In some embodiments, a first solution comes from a body of a liquid, such as, for example, an ocean, sea, canal, lagoon, reservoir, or the like. In some embodiments, a body of water comprises salt water or freshwater rich in ions. In some embodiments, a body of water is downstream of a water treatment plant in the form of household wastewater, an industrial site, such as, for example, a mining site, a production site of products comprising concrete, or the like. Typically, such bodies of water that are downstream of industrial sites are rich in calcium, therefore being able to saturate a medium comprising a calcium-sequestering protein. In some embodiments, the first solution is from a waste stream from a household, a factory, a construction site, or similar industrial site, such as, for example, a mining site, a production site of products comprising concrete, or the like. In some embodiments, the first solution comprises a solution configured to extract calcium from a waste stream, a body of liquid, or the like. For example, the solution configured to extract calcium can include one or more chemicals such as, for example, acids selected from acetic acid, citric acid, hydrochloric acid, phosphoric acid, or the like, chelating agents such as ethylenediaminetetraacetic acid (EDTA), or the like. Such solutions configured to extract calcium can allow for breaking up or dissolving materials to allow for calcium to be in suspension or dispersed in the solution for processing. In some embodiments, the calcium concentration in the first solution exceeds 25 mM either in original form of the solution or after concentrating the solution.

[0033] In some embodiments, a first solution is prepared to maximize an absorption of calcium by the at least one calcium-sequestering protein. In some embodiments, a first solution is adjusted to include one or more buffers. In some embodiments, a first solution is adjusted so

as to maintain a predetermined pH range. In some embodiments, the pH of the first solution is maintained to be above pH 6.0, above pH 7.0, above pH 7.5, or higher. In some embodiments, the pH of the first solution is maintained to range from pH 4 to pH 9. In some embodiments, a first solution comprises a buffer. Non-limiting examples of buffer includes phosphate buffered saline (PBS); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 2-(N-Morpholino)-ethanesulfonic acid (MES); piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES); N-(2-acetamido)-2-aminoethanesulfonic acid (ACES); 3-(N-morpholino)propanesulfonic acid (MOPS); 2-{[1,3-Dihydroxy-2-(hydroxymethyl)propan-2-yl]amino}ethane-1-sulfonic acid (TES); N,N-Bis(2-hydroxyethyl)glycine (Bicine); 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS or EPPS); N-[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]glycine (Tricine); and 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris). In some instances, a buffer may comprise Tris-HCl, VLB, CH_3COOH, TCEP, IsoAmp®, KCl, $MgSO_4$, KOAc, MgOAc, BSA, TCEP, Tween-20, Tween-80, Triton X-100, CHAPS, β-Mercaptoethanol, PMSF (Phenylmethylsulfonyl fluoride), or any combination thereof. [In some embodiments, a first solution includes calcium at a concentration above 0.1 mM, above 1 mM, above 10 mM, or above 20 mM. In some embodiments, a storage buffer includes (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (HEPES), sodium chloride (NaCl), calcium chloride ($CaCl_2$), tris(2-carboxyethyl)phosphine (TCEP), glycerol, or any combination thereof. In some embodiments, a buffer may comprise HEPES, 2-morpholinoethanesulfonic acid (MES), , ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), Tween 20, glycerol, or any combination thereof. In some instances, the buffer may comprise from 0 to 500 mM HEPES pH 6.8. In some embodiments, a storage buffer includes HEPES ranging from a pH ranging from about 6.8 to about 8.2. In some embodiments, the pH of the storage buffer ranges from pH 4 to pH 9. In some embodiments, a storage buffer includes 0 to 500 mM NaCl. In some embodiments, a storage buffer includes 0 to 500 mM $CaCl_2$. In some embodiments, a storage buffer includes 0 to 500 mM TCEP. In some embodiments, a storage buffer includes less than 1%, less than 5%, less than 10%, less than 20%, or less than 50% glycerol. In some embodiments, a first solution includes additives for modifying protein activity. In some embodiments, a first solution includes additives for stabilizing ions, such as, for example, KC1 or the like. In some embodiments, a first solution includes preservatives or protein stabilizers, such as, for example, glycerol, KOAc, or the like. In some embodiments, a first solution includes surfactants, such as, for example, Tween 20, Tween 80, or the like.

[0034] In some embodiments, a first predetermined amount of time is an amount of time to saturate a binding capacity of at least one calcium-sequestering protein. In some embodiments, a first predetermined amount of time is less than 1 minute, less than 5 minutes, less than 10 minutes, less than 30 minutes, less than 1 hour, less than 2 hours, less than 5 hours, less than 10 hours, or less than 20 hours.

[0035] At step 1002, the at least one calcium-sequestering protein is allowed to contact a second solution for a second predetermined amount of time sufficient to release calcium from the at least one calcium-sequestering protein. In some embodiments, a second solution is adjusted to allow for calcium captured by the at least one calcium-sequestering protein to be released into the second solution for precipitation or otherwise stored in the second solution. In some embodiments, a concentration of calcium in a second solution is less than a concentration of calcium in a first solution. In some embodiments, a second solution is prepared to maximize a release of calcium by the at least one calcium-sequestering protein. In some embodiments, a second solution is adjusted to include one or more buffers disclosed herein. In some embodiments, a second solution is adjusted so as to maintain a predetermined pH range. In some embodiments, the second solution is at a pH below pH 4.5. In some embodiments, a second solution comprises calcium below a predetermined concentration. In some embodiments, a second solution comprises a column configured to extract calcium from the second solution. In some embodiments, a column includes a column for chromatography or solid-phase extraction.

[0036] In some embodiments, a second solution comprises a buffer. In some embodiments, a buffer comprises one or more reagents or counterions, thereby allowing at least a portion of released calcium to precipitate. In some embodiments, a buffer lacks or includes calcium at a concentration below 2 mM. In some embodiments, an extraction buffer includes HCO_3-, Na-HCOO, Na-CH_3COO, $NaNaSO_4$, HEPES, Tris-HCl, Phosphate buffer, MES, NaCl, TCEP, glycerol, or any combination thereof. In some embodiments, a buffer includes a formate buffer, an acetic acid buffer, or the like. In some embodiments, a second solution includes additives for enhancing protein activity. In some embodiments, a second solution includes additives for stabilizing ions. In some embodiments, a second solution includes preservatives or protein stabilizers, such as, for example, glycerol, KOAc, or the like. In some embodiments, a second solution includes chelators. In some embodiments, a second solution includes surfactants, such as, for example, Tween 20, Tween 80, or the like. In some embodiments, a portion of calcium

released from the at least one calcium-sequestering protein comprises at least 1%, at least 10%, at least 50%, at least 75% or about 100% of calcium bound to the at least one calcium-sequestering protein. In some embodiments, a portion of calcium released is allowed to precipitate so as to form solid calcium carbonate ($CaCO_3$), calcium formate, calcium acetate or other calcium salts.

Variants of Calcium-Sequestering Proteins and Methods

[0037] In some embodiments, the present disclosure further provides variants of naturally-occurring calcium-sequestering proteins and methods of making thereof, wherein the variants are capable of sequestering calcium in solution with greater activity than naturally-occurring calcium-sequestering proteins.

[0038] A calcium-sequestering protein includes a protein capable of capturing calcium ions. In some embodiments, naturally-occurring calcium-sequestering proteins comprise calsequestrins, calmodulins, troponin C, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), phospholambans, sodium-calcium exchangers, L-type calcium channels, ryanodine, receptors, or the like or isoforms thereof. In some embodiments, the relative calcium concentrations of the first solution or the second solution is adjusted according to a binding constant of the naturally-occurring calcium-sequestering protein In some embodiments, the at least one calcium-sequestering protein includes a calsequestrin protein (e.g., CASQ1, CASQ2, or the like). In some embodiments, the at least one calcium-sequestering protein includes a variant of a calsequestrin protein, discussed in more detail below.

[0039] Calsequestrins bind and store calcium in the endoplasmic reticulum and release calcium during cellular events like movement, contraction, and division. In some embodiments, variants of calcium-sequestering proteins are engineered that are selective for calcium ions over magnesium and other divalent ions and are capable of sequestering large quantities of calcium.

[0040] In some embodiments, variants of natural calcium-sequestering proteins described herein include one or more amino acid sequence that allows for sequestering calcium in a solution. In some embodiments, one or more amino acid sequences that allow for sequestering calcium in a solution are at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to any number of SEQ ID NOS: 1-10 listed in Table 1. In some embodiments, one or more DNA sequences that allow for sequestering calcium in a solution are at least 70%, at least 75%, at least 80%, at least 85%, at

least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to any number of SEQ ID NOS: 11-12 listed in Table 2. In some embodiments, a protein or amino acid sequence or a DNA sequence is "human", but can be optimized for bacterial expression.

[0041] In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises one or more of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. SEQ ID NO: 1 includes an exemplary amino acid sequence at the beginning of a naturally-occurring calcium-sequestering protein CASQ1. SEQ ID NO: 2 and 4 include exemplary amino acid sequences at an end of a naturally-occurring calsequestrin protein CASQ1. SEQ ID NO: 3 includes an amino acid sequence from a middle portion of CASQ1.

[0042] In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NO: 1. In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NO: 2. In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NO: 4. In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NOs: 1 and 4. In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NOs: 1 and 2.

[0043] In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence that is at least 70%, at least 80%, or at least 90% identical to SEQ ID NO: 3. In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence of SEQ ID NO: 1 and an amino acid sequence at least 70%, at least 80%, or at least 90% identical to SEQ ID NO: 3. In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence of SEQ ID NO: 2 and an amino acid sequence at least 70%, at least 80%, or at least 90% identical to SEQ ID NO: 3. In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence of SEQ ID NO: 4 and an amino acid sequence at least 70%, at least 80%, or at least 90% identical to SEQ ID NO: 3. In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence of each of SEQ ID NOs: 1 and 2 and an amino acid sequence at least 70%, at least 80%, or at least 90% identical to SEQ ID NO: 3. In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence of each of SEQ ID NOs: 1 and 4 and an amino acid sequence at least 70%, at least 80%, or at least 90% identical to SEQ ID NO: 3. In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence of each of SEQ ID NOs: 1 and 3. In some embodiments, a variant of a calcium-

sequestering protein comprises an amino acid sequence of each of SEQ ID NOs: 1, 3 and 4. In some embodiments, a variant of a calcium-sequestering protein comprises an amino acid sequence of each of SEQ ID NOs: 1, 2 and 3. In some embodiments, variants of natural calcium-sequestering proteins described herein include one or more amino acid sequence that allows for one or more of purification, tracking, binding, or some other functionality discussed in more detail herein. In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises at least SEQ ID NOS. 5 and 6. SEQ ID NOS: 5 and 6 include amino acid sequences directed to a strep-tag and an enhanced green-fluorescent protein (eGFP) tag, respectively, one or more of which allow for purification, such as the strep-tag of a poly-His tag, a Factor-Xa or tracking such as GFP or another fluorescent protein. For example, a strep-tag allows for binding of the protein to a streptavidin moiety, which further allows for binding to biotin. In some embodiments, variants of natural calcium-sequestering proteins described herein include one or more amino acid sequence that allows for cleavage of a protein from one or more tags attached to the protein. In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NO: 7. SEQ ID NO: 7 is an amino acid sequence comprising a thrombin site for allowing cleavage of the amino acid sequence to allow for a strep-tag to remain bound to an additional sequence, e.g., an eGFP tag.

[0044] In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NO: 8. SEQ ID NO: 8 is an amino acid sequence comprising a calsequestrin protein, i.e., CASQ1. In some embodiments, an amino acid sequence comprises an amino acid sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 8.

[0045] In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NO: 9. SEQ ID NO: 9 is an amino acid sequence comprising each of SEQ ID NOS: 1-8. In some embodiments, an amino acid sequence comprises an amino acid sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 9.

[0046] In some embodiments, variants of natural calcium-sequestering proteins described herein include one or more amino acid sequence that allows for overexpression in a cell culture. In some embodiments, an amino acid sequence of a variant of a calcium-sequestering protein comprises SEQ ID NO: 10. SEQ ID NO: 10 is an amino acid sequence that includes a calsequestrin protein, i.e., CASQ1, and a BK DNA sequence codon optimized to *E. coli*

expression. In some embodiments, an amino acid sequence comprises an amino acid sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 10.

[0047] SEQ ID NOs: 11 and 12 are DNA sequences corresponding to each of SEQ ID Nos: 9 and 10, respectively. SEQ ID NOs: 11 and 12 are codon optimized for *E. coli* and comprise the genetic information for the whole expressed proteins, including the tags.

[0048] Table 1 provides illustrative amino acid sequences related to calsequestrin variants having calcium sequestering activity. Table 2 provides illustrative DNA sequences related to calsequestrin variants having calcium sequestering activity.

TABLE 1 – Example Protein Sequences

SEQ ID	SEQUENCE NAME	SEQUENCE
1	CASQ1 MONOMER 1	LDFPEYDGVDRVINVNAKNYKNVFKKYEVLALLYHEPPE DDKA
2	CASQ1 MONOMER II	DVLEGEINTEDDDDDDDD
3	CASQ1 MONOMER III	LDFPEYDGVDRVINVNAKNYKNVFKKYEVLAL LYHEPPEDDKASQRQFEMEELILELAAQVLEDK GVGFGLVDSEKDAAVAKKLGLTEVDSMYVFKG DEVIEYDGEFSADTIVEFLLDVLEDPVELIEGERE LQAFENIEDEIKLIGYFKSKDSEHYKAFEDAAEE FHPYIPFFATFDSKVAKKLTLKLNEIDFYEAFMEE PVTIPDKPNSEEEIVNFVEEHRRSTLRKLKPESM YETWEDDMDGIHIVAFAEEADPDGFEFLETLKA VAQDNTENPDLSIIWIDPDDFPLLVPYWEKTFDI DLSAPQIGVVNVTDADSVWMEMDDEEDLPSAE ELEDWLE

4	CASQ1 MONOMER IV	DVLEGEINTE
5	Strep tag II	MASWSHPQFEK
6	eGFP	MRGSQQQQQQGSMSKGEELFTGVVPILVELDGDVNGHK FSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTLVTTLTY GVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGT YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNF NSHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQ QNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLE FVTAAGITHGMDELYKG
7	Thrombin cut site	LVPRGSGAAQEG
8	CASQ1	LDFPEYDGVDRVINVNAKNYKNVFKKYEVLALLYHEPPE DDKASQRQFEMEELILELAAQVLEDKGVGFGLVDSEKD AAVAKKLGLTEVDSMYVFKGDEVIEYDGEFSADTIVEFL LDVLEDPVELIEGERELQAFENIEDEIKLIGYFKSKDSEIIY KAFEDAAEEFHPYIPFFATFDSKVAKKLTLKLNEIDFYEAF MEEPVTIPDKPNSEEEIVNFVEEHRRSTLRKLKPESMYET WEDDMDGIHIVAFAEEADPDGFEFLETLKAVAQDNTENP DLSIIWIDPDDFPLLVPYWEKTFDIDLSAPQIGVVNVTDA DSVWMEMDDEEDLPSAEELEDWLEDVLEGEINTEDDDD DDDD
9	CASQ1GFP	MASWSHPQFEKGALEVLFQGPGMRGSQQQQQQGSMSK GEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKL TLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKRH DFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLV NRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKN

		GIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDN HYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELY KGAAALVPRGSGAAQEGLDFPEYDGVDRVINVNAKNYK NVFKKYEVLALLYHEPPEDDKASQRQFEMEELILELAAQ VLEDKGVGFGLVDSEKDAAVAKKLGLTEVDSMYVFKGD EVIEYDGEFSADTIVEFLLDVLEDPVELIEGERELQAFENI EDEIKLIGYFKSKDSEHYKAFEDAAEEFHPYIPFFATFDSK VAKKLTLKLNEIDFYEAFMEEPVTIPDKPNSEEEIVNFVEE HRRSTLRKLKPESMYETWEDDMDGIHIVAFAEEADPDGF EFLETLKAVAQDNTENPDLSIIWIDPDDFPLLVPYWEKTF DIDLSAPQIGVVNVTDADSVWMEMDDEEDLPSAEELED WLEDVLEGEINTEDDDDDDDD
10	CAL+BK	MQEGLDFPEYDGVDRVINVNAKNYKNVFKKYEVLALLY HEPPEDDKASQRQFEMEELILELAAQVLEDKGVGFGLVD SEKDAAVAKKLGLTEVDSMYVFKGDEVIEYDGEFSADTI VEFLLDVLEDPVELIEGERELQAFENIEDEIKLIGYFKSKD SEHYKAFEDAAEEFHPYIPFFATFDSKVAKKLTLKLNEIDF YEAFMEEPVTIPDKPNSEEEIVNFVEEHRRSTLRKLKPES MYETWEDDMDGIHIVAFAEEADPDGFEFLETLKAVAQDN TENPDLSIIWIDPDDFPLLVPYWEKTFDIDLSAPQIGVVNV TDADSVWMEMDDEEDLPSAEELEDWLEDVLEGEINTGG GGGRPPGFSPFR

TABLE 2 – Example DNA Sequences

SEQ ID	SEQUENCE NAME	SEQUENCE
11	CASQ1GFP	ATGGCAAGCTGGAGCCACCCGCAGTTCGAAAAGGGTG CACTTGAAGTCCTCTTTCAGGGACCCGGGATGAGGGG ATCACAGCAGCAACAACAACAAGGTAGCATGAGCAAA GGTGAGGAATTATTTACCGGTGTGGTTCCGATTCTGGTA GAGCTGGATGGCGATGTCAACGGCCATAAATTCTCTGT

		TCGTGGCGAGGGTGAGGGCGATGCAACCAATGGTAAA CTCACTCTGAAATTTATTTGTACCACGGGTAAGCTGCCG GTCCCTTGGCCTACATTGGTCACGACCCTCACGTACGG CGTTCAGTGCTTTAGCCGTTATCCGGATCACATGAAAC GTCACGATTTCTTCAAGAGTGCTATGCCGGAAGGCTAC GTGCAAGAGCGCACCATCAGCTTTAAGGATGATGGCAC TTACAAGACCCGTGCAGAGGTGAAATTCGAGGGTGAT ACCTTGGTCAATCGTATTGAGCTGAAAGGTATCGACTT CAAAGAAGACGGAAACATTCTGGGTCACAAACTGGAG TACAACTTTAATTCCCACAACGTGTATATCACTGCCGAT AAACAGAAAAACGGGATTAAAGCGAATTTCAAGATCC GCCACAACGTGGAGGACGGCTCCGTTCAACTGGCGGA CCACTACCAGCAGAATACCCCGATCGGCGATGGTCCGG TTCTGCTGCCGGACAACCATTACCTGTCAACCCAGAGC GTGTTGTCGAAGGATCCGAATGAAAAGCGCGATCACAT GGTTTTGTTGGAGTTCGTTACGGCAGCCGGTATAACCC ATGGTATGGATGAGTTGTACAAAGGTGCCGCGGCGCTG GTGCCGCGTGGTAGCGGTGCGGCTCAAGAAGGTCTGG ATTTCCCAGAGTACGACGGTGTTGATCGTGTTATTAACG TAAACGCGAAAAACTATAAGAATGTGTTTAAAAAGTAT GAAGTTCTGGCGTTGTTGTACCATGAACCACCGGAAGA CGACAAAGCAAGCCAGCGCCAGTTTGAGATGGAGGAA CTGATTCTGGAGTTGGCTGCGCAAGTTCTCGAGGACAA GGGCGTGGGTTTTGGTCTGGTGGATTCCGAGAAGGAC GCTGCGGTGGCGAAAAAATTGGGCCTGACGGAGGTGG ACTCCATGTATGTTTTTAAGGGCGACGAGGTCATTGAAT ACGACGGCGAATTCAGCGCTGACACCATCGTGGAGTTC CTGCTTGACGTGCTTGAGGATCCAGTTGAATTGATTGA GGGCGAGAGAGAACTTCAAGCATTCGAGAACATCGAG GACGAAATTAAGTTAATCGGCTATTTTAAGAGCAAGGA CAGCGAACATTATAAAGCGTTCGAAGATGCGGCGGAA GAATTTCATCCGTACATCCCGTTTTTCGCGACCTTTGAT

		TCAAAGGTGGCCAAGAAGCTGACCCTGAAACTGAATG AAATCGATTTCTATGAAGCGTTCATGGAAGAGCCAGTT ACCATCCCGGATAAACCGAATTCTGAAGAAGAGATCGT GAACTTTGTTGAAGAGCACCGCCGTAGCACCCTGCGTA AACTGAAGCCGGAATCTATGTACGAGACGTGGGAAGA CGACATGGATGGCATCCACATTGTAGCGTTCGCGGAAG AGGCCGATCCGGATGGTTTTGAATTCCTGGAGACCCTG AAGGCAGTTGCCCAAGATAACACCGAAAATCCGGACT TAAGCATTATTTGGATCGATCCGGACGACTTCCCGCTGT TGGTGCCGTATTGGGAAAAAACCTTCGACATCGATCTG TCCGCACCGCAGATTGGCGTGGTGAACGTTACTGATGC TGATTCCGTTTGGATGGAAATGGACGACGAAGAGGACT TACCGAGCGCGGAAGAGCTGGAGGACTGGCTGGAGGA CGTGCTGGAAGGCGAGATCAACACCGAAGACGACGAC GATGACGACGACGACTAA
12	CAL+ BK	ATGGGCCATCATCATCATCATCATCATCATCACAGC AGCGGCCATATCGAAGGTCGTCATATGCAAGAAGGACT AGACTTTCCCGAGTATGATGGCGTGGACCGCGTGATCA ACGTCAACGCGAAAAACTATAAAAACGTTTTTAAGAA GTACGAGGTGCTTGCCCTGCTGTACCATGAACCGCCGG AAGACGACAAGGCGTCGCAACGTCAGTTTGAGATGGA AGAGCTGATCCTGGAGTTGGCAGCGCAGGTTCTGGAG GACAAAGGCGTAGGTTTTGGTCTGGTTGATTCCGAGAA GGACGCGGCGGTTGCTAAGAAACTGGGTCTCACCGAA GTCGATTCTATGTACGTTTTTAAGGGTGATGAGGTCATC GAATATGATGGCGAATTCTCCGCTGATACCATTGTCGAG TTCTTGCTGGACGTTCTGGAGGACCCGGTGGAACTCAT CGAAGGTGAACGTGAACTGCAGGCATTCGAGAACATT GAGGACGAGATCAAACTGATTGGCTATTTTAAAAGCAA AGACAGCGAGCACTACAAAGCGTTCGAAGACGCCGCG GAGGAGTTCCATCCGTATATTCCGTTCTTCGCCACCTTT

		GATTCTAAGGTGGCCAAAAAGCTAACTCTGAAACTCA ATGAAATCGACTTCTATGAAGCGTTTATGGAAGAGCCG GTTACCATCCCCGACAAGCCGAATAGCGAAGAAGAAA TCGTTAACTTCGTGGAAGAGCACCGCCGTAGCACCCTG CGCAAGCTGAAACCGGAGAGCATGTACGAAACCTGGG AGGACGACATGGATGGTATCCACATTGTGGCGTTTGCA GAAGAGGCGGACCCAGACGGCTTCGAGTTCTTGGAAA CGCTTAAGGCAGTCGCTCAAGATAATACGGAAAATCCG GATTTGTCCATTATTTGGATCGACCCGGATGATTTTCCG CTGTTAGTTCCGTACTGGGAAAAGACCTTTGATATCGA CCTGAGCGCTCCGCAAATTGGCGTGGTGAACGTGACT GATGCGGATAGCGTTTGGATGGAAATGGATGATGAGGA AGATCTGCCTTCTGCTGAGGAATTGGAGGACTGGCTGG AGGACGTGTTGGAGGGCGAGATTAACACCGGTGGTGG TGGCGGCCGTCCGCCAGGTTTCTCACCGTTTCGTTAA

[0049] One non-limiting example of a method for preparing a variant of a calcium-sequestering protein is to express nucleic acids encoding one or more peptides or polypeptides in a suitable host cell, such as a bacterial cell, a yeast cell, or other suitable cell, using methods well known in the art, and recovering the expressed peptide or polypeptide, again using well-known purification methods, as described herein. Calcium-sequestering proteins can be isolated directly from cells that have been transformed with expression vectors as described herein. Recombinantly expressed calcium-sequestering proteins can also be expressed as fusion proteins with appropriate affinity tags and affinity purified, if desired. A calcium-sequestering protein of the present disclosure can retain the affinity tag, if desired, or optionally the affinity tag can be removed from the calcium-sequestering protein using well known methods to remove an affinity tag, for example, using appropriate enzymatic or chemical cleavage. Thus, provided herein are calcium-sequestering proteins without or optionally with an affinity tag. Accordingly, in some embodiments, provided herein is a host cell expressing a variant of a calcium-sequestering protein disclosed herein. A calcium-sequestering protein or variant thereof can also be produced by chemical synthesis using a method of protein synthesis well known to one of skill in the art.

[0050] In some embodiments, a variant of a calcium-sequestering protein includes a peptide tag. In some embodiments, a peptide tag includes a purification peptide tag. In some embodiments, a peptide tag includes a heterologous polypeptide tag (i.e., the heterologous polypeptide is a detectable label) for ease of tracking and/or purification (e.g., a fluorescent protein, e.g., green fluorescent protein (GFP), enhanced GFP (eGFP), YFP, RFP, CFP, mCherry, tdTomato, and the like; a histidine tag, e.g., a 6XHis tag; a hemagglutinin (HA) tag; a FLAG tag; GST tag; a Myc tag; and the like). "Heterologous," as used herein, can refer to a nucleotide or polypeptide sequence that is not found in the native nucleic acid or protein, respectively. In some cases, a heterologous polypeptide may comprise a sequence different from a sequence of a given protein. In some embodiments, variants of natural calcium-sequestering proteins described herein are capable of attaching to sand, agarose beads and other substrates. The term "bead" is not limited to any particular size or shape. Beads may be uniform or non-uniform, spherical or non-spherical, regular, or irregular. In embodiments, beads are magnetic or paramagnetic. The surface of the beads may comprise a reaction partner for the functional moiety for immobilization (e.g., streptavidin coated beads for capture of targets comprising a biotin functionality). In some embodiments, the beads are microparticles or nanoparticles. In some embodiments, the one or more substrates may comprise a polymeric substance. In some embodiments, the one or more substrates may comprise a hydrogel. In some embodiments, the one or more substrates may comprise glass, PDMS, a hydrogel, plastic, magnetic beads, agarose beads, nitrocellulose, or any other support whose surface can be modified to bind proteins, peptides, or nucleic acids. In some embodiments, the one or more substrates may comprise any one or combination of substrates presented in this disclosure. In some embodiments, one or more purification tags can be used to adhere binding of the protein onto various substrates or be a tag used only for attachment, such as a silicon-binding tag for glass-based particles. In some embodiments, variants of natural calcium-sequestering proteins described herein are configured to be embedded in a substrate. In some embodiments, a substrate includes one or more membrane or membrane filter. In some embodiments, a substrate includes a plurality of beads. In some embodiments, at least one calcium-sequestering protein is embedded in a surface or a bulk of a substrate. In some embodiments, at least one calcium-sequestering protein is embedded in a substrate so as to cover at least 1%, at least 10%, at least 25%, at least 50%, at least 75%, or about 100% of a substrate.

[0051] In some embodiments, the present disclosure provides methods of making variants of a calcium-sequestering protein. In some embodiments, a method includes cultivating a cell overexpressed with a nucleic acid construct that encodes the variant of the calcium-sequestering protein. In some embodiments, a method includes inducing the cell to express the variant of the calcium-sequestering protein encoded by the nucleic acid construct. In some embodiments, a method includes lysing the cell. In some embodiments, a method includes purifying the variant of the calcium-sequestering protein from the lysed cell.

[0052] In some embodiments, a variant of a calcium-sequestering protein of exhibits calcium-sequestering activity that is at least 1%, at least 10%, at least 20%, at least 30%, at least 50%, at least 75% or at least 100% greater than a natural calcium-sequestering protein. In some embodiments, a natural calcium-sequestering protein includes a calsequestrin protein.

Extraction Devices and Methods

[0053] In some embodiments, the present disclosure provides extraction devices incorporating at least one calcium-sequestering agent, the extraction devices being adapted to receive solutions rich in calcium for capture and release of calcium. In some embodiments, the present disclosure further provides methods of use of extraction devices incorporating at least one calcium-sequestering agent.

[0054] As discussed above, there is a need for renewable techniques for capturing calcium. An extraction device described here allow for renewable application to calcium-rich solutions, such as ocean water and sodium hydroxide, thereby reducing reliance on mining. Such a technique significantly reduces the environmental impact compared to conventional methods.

[0055] Provided herein is an extraction device for capturing calcium. In some embodiments, an extraction device comprises a sample interface configured to receive a sample comprising calcium, such as a calcium-containing solution, and a calcium-sequestering agent. In some embodiments, a calcium-sequestering agent comprises a carrier and a calcium-sequestering protein. The calcium-sequestering protein can be a protein that sequesters or binds to calcium in the sample comprising calcium, such as the calcium-sequestering proteins as described above. In some embodiments, a carrier comprises a solution or a substrate.

[0056] In some embodiments, a calcium-sequestering agent comprises a calcium-sequestering protein dispersed in a carrier solution (e.g., distributed through a liquid phase such an aqueous solution). In some embodiments, the calcium-sequestering agent comprises one or more of a calcium-sequestering protein, a substrate, or a combination thereof in a solution, a suspension,

an emulsion, or the like. In some embodiments, the solution, suspension, or emulsion comprises the calcium-sequestering protein, a substrate, or a combination thereof in the form of a plurality of solid particles.

[0057] In some embodiments, a calcium-sequestering agent comprises a calcium-sequestering protein embedded on or within a carrier substrate (e.g., a bead, a filter, a membrane, or other substrate). In some embodiments, a substrate is embedded with a calcium-sequestering protein to provide for the substrate to capture at least a portion of the calcium from the sample. A calcium-sequestering protein allows for the substrate to entrap aqueous calcium ions from sea water or other calcium-rich solutions. The calcium ions can then be released by exposure to water or a low-calcium solution that can be precipitated with counterions and stored for later use.

[0058] In some embodiments, the sample interface includes a membrane filter. In some embodiments, the membrane filter includes a dialysis filter stack.

[0059] In some embodiments, at least one calcium-sequestering protein is embedded in a substrate. In some embodiments, a substrate includes one or more membranes or membrane filters. In some embodiments, at least one calcium-sequestering protein is embedded in a surface or a bulk of a substrate. In some embodiments, at least one calcium-sequestering protein is embedded in a substrate so as to cover at least 1%, at least 10%, at least 25%, at least 50%, at least 75%, or about 100% of the substrate.

[0060] In some embodiments, a substrate comprises sand, agarose beads, or the like. The term "bead" is not limited to any particular size or shape. Beads may be uniform or non-uniform, spherical or non-spherical, regular, or irregular. In embodiments, beads are magnetic or paramagnetic. The surface of the beads may comprise a reaction partner for the functional moiety for immobilization (e.g., streptavidin coated beads for capture of targets comprising a biotin functionality). In some embodiments, the beads are microparticles or nanoparticles. In some embodiments, the one or more substrates may comprise a polymeric substance. In some embodiments, the one or more substrates may comprise a hydrogel. In some embodiments, the one or more substrates may comprise glass, PDMS, a hydrogel, plastic, magnetic beads, agarose beads, nitrocellulose, or any other support whose surface can be modified to bind proteins, peptides, or nucleic acids. In some embodiments, the one or more substrates may comprise any one or combination of substrates presented in this disclosure.

[0061] In some embodiments, a calcium-sequestering protein is encapsulated in a dialysis chamber rather than attached to a bead or another matrix.

[0062] In some embodiments, a substrate includes one or more membranes or membrane filters or is in a column. In some embodiments, a substrate includes a plurality of beads. In some embodiments, at least one calcium-sequestering protein is embedded in a surface or the bulk of a substrate. In some embodiments, at least one calcium-sequestering protein is embedded in a substrate so as to cover at least 1%, at least 10%, at least 25%, at least 50%, at least 75%, or about 100% of a substrate.

[0063] Further described herein is an extraction system for capturing calcium. In some embodiments, an extraction system includes at least one input or inlet to receive a sample comprising calcium. In some embodiments, an extraction system includes at least one filter configured to filter or remove suspended solids and aggregates from the sample comprising calcium. In some embodiments, the filter is a vacuum-based filter. In some embodiments, the filter (1) comprises a pore size 0.22 μm. In some embodiments, the filter (1) comprises a polyether sulfone membrane or the like.

[0064] In some embodiments, an extraction system includes at least one chamber for containing the filtered sample. In some embodiments, the chamber is configured to store a solvent. In some embodiments, a chamber comprises glass, polypropylene, or the like.

[0065] In some embodiments, an extraction system includes at least one pump for driving flow from the sample comprising calcium from the at least one inlet through the at least one filter into the at least one chamber. In some embodiments, a pump can be further optimized to obtained a desired rate of calcium sequestration and release. For example, a pump can be adjusted for controlling a flow rate and/or contact time of the protein to the calcium containing solution or a solution that has a lower or absent concentration of calcium. A pump may also be adjusted to control a flow rate and/or a contact time between the stored calcium and a reaction substrate, described in more detail below, for precipitating the calcium.

[0066] In some embodiments, an extraction system includes at least one sensor configured to measure at least one analyte and/or a pH in the system. In some embodiments, the at least one analyte comprises calcium, magnesium, or any other ion or divalent ion in the solution. In some embodiments, the sensor comprises an electrode, a pH probe, or the like.

[0067] In some embodiments, an extraction system includes at least one valve configured to change flow through the system. For example, the at least one valve is configured to allow flow through a first flow loop comprising at least one calcium-sequestering portion, e.g., a dialysis portion. A dialysis portion can include at least one calcium-sequestering protein described herein so as to sequester calcium from the sample comprising calcium. A dialysis portion can

include a dialysis chamber, a dialysis tube, a dialysis cassette, or the like. A dialysis portion can include one or more of Snakeskin™ Dialysis tubing, SpectraPor® Tube-A-Lyzer®, a Slide-A-Lyzer® Pierce®, or the like. In some embodiments, the dialysis portion is within a flow column in fluid communication with the at least one valve. In some embodiments, the dialysis portion includes dialysis tubes housed in a casing and configured to disperse flow from the at least one valve to allow the dialysis tubes to last longer. In some embodiments, the dialysis portion comprises a pH resistant plastic. In some embodiments, dialysis portion comprises polypropylene or the like. In some embodiments, dialysis tubes of the dialysis portion comprise dialysis membranes, a custom material, or the like.

[0068] In some embodiments, the extraction system is configured to circulate the sample to keep protein in constant equilibrium based on its dissociation constant. The circulation and contact time can be based on the calcium concentration and the amount of calcium-binding protein in the dialysis portion. Levels of calcium can be measured in the dialysis portion using a calcium sensor. In some embodiments, the extraction system is configured to drain the dialysis portion.

[0069] In some embodiments, the at least one valve is configured to allow flow through a second flow loop comprising at least one collection portion. In some embodiments, the at least one valve is configured to cut off flow through the first loop to allow flow through the second flow loop. As an example, the at least one valve can be controlled to allow the sample to flow through the second loop once a predetermined amount of calcium has been sequestered in the dialysis portion. A collection portion can include a second solution described herein, e.g., a buffer solution that has a lower concentration of calcium in the solution than the sample comprising calcium. Using the solution with the lower concentration of calcium, the calcium-sequestering protein depolymerizes and releases the sequestered calcium. Circulating buffer results in a localized low concentration of calcium leading to release of the sequestered calcium in the protein according to its dissociation constant. In some embodiments, the at least one collection portion is configured to reach kinetic equilibrium with the sample comprising calcium to as to obtain an equilibrated solution. In some embodiments, the second solution has a slightly lower pH than the sample comprising calcium.

[0070] In some embodiments, the extraction system comprises at least one reaction chamber. In some embodiments, the at least one reaction chamber comprises at least one reaction substrate (e.g., Na_2CO_3, NaOH, or the like or a combination thereof) or is in fluid communication with a separate chamber comprising the at least one reaction substrate. In some

embodiments, multiple reaction substrates are contained together or contained separately within the same reaction chamber or multiple reaction chambers. As an example, after equilibrium is reached in the sample comprising calcium to obtain the equilibrated solution, the equilibrated solution can flow into the reaction chamber, and the reaction substrate that is in the reaction chamber or is piped into the reaction chamber can react with the calcium in the equilibrated solution. In some embodiments, the reaction comprises a precipitation reaction. As an example, precipitation of the calcium can be facilitated and scaled using a stoichiometric equation described in more detail below.

[0071] In some embodiments, the extraction system comprises at least one shutoff valve, wherein the shutoff valve can be positioned at one or more locations along the extraction system. In some embodiments, the tubing in the extraction system comprises a pH resistant plastic, e.g., polypropylene or the like.

[0072] FIG. 1B illustrates a block diagram of an extraction system 2000 with which some embodiments may operate for capturing calcium. The system 2000 can be adapted to receive solutions rich in calcium for capture and release of calcium for future use.

[0073] As illustrated in FIG. 1B, the extraction system 2000 can include a solution chamber 2001 for containing a received solution rich in calcium or a sample comprising calcium. The solution can be previously filtered or actively filtered in the solution chamber 2001. The solution chamber 2001 is in fluidic communication via a first loop 2002 with a calcium-sequestering portion 2003. The calcium-sequestering portion 2003 includes a calcium-sequestering agent configured to bind the calcium in the sample comprising calcium. In some embodiments, at least one calcium-sequestering agent comprises a substrate. A calcium-sequestering agent allows for the substrate to entrap aqueous calcium ions from sea water or other calcium-rich solutions. The fluidic communication via the first loop 2002 is facilitated via at least one first loop inlet 2002A and at least one first loop outlet 2002B. In some embodiments, the fluidic communication is controlled by one or more valve between the solution chamber 2001 and the first loop inlet 2002A. By circulating the solution rich in calcium through the first loop 2002, calcium can controllably be sequestered by the calcium-sequestering agent in the calcium-sequestering portion 2003 until the solution reaches equilibrium and/or the calcium has been sufficiently sequestered by the calcium-sequestering agent.

[0074] The extraction system 2000 further illustrates the calcium-sequestering portion 2003 in fluid communication via a second loop 2004 with a calcium-collection portion 2005. The

fluidic communication via the second loop 2004 is facilitated via at least one second loop inlet 2004A and at least one second loop outlet 2004B. Once the desired amount of calcium has been sequestered in the solution in the calcium-sequestering portion 2003, the solution in the calcium-sequestering portion 2003 controlled to flow through the second loop 2004. Such control can be facilitated by one or more valves. The calcium-collection portion 2005 comprises a second solution with a lower concentration of calcium than the solution from the calcium-sequestering portion 2003, such that the calcium-sequestering agent releases the sequestered calcium, such as, for example, via depolymerization of a calcium-sequestering protein. The second solution can be, for example, a buffer described herein. Circulating the solution through the calcium-collection portion 2005 results in a localized low concentration of calcium leading to release of the sequestered calcium in the calcium-sequestering agent . Again, kinetic equilibrium is reached with the solution. In other words, as the buffer circulates, because the second solution does not contain calcium or contains a substantially lower amount of calcium and has a slightly lower pH, the calcium-sequestering protein is configured to release the sequestered calcium according to its dissociation constant in the calcium-collection portion 2005.

[0075] The extraction system 2000 further illustrates the calcium-collection portion 2005 in fluid communication with a reaction chamber 2006. The reaction chamber 2006 includes a reaction substrate (e.g., Na_2CO_3) that is already in the reaction chamber 2006 or is piped into the reaction chamber 2006. The reaction substrate allows for precipitation of the calcium as described in more detail herein. Once precipitated, the calcium can be used as desired. Extraction system 2000 can be scaled accordingly.

[0076] In some embodiments, solutions can be driven through one or more of first loop 2002 or second loop 2004 as a continuous process. As shown in 1B, the extraction system 2000 can further include at least one pump 2007 to drive the solutions through one or more of the first loop 2002 or second loop 2004. In some embodiments, the at least one pump is a plurality of pumps.

[0077] In some embodiments, solutions can interact with one or more of first loop 2002 or second loop 2004 as a batch process. In such a process, solutions may enter the solution chamber 2001 or the calcium-sequestering portion 2003 and remain for predetermined amount of time, or, for example, until the solutions reach equilibrium with the calcium-sequestering agent. Once the predetermined amount of time has passed, the calcium-sequestering agent can be drained from the solution chamber 2001 or the calcium-sequestering portion 2003, and the

solutions can be subsequently drained into the calcium-collection portion 2005 or the solutions in the calcium-collection portion 2005 can enter into the solution chamber 2001 or the calcium-sequestering portion 2003 and remain for predetermined amount of time, or, for example, until the solutions reach equilibrium with the calcium-collection solutions. Once the predetermined amount of time has passed, the calcium-collection solutions can be drained from the solution chamber 2001, the calcium-sequestering portion 2003, or the calcium-collection portion 2005, and the solutions can be subsequently drained into the reaction chamber 2006 or the solutions in the reaction chamber 2006 can enter into the solution chamber 2001, the calcium-sequestering portion 2003, or the calcium-collection portion 2005 and remain for predetermined amount of time, or, for example, until the solutions reach equilibrium with a reaction substrate. Any number of these steps may be repeated as necessary for additional calcium collection.

[0078] In some embodiments, the calcium-sequestering portion 2003, the calcium-collection portion 2005, and/or the reaction chamber 2006 further includes one or more of sedimentation, a filter, or the like to remove the calcium-sequestering agent (e.g., the calcium-sequestering protein) from the solution.

[0079] Further provided herein is a method of capturing calcium from a calcium-containing solution. In some embodiments, the method includes providing an extraction device for capturing calcium, the extraction device includes a sample interface configured to receive a sample comprising calcium, such as a calcium-containing solution; and a substrate embedded with a calcium-sequestering protein configured to capture at least a portion of the calcium when the substrate is contacted with the sample interface containing a calcium-containing solution. A method further includes contacting the substrate with the sample interface containing a sample solution comprising a calcium-containing solution for a first predetermined amount of time, thereby allowing the calcium-sequestering protein to bind to the calcium. A method further includes contacting the substrate with a storage solution for a second predetermined amount of time, the second predetermined amount of time being until a predetermined amount of the calcium is released from the calcium-sequestering protein. In some embodiments, the predetermined amount of calcium comprises at least 5%, at least 10%, at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or at least 95% of the calcium.

[0080] In some embodiments, the calcium-containing solution comprises calcium at a concentration above 1 mM, above 10 mM, above 100 mM, 150 mM, above 300 mM, above 500 mM, etc.

[0081] In some embodiments, contacting the substrate with the sample interface containing a sample solution comprising calcium for a first predetermined amount of time, thereby allowing the substrate to bind to the calcium; and contacting the substrate with a storage solution for a second predetermined amount of time, the second predetermined amount of time being until at least 90% of the calcium is released from the substrate embedded with the calcium-sequestering protein.

Methods of Engineering Protein Variants

[0082] In some embodiments, variants may be engineered using a nucleic acid. In some embodiments, a nucleic acid is a double stranded nucleic acid (e.g., DNA). In some embodiments, a nucleic acid is a single stranded nucleic acid (e.g., a RNA, wherein the RNA comprises a mRNA, a rRNA, a tRNA, a non-coding RNA, a long non-coding RNA, a microRNA (miRNA), a small interfering RNA (siRNA), and a single-stranded RNA (ssRNA)).

[0083] In some embodiments, a nucleic acid described herein is a vector. In some embodiments, a nucleic acid comprises nucleotides in a range of from 5 to 100, 10 to 100, 20 to 100, 50 to 100, 70 to 100, or more nucleotides. In some embodiments, a nucleic acid comprises nucleotides in a range of from 0.1 kb to 5 kb, from 0.5 kb to 5 kb, from 1 kb to 5 kb, from 2 kb to 5 kb, from 3 kb to 5 kb, or from 4 kb to 5 kb. In some embodiments, a vector is a viral vector. In some embodiments, a viral vector is derived from one or more types of viruses, including but not limited to retroviruses (e.g., lentiviruses and γ-retroviruses), adenoviruses, arenaviruses, alphaviruses, adeno-associated viruses (AAVs), baculoviruses, vaccinia viruses, herpes simplex viruses and poxviruses. In some embodiments, a viral vector is an adeno-associated viral vector.

[0084] In some embodiments, a nucleic acid sequence of such variants may be designed and synthesized by molecular biology methods and the nucleic acid sequence introduced into a cell.

[0085] As used herein, a "cell" refers to a biological cell. Some non-limiting examples include: a prokaryotic cell, eukaryotic cell, a bacterial cell, an archaea cell, a cell of a single-cell eukaryotic organism, a protozoa cell, a cell from a plant, an algal cell, a fungal cell, a fungal protoplast cell, an animal cell, and the like. Sometimes a cell is not originating from a natural organism, e.g., a cell can be a synthetically made, sometimes termed an artificial cell or a non-naturally occurring cell.

[0086] In some embodiments, a "cell" refers to a microbial organism. As used herein, the terms "microbial," "microbial organism" or "microorganism" are intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea, and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

[0087] In some embodiments, a microbial organism includes a non-naturally occurring microbial organism. The non-naturally occurring microbial organisms of the present disclosure can contain stable genetic alterations, which refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

[0088] In the case of gene disruptions, a particularly useful stable genetic alteration is a gene deletion. The use of a gene deletion to introduce a stable genetic alteration is particularly useful to reduce the likelihood of a reversion to a phenotype prior to the genetic alteration. For example, if desired, stable growth-coupled production of a biochemical can be achieved, for example, by deletion of a gene encoding an enzyme catalyzing one or more reactions within a set of metabolic modifications. The stability of growth-coupled production of a biochemical can be further enhanced through multiple deletions, significantly reducing the likelihood of multiple compensatory reversions occurring for each disrupted activity.

[0089] Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein are described with reference to a suitable source or host organism such as *Escherichia coli*, yeast, or other organisms disclosed herein and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes encoding enzymes for their corresponding metabolic reactions for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than

the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

[0090] Generally, a host microbial organism is selected such that it produces a variant of a calcium-sequestering protein or a precursor thereof, either as a naturally produced molecule or as an engineered product that either provides *de novo* production of a desired variant or precursor or increased production of a variant or precursor naturally produced by the host microbial organism. For example, there are various precursors that are produced naturally in a host organism such as *E. coli*. A host organism can be engineered to increase production of a variant, as disclosed herein, or a precursor. In addition, a microbial organism that has been engineered to produce a desired variant can be used as a host organism and further engineered to express calcium-sequestering proteins.

Exemplary Embodiments

[0091] The following specific examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and systems of the present disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure.

Example 1: Method of making calsequestrin variant

[0092] The purpose of this example is to provide an exemplary process for generating a variant of calsequestrin having SEQ ID NO: 7, referred herein as CASQ1GFP which has high calcium binding affinity but a relatively weak dissociation constant.

[0093] The DNA of calsequestrin variants according were introduced into bacteria (BL21).

[0094] *Preculture prep*

[0095] A preculture was grown at 25°C. Precultures contained 0.4% glucose (filtered).

[0096] *Induction*

[0097] 2L of LB was induced with 1mM isopropyl β- d-1-thiogalactopyranoside (IPTG) and placed in a shaker for 5 hours at 25°C.

[0098] *Lyse and sonicate*

[0099] Pellets containing the BL21 cells overexpressed were purified starting with cellular lysis by sonication 30 min total time, 15 seconds on and 15 seconds off at a 50% amplitude.

[0100] *Prep for purification*

[0101] After sonication the resulting lysate was spun down again at 4000 RPM for 30 minutes and the supernatant was removed.

[0102] *Purification 1*

[0103] Purification was performed using a Strep-tactin XT Sepharose resin (i.e., chromatography resin) purification protocol.

[0104] Strep-tactin XT Sepharose resin was equilibrated by adding 5 column volumes (CV) of Lysis buffer (100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1% Triton X-100). Next, supernatant from the prep was placed in a conical tube or a sealed column with the equilibrated Strep-tactin XT. The conical tube or sealed column was shaken at 4°C for 1 hour. The conical tube or sealed column was washed with Lysis buffer 5-10 CV.

[0105] The conical tube or sealed column was eluted with 5 CV of elution buffer (100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 25 mM Biotin). FIG. 2 illustrates purification of the resulting CASQ1GFP using the above purification 1 protocol, including wash steps 1-4 and Elution steps 1-5. The Streptactin TX purification is a 1 step purification system. Nonspecific binding was prevented using 2 elution buffers, such as 100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 2 mM Biotin followed by 100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 25 mM Biotin.

[0106] *Purification 2*

[0107] Strep-tactin XT Sepharose resin (i.e., chromatography resin) was equilibrated by adding 5 column volumes (CV) of Lysis buffer (100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1% Triton X-100). Next, the supernatant from the prep was placed in a conical tube or a sealed column with the equilibrated Strep-tactin XT. The conical tube or sealed column was shaken at 4°C for 1 hour. The conical tube or

sealed column was washed with wash buffer (100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1% Triton X-100) 5-10 CV.

[0108] The conical tube or sealed column was eluted with 20 CV of elution buffer (100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 20 mM Biotin). FIG. 3 illustrates purification of the resulting CASQ1GFP using the above purification 2 protocol.

[0109] *Digest*

[0110] 2 units of Biotin-thrombin were incubated with CASQ1GFP overnight at 4°C 17-20 hours. Thrombin site was cleaved between R and G leaving the strep tag II (i.e., SEQ ID NO: 3) on the GFP. The following day the digested protein was ran through the Strep-tactin column. Both eGFP (i.e., SEQ ID NO: 4) and the biotin-thrombin bound the column and the CASQ1 came out in the flowthrough and the wash.

Example 2: Method of making calsequestrin variant

[0111] The purpose of this example is to provide an exemplary process and results for a variant of calsequestrin having SEQ ID NO: 10, referred herein as CAL+BK.

[0112] *Lyse and sonicate*

[0113] The pellets containing the BL21 cells overexpressed were purified starting with cellular lysis by sonication 45 min total time, 15 seconds on and 30 seconds off at a 50% amplitude.

[0114] *Purification*

[0115] Purification was performed using a Ni-NTA purification column protocol.

[0116] The Ni-NTA agarose Resin bottle was gently shaken to completely resuspend the slurry and transfer to the column. Resin was allowed to settle. Bottom plug of the column was opened, and storage buffer was allowed to flow through and out.

[0117] The resin was equilibrated by adding 5 column volumes (CV) of Lysis buffer including 50 mM HEPES pH 8.0, 300 mM NaCl, 10mM $CaCl_2$ 0.5 mM TCEP, 10% glycerol, and 10 mM imidazole.

[0118] 10 CV of Wash buffer was added to the column and allowed to flow through. Flow through was monitored with absorbance at 280 nm to ensure complete removal of unbound

lysate. Coomassie Bradford reagent may also be used. Wash buffer: 50 mM HEPES pH 8.0, 300 mM NaCl, 10mM $CaCl_2$, 0.5 mM TCEP, 10% glycerol, and 50mM imidazole.

[0119] 2 CV of 50 mM HEPES pH 8.0, 300 mM NaCl, 10mM $CaCl_2$, 0.5 mM TCEP, 10% glycerol, and 100mM imidazole were eluted through the column (E1).

[0120] 2 CV of 50 mM HEPES pH 8.0, 300 mM NaCl, 10mM $CaCl_2$, 0.5 mM TCEP, 10% glycerol, and 250mM imidazole were eluted through the column (E2).

[0121] 5 CV 50 mM HEPES pH 8.0, 300 mM NaCl, 10mM $CaCl_2$, 0.5 mM TCEP, 10% glycerol, and 500mM imidazole were eluted through the column. Each CV after is an elution excluded from the gel.

[0122] FIG. 4 illustrates purification of the resulting CAL+BK using the above purification protocol.

Example 3: Capturing calcium with extraction device

[0123] The purpose of this example is to provide an exemplary process for capturing calcium with an extraction device (i.e., "dialysis cassette") including a variant of a calcium-sequestering protein, i.e., CAL+BK.

[0124] *Preliminary Data*

[0125] Storage buffer containing 10 mM calcium: 50 mM HEPES pH 8.0, 300 mM NaCl, 10mM $CaCl_2$, 0.5 mM TCEP, and 10% glycerol.

[0126] Extraction buffer containing no calcium: 50 mM HEPES pH 8.0, 300 mM NaCl, 0.5 mM TCEP, and 10% glycerol.

[0127] *Experiment 1:*

[0128] 13.31 μM CAL+BK was loaded into a 1 ml dialysis cassette and was placed in 150 ml of 50 mM HEPES pH 8.0, 300 mM NaCl, 0.5 mM TCEP, and 10% glycerol. The calcium contribution from storage buffer to the bulk solution was 17.15 μM final conc at equilibrium. The cassette was placed in the Extraction buffer overnight.

[0129] 258 μL of 13.31 μM CAL+BK was loaded into a 1 ml dialysis cassette and was placed in a beaker containing 150 ml of 50 mM HEPES pH 8.0, 300 mM NaCl, 0.5 mM TCEP, and

10% glycerol. The calcium contribution from storage buffer (same buffer but containing 10 mM $CaCl_2$) to the bulk solution was 17.15 μM final concentration at equilibrium. Calcium ion concentrations were measured using a calcium sensitive electrode.

[0130] FIG. 5 illustrates calcium concentrations in the storage buffer. In the experiment, this dialysis cassette containing 258 μL of CAL+BK was allowed to reach equilibrium with the bulk buffer overnight. FIG. 5 illustrates initial calcium concentrations that reflect the amount in the storage buffer. After placing the dialysis cassette in a low calcium solution, calcium is released from the protein and the concentration of the buffer increases. The change in calcium concentration from before extraction and after according to the electrode was 189.64 μM. When subtracting the contribution from the storage buffer the protein changed the total concentration of the bulk solution by 172.49 μM. This shows that that 0.15 mg of CAL+BK adds 1.14 mg of calcium to the bulk solution.

[0131] *Experiment 2:*

[0132] FIG. 6A illustrates dynamic light scattering (DLS) data of CAL+BK. 100 mM EDTA and 4 μM of CAL+BK was placed in a cuvette and DLS reads were taken over the course of 15 minutes. In other words, FIG. 6A shows the number of particles and how their hydrodynamic diameter changes over time. FIG. 6B illustrates DLS data of CAL+BK chelated in pH 4.7 buffer with 100 mM EDTA and run through an S200 column to desalt it into new storage buffer, i.e., 50 mM HEPES pH 8.0, 300 mM NaCl, 0.5 mM TCEP, and 10% glycerol.

[0133] *Experiment 3:*

[0134] FIG. 7 illustrates DLS correlation data of CAL+BK in storage buffer at different pH levels. Protein batch F.1 CAL+BK was in storage buffer containing 10 mM $CaCl_2$. The CAL+BK was buffer exchanged into pH 6.5 buffer, then exchanged again back into pH 8.2. FIG. 7 shows that the CAL+BK was dissociated based on pH. Specifically, changing the pH level from 8 to 6.5 changed the net charge from -80 to -74. The theoretical isoelectric point (PI) is 4.5. The DLS correlation data thus showed that changing the pH can result in the polymer coming apart and calcium being released from the polymer.

[0135] *Experiment 4:*

[0136] FIG. 8 illustrates DLS data for CAL + BK. Protein batch F.1 CAL+BK was in storage buffer containing 10 mM $CaCl_2$. The CAL+BK was buffer exchanged into pH 8 buffer, which

removes calcium from the buffer solution as seen in FIG. 8 by the change in the diameter between the peaks. Adding 100 μM of nickel resulted in a slight shift. Adding 10 mM Ca resulted in a shift consistent with refolding of the protein to its monomer form.

[0137] *Experiment 5:*

[0138] This experiment shows the modulation of the oligomeric state of CASQ1GFP in 50 mM HEPES pH 8.0, 500 mM NaCl, 0.5 mM TCEP, 10% glycerol, 10 mM CaCl2. FIGS. 9 and 10 illustrate trials performed in each of the buffer mentioned above and ocean water, respectively. 400 μL of 0.418 mg/ml of protein was placed in 400 mL of each solution. FIGS. 9-10 illustrate the ability of CASQ1GFP to capture calcium ions from solution, and that this ability is similar for different solutions. FIG. 9 illustrates a 118.33 ppm change in calcium concentration from a 250-ppm buffer. This means the protein changed the concentration of the solution by 2.95 mM. In FIG. 10, the ppm change in the ocean water was 113.017, signifying a concentration change of 2.82 mM. This means the protein captured 47.33 mg of calcium from the 250-ppm solution, and 45.21 mg from the ocean water.

[0139] *Experiment 6:*

[0140] This experiment shows inductively coupled plasma – mass spectrometry (ICP-MP) results of CASQ1GFP to different solutions in testing selectivity for calcium ions over magnesium.

[0141] 1 mL of 0.832 mg/mL CASQ1GFP (11.32 μM) was exposed to circulating solutions of 1:1 Ca:Mg, 1:0 Ca:Mg, or ocean water, each for about 20 minutes. The results comparing the change in Mg, percent capture of Mg, change in Ca, and percent capture of Ca are provided in Table 2, and ICP-MS stoichiometry are provided in Table 3.

[0142] Table 2 provides ICP-MS results for CASQ1GFP exposed to different capture solutions.

	Delta Mg (ppm)	%capture Mg (ppm)	Delta Ca (ppm)	%capture Ca (ppm)
Ocean water	751.75	63%	115.32	62%
1:1 capture	5.94	31%	1.29	3%
1:0 capture	-	-	9.53	27%

[0143] Table 3 provides stoichiometry of either Ca or Mg ions for each circulating solution.

	Ca ion per protein	Mg ion per protein	ppm Mg	ppm Ca
Ocean water	250	2733	1188	185
1:1 capture	2.82	21.6	19	38.4
1:0 capture	21		0.5	35.9

[0144] The results in Table 2 and Table 3 indicate the Mg is inhibitory at low concentrations to an extent, but as the concentration of Ca is raised, the impact of Mg is diminished. This is further evidenced by the electrode data using the 250 ppm Ca solution in FIG. 9 as compared to the ocean water in FIG. 10.

Example 4: Extraction system for capturing calcium

[0145] The purpose of this example is to provide an exemplary extraction system for capturing calcium that includes a variant of a calcium-sequestering protein.

[0146] In some embodiments, described herein is a method for harvesting metals from an aqueous source such as seawater includes populating a membrane filter with a binding protein having an affinity for a target metal and a weaker dissociation with the target metal, and passing an aqueous solution including the target metal through the membrane filter. A valve and filter apparatus terminates a flow of the aqueous solution and passes a buffer solution through the membrane filter for harvesting the target metal. In a particular configuration, the membrane filter is a dialysis filter stack. One particular protein used for the binding protein is calsequestrin or other highly negative charged protein with a particular affinity for positively charged metals such as calcium, uranium and gold. The source aqueous solution has a concentration of the target metal, such that harvesting the bound target metal occurs upon reaching a saturation based on the concentration of the aqueous solution as the metal binds with the binding protein.

[0147] An example herein includes a dialysis-based system that will be used with a calcium binding protein to sequester large quantities of calcium. This system can use ocean water and/or other calcium rich aqueous solutions. Calcium can be sequestered, released, and precipitated into a solid form. The product allows the calcium captured by the binding protein to be purified, concentrated and used for other applications. This device functions based on the dissociation constant of calcium binding proteins and uses dialysis for the release of aqueous calcium. This release is followed by introduction of dissolved carbon dioxide and sodium hydroxide to lower the pH, precipitating the released calcium as a salt.

[0148] Calcium is a fundamental material for development of structural materials and in providing strength, and is a required component for many industrial processes, most notably in the production of structural building materials. Global annual cement production is estimated at 4 billion tons, and roughly 50% of this mass is elemental calcium. Current methods of cement production rely on expensive limestone quarrying operations that depend on heavy machinery, high labor costs, and continued extraction from depletable deposits.

[0149] The systems described herein can isolate and precipitate calcium cost-effectively, thereby meeting the need for sustainable resource recovery in industries from chemical processing to construction materials. In particular, the systems herein can provide a renewable method for calcium production from ocean water and other calcium-rich aqueous solutions and does so with minimal maintenance and few labor hours. In addition to construction materials, the techniques described herein for extracting calcium can be used in other chemical and industrial applications, adding redundancy in the event of potential downturns in specific markets. Regarding possible business models for the systems herein, one option can include building a facility with large-scale device versions to capture ice and then sell the calcium directly; another option can include developing a modular version of the device that could be sold or leased to individual operators with acute needs for calcium production. Regardless, there are some consumable components for replacement, which may also generate recurring revenue depending on the chosen business model.

[0150] Calcium is traditionally produced by heating limestone with aluminum, a process that requires the quarrying of both materials. The techniques described herein are renewable, utilizing sodium hydroxide and calcium-rich solvents such as ocean water, solvents from dissolved recycled materials or wastewater, thereby eliminating the need for mining. This method significantly reduces the environmental impact and continued capital expenditure

compared to conventional methods, given the availability of the calcium-rich feedstock. There is a need for affordably and efficiently producing calcium at-scale without the need for limestone quarrying.

[0151] Described herein are dialysis-based systems that can be used with a calcium binding protein to sequester large quantities of calcium. This system can use ocean water and/or other calcium rich aqueous solutions. Calcium can be sequestered, released, and precipitated into a solid form. The product allows the calcium captured by the binding protein to be purified, concentrated and used for other applications. This device functions based on the dissociation constant of calcium binding proteins and uses dialysis for the release of aqueous calcium. This release is followed by introduction of dissolved carbon dioxide and sodium hydroxide to lower the pH, precipitating the released calcium as a salt.

[0152] In some embodiments, the techniques described herein can include using the chemical reaction $CaCl_2 + Na_2CO_3$, which results in precipitate solid $CaCO_3$. As an example of a cost/benefit analysis, by comparing inputs and outputs, consider Millipore ACS 99% pure Na_2CO_3: 12 kg costs $598, or $0.0498 per gram. Using the dialysis and capture method described herein, the same amount of $CaCO_3$, which typically costs $887 for 12 kg or $0.0739 per gram, can be obtained more efficiently. For the application using seawater, this is not a direct 1:1 reaction because ocean water contains dissolved CO_2 that can also supplement this reaction making it more efficient. The systems and methods described herein allow for calcium-sequestration using proteins in a manner that is rechargeable and reusable, and allows for the release of calcium and precipitation easier by carrying it out sequentially.

[0153] FIG. 11A illustrates an extraction system according to an exemplary embodiment of this disclosure. A basic control outline of FIG. 11A is as follows: Ocean water or a high calcium-containing solution is filtered through filter (1) into a water chamber (2) to remove suspended solids and aggregates. An example of the filter, water chamber (2) and a pump for driving flow from a calcium-containing solution through the filter is illustrated in FIG. 11B. Calcium concentration can be measured by a standard calcium electrode (not shown). The water chamber is connected to a valve 1 (3) that is used to change flow between loop 1 and loop 2 (5) along with valve 2. Once the water has been filtered it circulates around loop 1 through a dialysis chamber (4). Nonlimiting examples of the dialysis chamber are illustrated in FIG. 11C (left: Snakeskin™ Dialysis tubing; middle: SpectraPor® Tube-A-Lyzer®; right: Dialysis cassettes, Slide-A-Lyzer® Pierce®), FIG. 11D, and FIG. 11E. FIG. 11D illustrates a

schematic view of a dialysis chamber in a SpectraPor® Tube-A-Lyzer®, where the dialysis tube is clamped at both ends and placed in a flow column. FIG. 11E illustrates a schematic view of a dialysis cassette, which clamps dialysis tubing and can fit into a flow column with valves to change between the calcium-containing solution (e.g., ocean water) and collection buffer loops. The dialysis cassette includes dialysis tubes housed in a casing and configured to disperse flow from valve 1 to allow the dialysis tubes to last longer.

[0154] The solution circulates to keep protein in constant equilibrium based on its dissociation constant. The circulation and contact time are based on the calcium concentration and the amount of calcium-binding protein in the dialysis chamber. Levels of calcium are measured in the dialysis chamber using a calcium electrode. Once the optimal amount of calcium has been sequestered, the dialysis chamber is drained and the valves (i.e., valve 1 and valve 2) will change to allow flow through loop 2 (5). Here, by lowering calcium in the solution, the protein depolymerizes and releases the sequestered calcium. Circulating buffer results in a localized low concentration of calcium leading to release of the sequestered calcium in the protein. Over time, kinetic equilibrium is reached with the bulk solution. In other words, the buffer will circulate, but because this solution does not contain calcium or contains a substantially lower amount of calcium and will have a slightly lower pH, the protein is configured to release the sequestered calcium according to its dissociation constant.

[0155] In some embodiments, after equilibrium is reached in the bulk solution, the solution is piped into a reaction chamber, and a reaction substrate (e.g., Na_2CO_3) is then piped into the reaction chamber. As an example, precipitation of the calcium can be facilitated and scaled using the following stoichiometric equations:

[0156] $$Ca^{2+}(aq) + CO_3^{2-}(aq) \rightarrow CaCO_3(s) \quad (1)$$

[0157] $$Ca^{2+}(aq) + 2OH^{-}(aq) \rightarrow Ca(OH)_2(s) \quad (2)$$

[0158] Regarding equation 1, adding a reaction substrate (e.g., Na_2CO_3) to the calcium-containing solution can create solid precipitates in a 1:1 ratio. In some embodiments, heating the solution can result in the dissolved CO_2 to react with the calcium and make $CaCO_3$. Regarding equation 2, this reaction can be facilitated by titrating the solution in NaOH and obtaining a resulting solution with a relatively higher pH. Exemplary reagents for facilitating equations 1 and 2 are relatively cheap for further scaling. For example, regarding equation (1),

$CaCl_2$ and Na_2CO_3 can precipitate out $CaCO_3$; here, Na_2CO_3 can be obtained at 12 kg for about $598 (i.e., ~$0.0498/g), $CaCO_3$ can be obtained at 12 kg for about $887 (i.e., ~$0.0739/g). Regarding equation (2), $CaCl_2$ and NaOH can precipitate out $CaOH_2$, where one could use 12.96 kg of NaOH to make 12 kg of $CaOH_2$; here, NaOH can be obtained at 12 kg for about $50.20 (i.e., ~$0.0418/g), and Ca_2OH_2 can be obtained at 2.5 kg for about $220 (i.e., ~$0.088/g). Overall, the advantages of obtaining $CaOH_2$ include: NaOH is relatively cheap, the pH would change is precipitates continue to form, and $CaOH_2$ is sparingly soluble in water at ~1.73 g/L. Further, the advantages of obtaining $CaCO_3$ include: dissolve CO_2 in the air can react when heated, $CaCO_3$ is obtained at a 1:1 molar ratio, other ions may hinder precipitation (e.g., Mg_{2+}, PO_{*-}, SO_{2-}, or HCO_3-), $CaCO_3$ has a solubility in water of ~0.13 g/L, and NaOH can be used to maintain the pH above 8.3 to reduce the concentration of bicarbonate ions. Additional considerations for facilitating equations (1) and/or (2) include adjusting the pumping system, the extraction method for precipitated calcium, fluidics and layout, equilibrium kinetics of calsequestrin and stability in ocean water, rates and sampling of the reaction chamber, and general conditions.

[0159] As illustrated in FIG. 11A, the solution containing the calcium is stored in the buffer container (6) which moves into the calcium reaction chamber (7) where the pH is raised and Na_2CO_3 is added from a substrate container (not shown). Under these conditions, calcium is insoluble and precipitates out of the solution.

[0160] In some embodiments, the filter (1) is a vacuum-based filter. In some embodiments, the filter (1) comprises a pore size 0.22 μm. In some embodiments, the filter (1) comprises a polyether sulfone membrane or the like.

[0161] In some embodiments, the water chamber (2) is configured to store the solvent. In some embodiments, water chamber (2) comprises glass, polypropylene, or the like.

[0162] In some embodiments, valve 1 (3) is configured to change between loop 1 and loop 2. In some embodiments, the tubing in the extraction system comprises polypropylene or the like.

[0163] In some embodiments, dialysis chamber (4) comprises a pH resistant plastic. In some embodiments, dialysis chamber (4) comprises polypropylene or the like. In some embodiments, dialysis tubes of the dialysis chamber (4) comprise dialysis membranes, a custom material, or the like.

[0164] In some embodiments, loop 2 (5) comprises a pH resistant plastic. In some embodiments, loop 2 (5) comprises polypropylene or the like.

[0165] In some embodiments, the buffer container (6) comprises the same material as the first chamber. In some embodiments, the buffer container (6) comprises a pH probe (e.g., an ionic straight pH probe or the like) for measuring the and/or aiding in controlling the pH.

[0166] In some embodiments, the reaction chamber (7) is connected to the buffer container (6). In some embodiments, the reaction chamber (7) is made from a similar material as the buffer container (6). The reaction chamber (7) may be monitored. In some embodiments, the substrate chamber is split and filled with NaOH and Na_2CO_3 solutions that are piped into the reaction chamber to precipitate solid $CaCO_3$.

[0167] In some embodiments, a pump can be further optimized to obtained a desired rate of calcium sequestration and release. For example, a pump can be adjusted for controlling a flow rate and/or contact time of the protein to the calcium containing solution or a solution that has a lower or absent concentration of calcium. A pump may also be adjusted to control a flow rate and/or a contact time between the stored calcium and the substrate for precipitating the calcium.

Example 5: System for capturing, storing and releasing calcium

[0168] The purpose of this example is to provide an exemplary case study of a system for capturing, storing and releasing calcium that includes a variant of a calcium-sequestering protein.

[0169] Introduction

[0170] Calcium is a key component in various aspects of daily life. While most people are familiar with its dietary needs, calcium plays an important role in neutralizing acidic soils, improves load-bearing capacity in roadbeds and foundations, enhances the mechanical properties of asphalt, boosts wear resistance and structural integrity of flooring materials, improves coverage, adhesion, and weather resistance of paints and coatings, enhances bonding strengths in construction-grade adhesives, gives paper its brightness and smoothness and regulates the pH in water supplies. Calcium is also crucial component in construction materials, providing the necessary strength and durability for building structures. Thus, there is a need for calcium sources for all of these purposes.

[0171] Calcium is acquired primarily from naturally occurring minerals, with limestone (calcium carbonate) followed by chalk, marble gypsum and dolomite. However, these quarried materials must be processed in a manner that contributes to its costs. Presently, the cost of calcium carbonate, commonly used as a filler in construction materials, can range from $50 to $200 per metric ton, depending on its purity and specific application. This cost is a significant factor in the overall expense of construction projects, influencing material selection and budgeting.

[0172] Aside from financial costs, calcium acquisition involves energetic and environmental costs. Concrete, which is the most widely used material in the world, relies on an energy-intensive conversion of lyme to calcium ions and/or salt that allow the formation of calcium oxides, carbonates and silicates as the material cures. This conversion involves grinding limestone to a fine powder and heating in a kiln at 2632°C. New-age green materials that strive to remove this step rely on enzymes that convert calcium ions to calcium carbonate. However, the cost of the calcium sources for these materials can prohibit widespread use.

[0173] Because calcium mediates many critical cell functions such as neural transmission and muscle contraction, nature has developed mechanisms to sequester, store and release calcium. Described herein is a biologically-based, rechargeable method to acquire calcium from natural and recycled materials that produces calcium salts at a low energetic and environmental cost. This method involves the use of natural cellular proteins that can be inexpensively manufacturing by bacterial systems.

[0174] Further described herein is a diffusion-based device to capture, store and release calcium based on proteins human Calsequestrin-1 (CASQ). In living systems, muscle contraction involves the release of large amounts of calcium from cellular stores that cause contraction of muscle fibers. CASQ can be the main calcium storage protein in human skeletal muscle. CASQ has a high calcium binding capacity but a low affinity allowing it to sequester large amounts of calcium in storage areas in the cells that can subsequently spill into low calcium areas of the cell when the cell receives signals to contract. Previous biophysical characterization of CASQ1 has shown it to have a net charge of -80 under physiological conditions thereby enabling it to bind many calcium ions electrostatically. In the absence of calcium, CASQ1 has no organized structure and fluctuates between random configurations. However, as shown in FIGS. 12A-12B, when CASQ binds calcium ions, the protein takes on

a uniform structure. Additionally, as more calcium ions bind, CASQ1(Ca2+) can aggregate to highly branched polymer chain structures.

[0175] The techniques described herein take advantage of CASQ1's high-capacity calcium binding by containing suspended CASQ1 monomers in a dialysis membrane that allows free diffusion of water and ions through the membrane thereby furthering CASQ's ability to polymerize into large complexes when calcium is present, and disassemble when calcium levels are lowered. This method can be used to reversibly bind and release calcium from sources such as ocean water and recycled concrete and can be used as an inexpensive and green method for sustainability producing calcium.

[0176] Materials and methods

[0177] Plasmid Constructs—Expression plasmids, pET-52b(+) CASQ1GFP, and pET-16b CASQ1

[0178] The initial step involves obtaining a plasmid containing the CASQ1 gene commercially. This gene was cloned into a pET-52b(+) vector containing the gene for eGFP followed by a thrombin cut site with CASQ1 flanking it. This fusing protein contains a strep tag II affinity tag in the N terminal end of the eGFP. This plasmid was then transformed into Bl21 cells for expression and 5 alpha cells for plasmid production and additional cloning if needed.

[0179] *Purification*

[0180] CASQ1GFP was purified using the Escherichia coli strain BL21 (DE3). The BL21 (DE3) was grown at 37°C in Luria-Bertani (LB) up to an A600 of 0.6 and induced with 1 mM isopropyl- D-thiogalactopyranoside. After induction the culture was incubated at 17°C for 20 hours. The overexpressed cells were then lysed in 100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF. The cell lysis was then spun down at 4000RPM and the supernatant was loaded into a column containing Strep-tactin XT Sepharose resin. The column was washed with 100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, and 1 mM DTT. Following this The CASQ1GFP was eluted with 100 mM Tris-HCl pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT, and 25 mM Biotin.

[0181] *DLS Dynamic light scattering*

[0182] Dynamic light scattering (DLS) was used to determine the average size of the Protein polymers (Zetasizer Nano ZS, Malvern, Worcestershire, UK). The instrument uses a 633 nm

He-Ne laser and detects scattered light at a backscatter angle of 173°. The protein was compared with titrations of calcium, EDTA, and storage buffer.

[0183] *Calcium sequestration Device*

[0184] 58.1 µM of CASQ1GFP was placed in a dialysis cassette and 400 ml of ocean water was run through the system for 20 minutes followed by 500 ml of 50 mM citric acid pH 4.5, and 100 mM NaCl for 20 minutes to release the calcium. The ocean water was then circulated again followed again by the release buffer. Samples were taken and analyzed by ICP-MS.

[0185] *Zeta potential*

[0186] CASQ1-GFP was prepared to a final concentration of 18 µM by dialysis into a low-salt buffer consisting of 10 mM Tris-HCl pH 7.5, and 10 mM NaCl. For calcium titration experiments, aliquots of a concentrated $CaCl_2$ stock solution were added to the protein sample to achieve final calcium concentrations of 0.5 mM, 1 mM, and 5 mM.

[0187] The zeta potential (ζ) of the particles was measured by Electrophoretic Light Scattering (ELS) using a Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK). Immediately after preparation, samples were loaded into a disposable folded capillary cell (DTS1070) and allowed to thermally equilibrate inside the instrument at 25 °C for 120 seconds. The instrument measured the electrophoretic mobility, which was then automatically converted to zeta potential using the Smoluchowski model. For each calcium concentration, four replicate measurements were performed and averaged.

[0188] *Calcium selective electrode*

[0189] The concentration of free calcium ions (Ca^{2+}) in aqueous samples was determined potentiometrically using a Hanna Instruments HI4104 combination calcium ion-selective electrode (ISE). All measurements were conducted at ambient temperature. while stirring the solution gently with a magnetic stir bar to ensure homogeneity.

[0190] 4.74 mg/ml of CASQ1GFP was placed in 0.4 ml dialysis cassette and equilibrated with 500 ml of 50 mM HEPES pH 8.0, 500 mM NaCl, 0.5 mM TCEP, 10% glycerol, 5 mM $CaCl_2$. The same concentration and volume was also placed in 400 ml of ocean water and allowed to reach equilibrium.

[0191] *ICP-MS*

[0192] CASQ1GFP was dialyzed into a storage buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5mM $CaCl_2$ and 5% glycerol. The resulting CASQ1GFP concentration was 34.3 µM. 300 ul of this was placed in a new cassette, and equilibrium dialysis was preformed into 40 ml of the following buffers; buffer 1: 100 mM Citric acid pH 4.5, 5% glycerol, and 200 mM NaCl, buffer 2: 50 mM Formic acid pH 4.5, 5% glycerol, and 200 mM NaCl and buffer 3:50 mM acetic pH 4.5, 5% glycerol, and 200 mM NaCl. Samples of each solution were taken before and after to measure the change in ionic concentration.

[0193] *Circular dichroism (CD)*

[0194] CASQ1GFP's secondary structure was explored using a Jasco J-1500 circular dichroism spectrophotometer (CD) with a 1 cm path length. This experiment was done by titrating calcium using 16 µM of CASQ1GFP. The CASQ1GFP was dialyzed into 10 mM Tris-HCl pH 7.5, 10 mM NaCl, and 25 mM EDTA. $CaCl_2$ titrations were performed by incremental addition of $CaCl_2$ to achieve molar ratios of up to threefold excess relative to EDTA. CD spectra were collected from 200 to 260 nm, with each spectrum averaged over multiple scans and corrected for buffer background.

[0195] Results

[0196] *Method Overview.*

[0197] On aspect of the diffusion-based calcium sequestration method described herein lies in the high calcium binding capacity of calcium to CASQ which allows it to sequester many molecules that are readily released because of its weak dissociation constant. Specifically, it has been reported that each molecule of CASQ1 binds ~80 moles of Ca^{2+} with a dissociation constant (Kd) of calcium is ~ 1 mM. This high calcium binding capacity is due to a combination of primary binding sites in the monomers, that allow for the protein to fold into a globular structure, and then assembly of these monomers into a polymer that generates additional sites, as described below.

[0198] To maximize calcium binding of CASQ1, the protein can be sequestered in a dialysis membrane that can allow exposure of the protein to different solutions but keep the protein molecules freely diffusing allowing for polymerization. Thus, if a solution high in calcium is added to a concentrated solution of CASQ monomers, CASQ1 can bind calcium and polymerize. After, if the solution is exchanged for a calcium solution with a low concentration

calcium solution, the bound calcium can be released from CASQ through simple thermodynamics.

[0199] *Characterization of CASQ1 properties*

[0200] CASQ1 monomers have been reported to bind ~80 calcium cations. To better quantify this binding under our conditions, a structural model was first generated using AlphaFold (FIG. 12A-12B). FIGS. 12A-12B illustrate a side by side of the AlphaFold predicted structure of CASQ1 with and without GFP. Comparison of the predicted structures suggest that the GFP tag does not bind calcium or interferes with calcium binding. In particular, this model predicts that each GFP-CASQ1 monomer binds ~54 calcium atoms mainly on the protein surface. This number is lower than previous reports and lower than what was found experimentally (described in more detail below) which was interpreted as being due to an increase in the number of binding sites as oligomeric complexes form.

[0201] CASQ1 was expressed in bacteria and purified using standard protocols (described in more detail in Methods). Additionally, a fluorescence tag was attached to the proteins (GFP) to better follow the protein's solution properties.

[0202] FIGS. 13A-13B illustrate the Alpha fold 3 structure of CASQ1GFP with 54 calcium ions bound (FIG. 13A), and the CD spectrum for CASQ1GFP in the presence of 25 mM EDTA are consistent with partially unfolded proteins (FIG. 13B), respectively. The trend of diminishing ellipticity and a less defined secondary structure. Upon the addition of equimolar Ca^{2-} (25 mM), the CD spectrum showed the appearance of secondary structure.

[0203] The ability of calcium to induce structure by circular dichroism was analyzed. The CD spectrum for CASQ1GFP in the presence of the calcium chelator EDTA (25 mM) was consistent with partially unfolded state (FIGS. 13A-13B) and the loss of free calcium diminishes ellipticity and a less defined secondary structure. Upon the addition of equimolar Ca^{2-} (25 mM), the CD spectrum transitioned to spectral fingerprints that correspond to helical and beta strand conformations.

[0204] FIGS. 14A-14B illustrate calcium selective electrode readings before and after dialysis into a calcium containing buffer (FIG. 14A) and a calcium release buffer (FIG. 14B). Here, calcium binding and release by CASQ1-GFP using a calcium electrode was analyzed. In these studies, the protein was placed in dialysis tubing and then placed in a beaker with a calcium containing calcium. Initially the calcium level is high, (250 ppm) but after 2 hours the calcium

concentration is reduced to 128.99 ppm. The figure below shows a calcium sensing electrode that is placed in a sample before dialysis then washed and placed in a sample after dialysis. Replacing the solution with water allows the release of calcium as seen by an increase to ~150 ppm (FIG. 14B).

[0205] FIGS. 15A-15C illustrate (left) normalized DLS autocorrelation curves of CASQ1GFP, and (right) the corresponding fitted hydrodynamic size distributions for the indicated samples. Regarding the left panels of FIGS. 15A-15C, the autocorrelation curves of CASQ1GFP are illustrated at increasing calcium concentrations. This figure shows the autocorrelation functions of CASQGFP in the absence of Ca^{2+} and in the presence of 500 μM, 1 mM, and 5 mM Ca^{2+}. Increasing Ca^{2+} shifted the decay of the correlation function to longer lag times. This indicates slower diffusion and therefore a larger apparent hydrodynamic radius. The progressive rightwards shift at 1 mM and 5 mM are consistent with calcium dependent assembly of the oligomeric form of CASQ1.

[0206] Regarding the right panels of FIGS. 15A-15C, DLS data is illustrated showing the impact of excess EDGA on the CASQ1 polymer overtime. In particular, to determine the oligomeric state of the CASQ1-GFP complexes with calcium binding, dynamic light scattering (DLS) was used, as DLS can assess the size distribution of species in solution. At high calcium levels (5 mM) two major peaks were observed: one centered around 1000 nm and another centered around 100 nm. Correlating these sizes to previously reported oligomerization patterns suggested that the 100 nm band corresponds to CASQ1 in a rope-like linear polymer whereas the 1000 nm peak corresponds to larger intertwined polymer chains. When the free calcium was lowered by the chelator of EDTA, these peaks disappeared and were replaced by peaks corresponding to lower oligomeric states until a final one at 5 nm corresponding to the monomer was seen. Reversibility of oligomerization was observed by the increase in size when calcium was added to the solution.

[0207] The electronic state of CASQ1GFP oligomers were also characterized by their zeta potential (ZP) and electrophoretic mobility (μ) in the absence of calcium ions and upon the adding of calcium chloride ($CaCl_2$). FIGS. 16A-16B illustrate the zeta potential and electrophoretic mobility of CASQ1GFP in response to increasing $CaCl_2$ concentrations (FIG. 16A) and the corresponding data curves (FIG. 16B). The top panel shows the distribution of apparent zeta potentials, and the bottom panel displays the corresponding electrophoretic mobility distributions. The red trace represents CASQ1GFP no calcium baseline. The green,

blue, and black traces correspond to 0.5 mM, 1 mM, and 5 mM $CaCl_2$, respectively. Progressive Ca^2 addition lead to a reduction in negative surface charge on the CASQ1GFP. This was reflected by the shift in zeta potential from –34.4 mV (no calcium) to –17.7 mV. Electrophoretic mobility values follow a similar trend, while conductivity increases with Ca^{2+} concentration. Sample characteristics are provided in Table 4 below.

[0208] Table 4: Sample characteristics for DLS data.

Sample Name	ZP (mV)	Mob (µmcm/Vs)	Cond (mS/cm)
CASQ1GFP	-34.4	-2.699	4.09
CASQ1GFP 500 uM Ca2+	-29.5	-2.31	4.34
CASQ1GFP 1 mM Ca2+	-27.5	-2.157	4.61
CASQ1GFP 5 mM Ca2+	-17.7	-1.39	5.69

[0209] Without calcium, CASQ1GFP was found to have a ZP of -34.4 mV, and the electrophoretic mobility of -2.699 µm·cm/V·s. Upon the addition of 500 µm, $CaCl_2$ both the ZP and the electrophoretic mobility shifted towards less negative values. This trend continued at the 1 mM and 5 mM titration conditions, indicating calcium binding resulting in a partial neutralization of the surface charge. The resulting final ZP and electrophoretic mobility at 5 mM $CaCl_2$ were –17.7 mV and 1.39 µm·cm/V·s respectively. This change showed a 48.5% reduction in surface charge.

[0210] The ZP distributions are monomodal shifts right as the surface change becomes positive. Without calcium, CASQ1GFP (red) is very narrow and symmetric relating to a uniform surface charge. As the calcium concentration increases, the peaks broaden. This broadening corresponds to the appearance of different protein/calcium complexes to due to aggregation of the protein as well as binding of several Ca2+ ions on the surface. Additionally, the decrease in mobility indicates a reduction in electrostatic repulsion.

[0211] Fluorescence correlation spectroscopy (FCS) was used to determine the equilibrium binding of calcium to CASQ1-GFP. This method correlated the time that a fluorophore diffuses in and out of a confocal volume, where the diffusion is related to molecular size. For the monomeric protein (i.e. CASQ1-GFP without calcium), the time for autocorrelation is 10+2, and as calcium is added this time increases two orders of magnitude consistent with increased size. These results are consistent with assembly of the protein with calcium.

[0212] *Dialysis device to sequester calcium*

[0213] After characterizing CASQ1 calcium and release properties, CASQ1 was used to sequester calcium. As a proof of principle, calcium was isolated from ocean water through the device shown in FIG. 11A, which illustrates the dialysis based device for reversible calcium capture and recovery. Here, feed stock is first filtered through a .22 μm membrane and circulated through a loop containing a dialysis cassette loaded with CASQ1. Calcium ions diffuse across the dialysis membrane and are sequestered by CASQ1 inside the cassette. This device uses a series of peristaltic pumps and chambers to allow CASQ1GFP to polymerize and collect calcium from an aqueous solution, which were tested using ocean water or a lab solvent (50 mM HEPES pH 8.0, 500 mM NaCl, 10 % glycerol and 10 mM $CaCl_2$).

[0214] Ocean water collected in Cape Cod was passed through a polyether sulfone membrane (1) with a pore size 0.22 μm to remove solids and aggregates. The ocean water was then passed into a polypropylene water chamber (2) which is connected to valve (3) that is used to change solvents using valve 2. Once the water has been filtered, it circulates around loop 1 through the dialysis chamber (4) which contains purified CASQ1 placed in a commercial dialysis chamber (i.e., Slide-a-Lyzer®) that allows calcium binding and protein oligomerization. Based on our characterization of the kinetics of calcium binding to CASQ1, we circulate the solvent around the dialysis chamber to maximize calcium binding. Once the time is optimized, the dialysis chamber is drained, and valves change to loop 2 to introduce a low calcium buffer at a slightly lower pH (5) that allows for calcium release. The reduced pH neutralizes the anionic groups that bind calcium, thus making the calcium binding weaker. Again, the buffer circulates, but because this solution does not contain calcium and the dissociation constant is weaker, CASQ1 releases bound calcium into the solvent. The solution containing the calcium is stored in the buffer container (6) which moves into the calcium reaction chamber (7) where the pH is raised and Na_2CO_3 or another salt is added to precipitate calcium.

[0215] The system was tested by first running 400 ml of ocean water for 20 minutes circulating through loop-1 in blue to allow calcium binding to CASQ1 contained in the dialysis bag. Once this was complete, the valves are changed to the loop-2 in orange and 500 ml of 50 mM citric acid pH 4.5, and 100 mM NaCl buffer circulated for 20 minutes to release the calcium for the polymerized CASQ1 protein. The same ocean water is then circulated followed by the release buffer. Samples are taken and analyzed by ICP-MS. The same cycle was repeated.

[0216] Success of the system was determined using ICP-MS to analyze calcium levels. FIG. 17 illustrates ppm values determined by ICP-MS the calcium capture and release. Samples for

each cycle were taken at the 20-minute mark. 58.1 μM CASQ1 was found to capture approximately 247 calcium ions per protein molecule, indicating high calcium-sequestration capacity. Analysis of the solvent after release shows substantially lower values, 23, 61 and 30 calcium ions per protein molecule for citric, formic, and acetic acid, respectively. These results indicate that while calcium capture is highly effective, only partial recovery is achieved under the tested release conditions. Formic acid produced the highest calcium release, suggesting it was the most effective of the acids tested for regenerating CASQ1. The inefficiency of release was attributed to the stable binding of calcium ions to the monomers; thus conditions can be optimized to enhance the overall system efficiency. Nevertheless, this study supported the feasibility of the dialysis-based calcium capture system but show that optimization of the release step can improve total calcium recovery.

[0217] Discussion

[0218] The DLS data supports the model that CASQ1GFP starts out as a linear peptide and folds in the presence of calcium.

Illustrative Embodiments:

[0219] Embodiment 1. A method for harvesting metals from an aqueous source, comprising: populating a membrane filter with a binding protein having an affinity for a target metal and a weaker dissociation with the target metal; passing an aqueous solution including the target metal through the membrane filter; and terminating a flow of the aqueous solution and passing a buffer solution through the membrane filter for harvesting the target metal.

[0220] Embodiment 2. The method of Embodiment 1, wherein the membrane filter is a dialysis filter stack.

[0221] Embodiment 3. The method of Embodiment 1, wherein the binding protein is calsequestrin.

[0222] Embodiment 4. The method of Embodiment 1, wherein the aqueous solution has a concentration of the target metal, further comprising harvesting the bound target metal upon reaching a saturation based on the concentration.

[0223] Embodiment 5. A method of capturing calcium, the method comprising: contacting at least one calcium-sequestering protein to a first calcium-containing solution for a first predetermined amount of time, thereby allowing the at least one calcium-sequestering protein to capture calcium in the first calcium-containing solution; contacting the at least one calcium-sequestering protein with a second solution for a second predetermined amount of time, the second predetermined amount of time being until a portion of the captured calcium is released from the at least one calcium-sequestering protein.

[0224] Embodiment 6. The method of Embodiment 5, wherein the second solution further comprises one or more counterions to provide for at least a portion of the released calcium to precipitate.

[0225] Embodiment 7. The method of Embodiment 5 or Embodiment 6, wherein the first calcium-containing solution comprises calcium at a concentration above 150 mM.

[0226] Embodiment 8. The method of any one of Embodiments 4-Embodiment 7, wherein the portion of the captured calcium released from the at least one calcium-sequestering protein comprises at least 50% of the calcium captured by the at least one calcium-sequestering protein.

[0227] Embodiment 9. The method of any one of Embodiments 4-Embodiment 8, wherein the at least one calcium-sequestering protein comprises a calsequestrin protein.

[0228] Embodiment 10. The method of any one of Embodiments 4-Embodiment 9, wherein the at least one calcium-sequestering protein comprises a variant of a calsequestrin protein.

[0229] Embodiment 11. The method of Embodiment 10, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

[0230] Embodiment 12. The method of Embodiment 10 or Embodiment 11, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

[0231] Embodiment 13. The method of any one of Embodiments Embodiment 10-Embodiment 12, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

[0232] Embodiment 14. The method of any one of Embodiments 4-Embodiment 13, wherein the calcium-sequestering protein is bound to a sample interface.

[0233] Embodiment 15. The method of any one of Embodiments 4-Embodiment 14, wherein the sample interface comprises a membrane filter.

[0234] Embodiment 16. The method of Embodiment 15, wherein the membrane filter comprises a dialysis filter stack.

[0235] Embodiment 17. An extraction device for capturing calcium from a calcium-containing solution, the extraction device comprising: a sample interface configured to receive a calcium-containing solution; a substrate embedded with a calcium-sequestering protein configured to capture at least a portion of calcium from the calcium-containing solution when contacted with the sample interface containing the calcium-containing solution.

[0236] Embodiment 18. The extraction device of Embodiment 17, wherein the sample interface comprises a membrane filter.

[0237] Embodiment 19. The extraction device of Embodiment 18 or Embodiment 18, wherein the membrane filter comprises a dialysis filter stack.

[0238] Embodiment 20. A method of capturing calcium, the method comprising: providing an extraction device for capturing calcium from a calcium-containing solution, the extraction device comprising: a sample interface configured to receive a calcium-containing solution; and a substrate embedded with a calcium-sequestering protein configured to capture at least a portion of calcium from the calcium-containing when contacted with the sample interface containing the calcium-containing solution; contacting the substrate with the sample interface comprising the calcium-containing solution for a first predetermined amount of time, thereby allowing the calcium-sequestering protein to capture at least a portion of calcium from the calcium-containing solution; and contacting the sample interface with a storage solution for a second predetermined amount of time, the second predetermined amount of time being until a predetermined amount of the calcium is released from the calcium-sequestering protein.

[0239] Embodiment 21. The method of Embodiment 20, wherein the storage solution further comprises one or more counterions to provide for at least a portion of the released calcium to precipitate.

[0240] Embodiment 22. The method of Embodiment 20 or Embodiment 21, wherein the calcium-containing solution comprises calcium at a concentration above 150 mM.

[0241] Embodiment 23. The method of any one of Embodiments Embodiment 20-Embodiment 22, wherein the portion of the captured calcium released from the at least one calcium-sequestering protein comprises at least 50% of the calcium captured by the at least one calcium-sequestering protein.

[0242] Embodiment 24. The method of any one of Embodiments Embodiment 20-Embodiment 23, wherein the at least one calcium-sequestering protein comprises a calsequestrin protein.

[0243] Embodiment 25. The method of any one of Embodiments Embodiment 20-Embodiment 24, wherein the at least one calcium-sequestering protein comprises a variant of a calsequestrin protein.

[0244] Embodiment 26. The method of Embodiment 25, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

[0245] Embodiment 27. The method of Embodiment 25 or Embodiment 26, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

[0246] Embodiment 28. The method of any one of Embodiments Embodiment 25-Embodiment 27, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

[0247] Embodiment 29. The method of any one of Embodiments Embodiment 20-Embodiment 28, wherein the substrate comprises a membrane filter.

[0248] Embodiment 30. The method of Embodiment 29, wherein the membrane filter comprises a dialysis filter stack.

[0249] Embodiment 31. A variant of a calsequestrin protein, wherein the variant exhibits calcium-sequestering activity that is at least 10% greater than the calsequestrin protein.

[0250] Embodiment 32. The variant of a calsequestrin protein of Embodiment 31, wherein the variant has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

[0251] Embodiment 33. The variant of a calsequestrin protein of Embodiment 31 or Embodiment 32, wherein the variant has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

[0252] Embodiment 34. The variant of a calsequestrin protein of any one of Embodiments Embodiment 31-Embodiment 33, wherein the variant has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

[0253] Embodiment 35. A method for producing a variant of a calsequestrin protein, the method comprising: cultivating a cell overexpressed with a nucleic acid construct that encodes the variant of the calsequestrin protein; inducing the cell to express the variant of the calsequestrin protein encoded by the nucleic acid construct; lysing the cell; and purifying the variant of the calsequestrin protein from the lysed cell, wherein the variant exhibits calcium-sequestering activity that is at least 10% greater than the calsequestrin protein.

[0254] Embodiment 32. A system for producing a variant of a calsequestrin protein, the system comprising: a solution chamber comprising a calcium-sequestering agent configured to bind to calcium; a first loop in fluid communication with the solution chamber, wherein the first loop is configured to pass a first solution through the solution chamber, the first solution comprising calcium such that when the first solution is passed through the solution chamber, the calcium-sequestering agent captures the calcium from the first solution; and a second loop in fluid communication with the solution chamber, wherein the second loop is configured to pass a second solution through the solution chamber to extract the captured calcium from the agent.

[0255] Embodiment 33. The system of Embodiment 32, wherein the first solution is filtered prior to entering the solution chamber.

[0256] Embodiment 34. The system of Embodiment 32 or Embodiment 33, wherein the calcium-sequestering agent comprises a calcium-sequestering protein dispersed in solution or a calcium-sequestering protein embedded within a sequestering substrate.

[0257] Embodiment 35. The system of Embodiment 34, wherein the sequestering substrate in calcium-sequestering portion includes one or more membranes, membrane filters, a plurality of beads, is housed within a column, or a combination thereof.

[0258] Embodiment 36. The system of any one of Embodiment 32-Embodiment 35, further comprising a reaction portion in fluid communication with the solution chamber, wherein the reaction portion comprises a reaction substrate that is configured to allow for precipitation of the extracted calcium.

[0259] Embodiment 37. The system of Embodiment 36, wherein the reaction substrate comprises Na2CO3, NaOH, or a combination thereof.

[0260] Embodiment 38. The system of Embodiment 36 or Embodiment 37, wherein the reaction portion comprises one or more of sedimentation, a filter, or a combination thereof to precipitate the extracted calcium.

[0261] Embodiment 39. The system of any one of Embodiment 32-Embodiment 38, wherein the first solution comprises calcium at a concentration above 150 mM.

[0262] Embodiment 40. The system of any one of Embodiment 32-Embodiment 39, wherein the calcium-sequestering agent comprises a calsequestrin protein.

[0263] Embodiment 41. The system of any one of Embodiment 32-Embodiment 39, wherein the calcium-sequestering agent comprises a variant of a calsequestrin protein.

[0264] Embodiment 42. The system of Embodiment 41, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

[0265] Embodiment 43. The system of Embodiment 41 or Embodiment 42, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

[0266] Embodiment 44. The system of any one of Embodiment 41-Embodiment 43, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

[0267]

[0268] As used herein, "about" and its grammatical equivalents in relation to a reference numerical value and its grammatical equivalents as used herein can include a range of values plus or minus 10% from that value. For example, the amount "about 10" includes amounts from 9 to 11. The term "about" in relation to a reference numerical value can also include a range of values plus or minus 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% from that value.

[0269] As used herein, a "cell" refers to a biological cell. Some non-limiting examples include: a prokaryotic cell, eukaryotic cell, a bacterial cell, an archaea cell, a cell of a single-cell eukaryotic organism, a protozoa cell, a cell from a plant, an algal cell, a fungal cell, a fungal protoplast cell, an animal cell, and the like. Sometimes a cell is not originating from a natural organism, e.g., a cell can be a synthetically made, sometimes termed an artificial cell.

[0270] As used herein, a "plurality" contains at least 2 members. In certain cases, a plurality may have at least 10, at least 100, at least 100, at least 10,000, at least 100,000, at least 10^6, at least 10^7, at least 10^8 or at least 10^9 or more members.

[0271] As used herein, when a quantitative characteristic (e.g., largest lateral dimension) is described as "in a range of," when accompanied by a smaller value and a larger value, this refers to the quantitative characteristic having a value between the smaller value and the larger value or equal to the smaller value of the larger value.

[0272] As used herein, the characterizing term "uniform" in referencing a quantity (e.g., a distance, a thickness, a dimension (e.g., a largest lateral dimension)) refers to a variation in that quantity by no more than 10% more or less than the stated value or an average of that quantity (e.g., no more than 5% more or less, no more than 1% more or less, no more than 0.1% more or less than the stated value or an average of that quantity).

[0273] Although various features of the disclosure may be described in the context of a single embodiment, the features can also be provided separately or in any suitable combination. Conversely, although the disclosure may be described herein in the context of separate

embodiments for clarity, various aspects and embodiments can be implemented in a single embodiment.

[0274] From the foregoing description, it will be apparent that variations and modifications may be made to the embodiments described herein to adopt it to various usages and conditions. Such other embodiments are also within the scope of the following claims.

[0275] Numerous modifications and alternative embodiments of the present disclosure will be apparent to those skilled in the art in view of the foregoing description. Accordingly, this description is to be construed as illustrative only and is for the purpose of teaching those skilled in the art the best mode for carrying out the present disclosure. Details of the structure may vary substantially without departing from the spirit of the present disclosure, and exclusive use of all modifications that come within the scope of any appended claims is reserved. Within this specification, embodiments have been described in a way which enables a clear and concise specification to be written, but it is intended and will be appreciated that embodiments may be variously combined or separated without parting from the scope of the present disclosure. It is intended that the present disclosure be limited only to the extent required by any appended claims and the applicable rules of law.

[0276] As utilized herein, the terms "comprise" and "comprising" are intended to be construed as being inclusive, not exclusive. As utilized herein, the terms "exemplary", "example", and "illustrative", are intended to mean "serving as an example, instance, or illustration" and should not be construed as indicating, or not indicating, a preferred or advantageous configuration relative to other configurations. As utilized herein, the terms "about", "generally", and "approximately" are intended to cover variations that may existing in the upper and lower limits of the ranges of subjective or objective values, such as variations in properties, parameters, sizes, and dimensions. In one non-limiting example, the terms "about", "generally", and "approximately" mean at, or plus 10 percent or less, or minus 10 percent or less. In one nonlimiting example, the terms "about", "generally", and "approximately" mean sufficiently close to be deemed by one of skill in the art in the relevant field to be included. As utilized herein, the term "substantially" refers to the complete or nearly complete extend or degree of an action, characteristic, property, state, structure, item, or result, as would be appreciated by one of skill in the art. For example, an object that is "substantially" circular would mean that the object is either completely a circle to mathematically determinable limits, or nearly a circle as would be recognized or understood by one of skill in the art. The exact allowable degree of

deviation from absolute completeness may in some instances depend on the specific context. However, in general, the nearness of completion will be so as to have the same overall result as if absolute and total completion were achieved or obtained. The use of "substantially" is equally applicable when utilized in a negative connotation to refer to the complete or near complete lack of an action, characteristic, property, state, structure, item, or result, as would be appreciated by one of skill in the art. The use of the terminology X "or" Y herein should be interpreted as meaning either "X" or "Y" individually, or both "X and Y" together.

INCORPORATION BY REFERENCE

[0277] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. Absent any indication otherwise, publications, patents, and patent applications mentioned in this specification are incorporated herein by reference in their entireties.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of capturing calcium, the method comprising:

contacting at least one calcium-sequestering protein to a first calcium-containing solution for a first predetermined amount of time, thereby allowing the at least one calcium-sequestering protein to capture calcium in the first calcium-containing solution;

contacting the at least one calcium-sequestering protein with a second solution for a second predetermined amount of time, the second predetermined amount of time being until a portion of the captured calcium is released from the at least one calcium-sequestering protein.

2. The method of claim 1, wherein the second solution further comprises one or more counterions to provide for at least a portion of the released calcium to precipitate.

3. The method of claim 1 or claim 2, wherein the first calcium-containing solution comprises calcium at a concentration above 150 mM.

4. The method of any one of claims 1-3, wherein the portion of the captured calcium released from the at least one calcium-sequestering protein comprises at least 50% of the calcium captured by the at least one calcium-sequestering protein.

5. The method of any one of claims 1-4, wherein the at least one calcium-sequestering protein comprises a calsequestrin protein.

6. The method of any one of claims 1-5, wherein the at least one calcium-sequestering protein comprises a variant of a calsequestrin protein.

7. The method of claim 6, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

8. The method of claim 6 or claim 7, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

9. The method of any one of claims 6-8, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

10. The method of any one of claims 1-9, wherein the calcium-sequestering protein is bound to a sample interface.

11. The method of claim 10, wherein the sample interface comprises a membrane filter.

12. The method of claim 11, wherein the membrane filter comprises a dialysis filter stack.

13. An extraction device for capturing calcium from a calcium-containing solution, the extraction device comprising:

a sample interface configured to receive a calcium-containing solution;

a substrate embedded with a calcium-sequestering protein configured to capture at least a portion of calcium from the calcium-containing solution when contacted with the sample interface containing the calcium-containing solution.

14. The extraction device of claim 13, wherein the sample interface comprises a membrane filter.

15. The extraction device of claim 14 or claim 14, wherein the membrane filter comprises a dialysis filter stack.

16. A method of capturing calcium, the method comprising:

providing an extraction device for capturing calcium from a calcium-containing solution, the extraction device comprising:

a sample interface configured to receive a calcium-containing solution; and

a substrate embedded with a calcium-sequestering protein configured to capture at least a portion of calcium from the calcium-containing when contacted with the sample interface containing the calcium-containing solution;

contacting the substrate with the sample interface comprising the calcium-containing solution for a first predetermined amount of time, thereby allowing the calcium-sequestering protein to capture at least a portion of calcium from the calcium-containing solution; and

contacting the sample interface with a storage solution for a second predetermined amount of time, the second predetermined amount of time being until a predetermined amount of the calcium is released from the calcium-sequestering protein.

17. The method of claim 16, wherein the storage solution further comprises one or more counterions to provide for at least a portion of the released calcium to precipitate.

18. The method of claim 16 or claim 17, wherein the calcium-containing solution comprises calcium at a concentration above 150 mM.

19. The method of any one of claims 16-18, wherein the portion of the captured calcium released from the calcium-sequestering protein comprises at least 50% of the calcium captured by the at least one calcium-sequestering protein.

20. The method of any one of claims 16-19, wherein the calcium-sequestering protein comprises a calsequestrin protein.

21. The method of any one of claims 16-20, wherein the calcium-sequestering protein comprises a variant of a calsequestrin protein.

22. The method of claim 21, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

23. The method of claim 21 or claim 22, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

24. The method of any one of claims 21-23, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

25. The method of any one of claims 16-24, wherein the substrate comprises a membrane filter.

26. The method of claim 25, wherein the membrane filter comprises a dialysis filter stack.

27. A variant of a calsequestrin protein, wherein the variant exhibits calcium-sequestering activity that is at least 10% greater than the calsequestrin protein.

28. The variant of a calsequestrin protein of claim 27, wherein the variant has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

29. The variant of a calsequestrin protein of claim 27 or claim 28, wherein the variant has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

30. The variant of a calsequestrin protein of any one of claims 27-29, wherein the variant has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

31. A method for producing a variant of a calsequestrin protein, the method comprising:

cultivating a cell overexpressed with a nucleic acid construct that encodes the variant of the calsequestrin protein;

inducing the cell to express the variant of the calsequestrin protein encoded by the nucleic acid construct;

lysing the cell; and

purifying the variant of the calsequestrin protein from the lysed cell, wherein the variant exhibits calcium-sequestering activity that is at least 10% greater than the calsequestrin protein.

32. A system for producing a variant of a calsequestrin protein, the system comprising

a solution chamber comprising a calcium-sequestering agent configured to bind to calcium;

a first loop in fluid communication with the solution chamber, wherein the first loop is configured to pass a first solution through the solution chamber, the first solution comprising

calcium such that when the first solution is passed through the solution chamber, the calcium-sequestering agent captures the calcium from the first solution; and

a second loop in fluid communication with the solution chamber, wherein the second loop is configured to pass a second solution through the solution chamber to extract the captured calcium from the agent.

33. The system of claim 32, wherein the first solution is filtered prior to entering the solution chamber.

34. The system of claim 32 or claim 33, wherein the calcium-sequestering agent comprises a calcium-sequestering protein dispersed in solution or a calcium-sequestering protein embedded within a sequestering substrate.

35. The system of claim 34, wherein the sequestering substrate in calcium-sequestering portion includes one or more membranes, membrane filters, a plurality of beads, is housed within a column, or a combination thereof.

36. The system of any one of claims 32-35, further comprising a reaction portion in fluid communication with the solution chamber, wherein the reaction portion comprises a reaction substrate that is configured to allow for precipitation of the extracted calcium.

37. The system of claim 36, wherein the reaction substrate comprises Na_2CO_3, NaOH, or a combination thereof.

38. The system of claim 36 or claim 37, wherein the reaction portion comprises one or more of sedimentation, a filter, or a combination thereof to precipitate the extracted calcium.

39. The system of any one of claims 32-38, wherein the first solution comprises calcium at a concentration above 150 mM.

40. The system of any one of claims 32-39, wherein the calcium-sequestering agent comprises a calsequestrin protein.

41. The system of any one of claims 32-39, wherein the calcium-sequestering agent comprises a variant of a calsequestrin protein.

42. The system of claim 41, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 1.

43. The system of claim 41 or claim 42, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2.

44. The system of any one of claims 41-43, wherein the variant of the calsequestrin protein has an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 7.

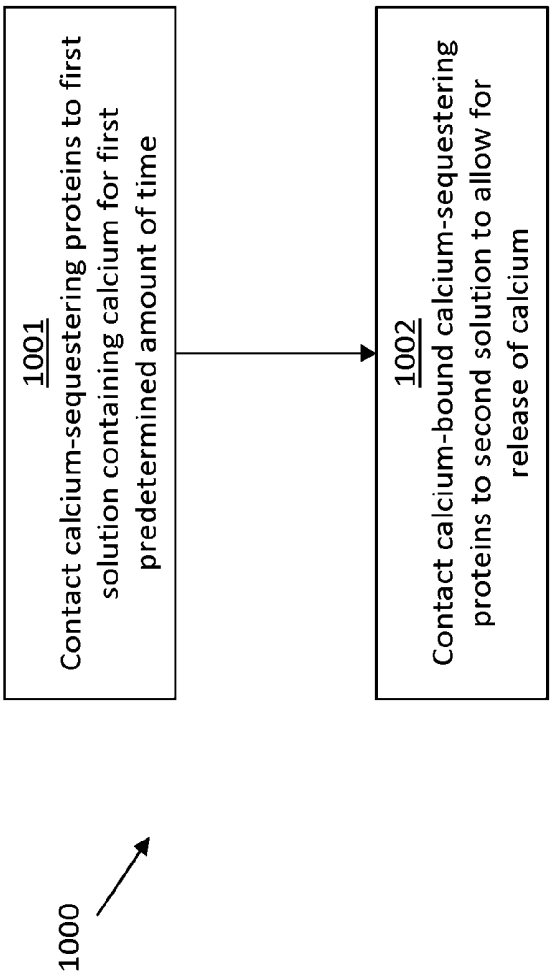

1001
Contact calcium-sequestering proteins to first solution containing calcium for first predetermined amount of time
1002
Contact calcium-bound calcium-sequestering proteins to second solution to allow for release of calcium
1000

FIG. 1A

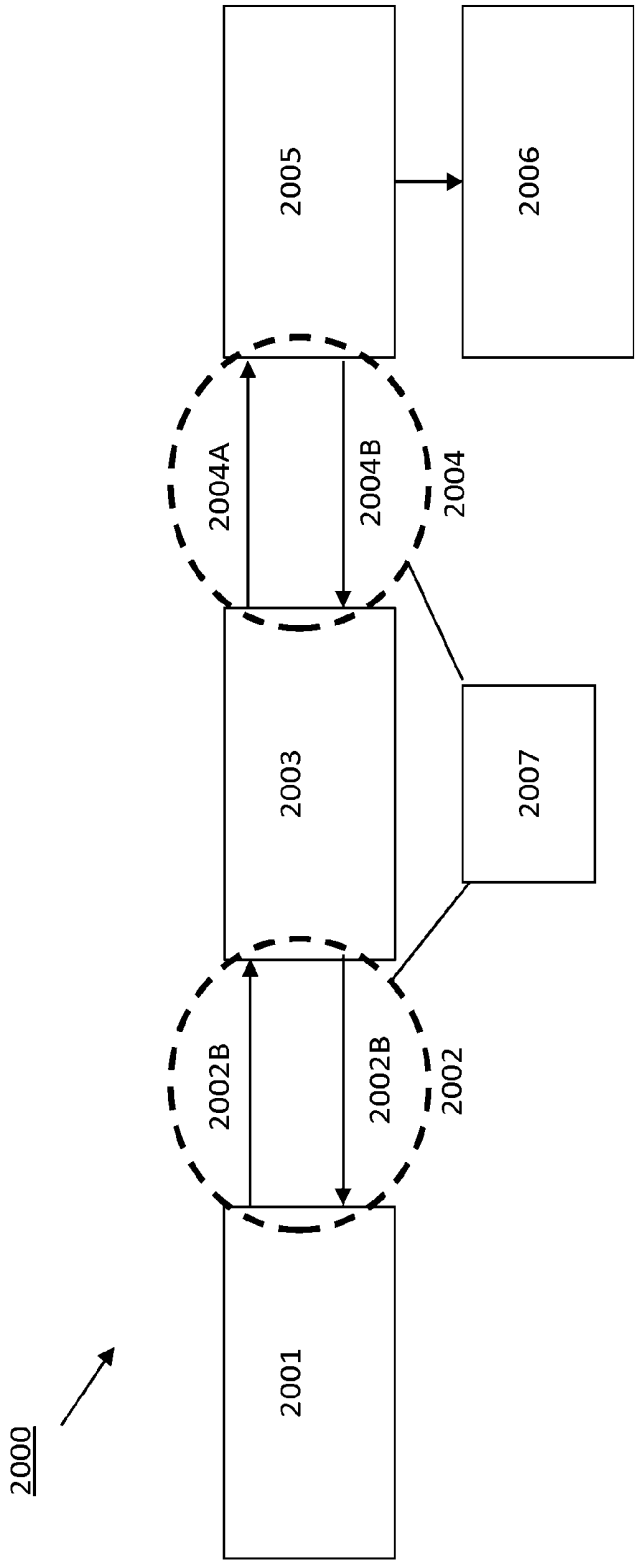

2005
2006
2004A
2004B
2004
2003
2007
2002B
2002B
2002
2001
2000

FIG. 1B

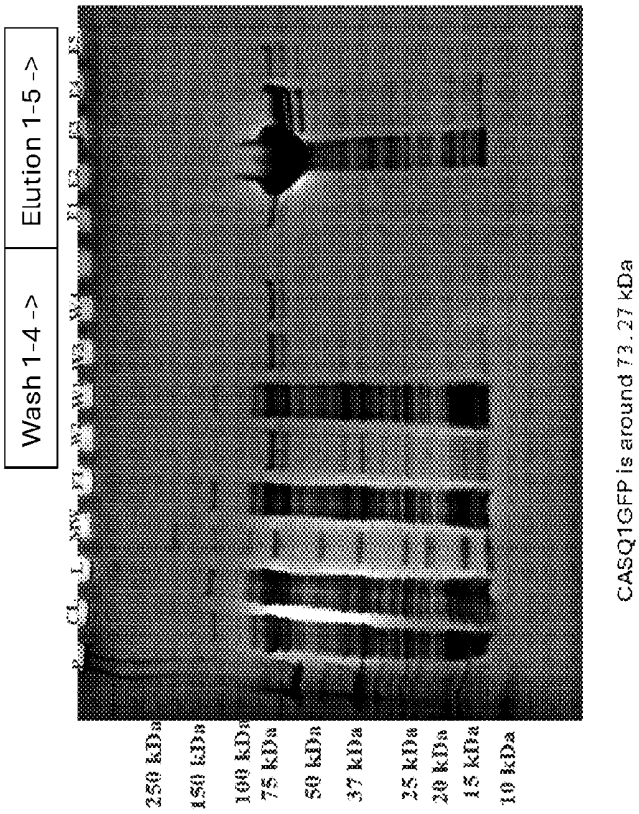

Wash 1-4 ->
Elution 1-5 ->
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
CASQ1GFP is around 73 . 27 kDa

FIG. 2

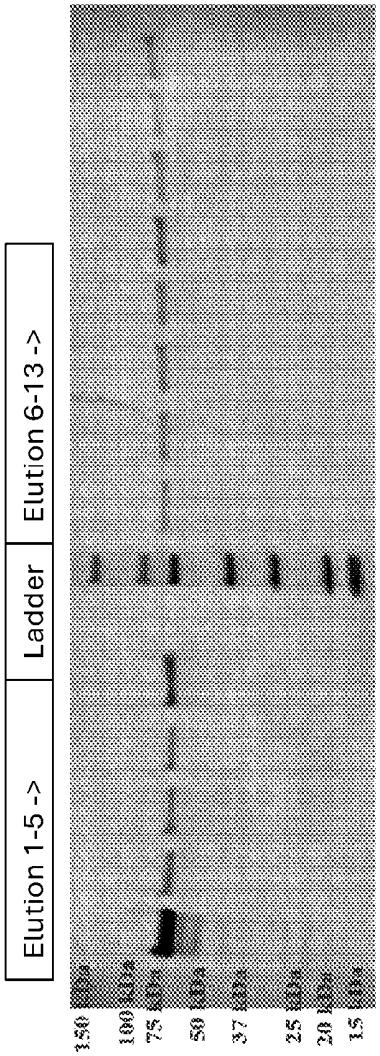


FIG. 3

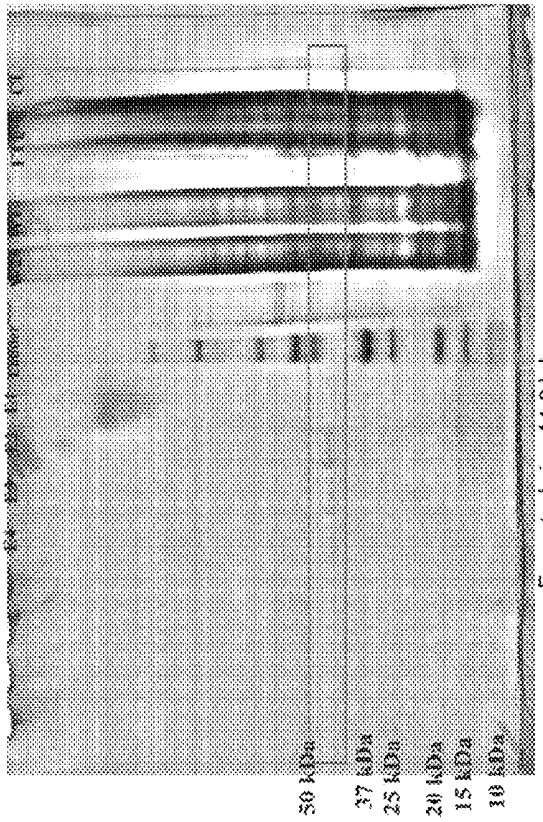


FIG. 4

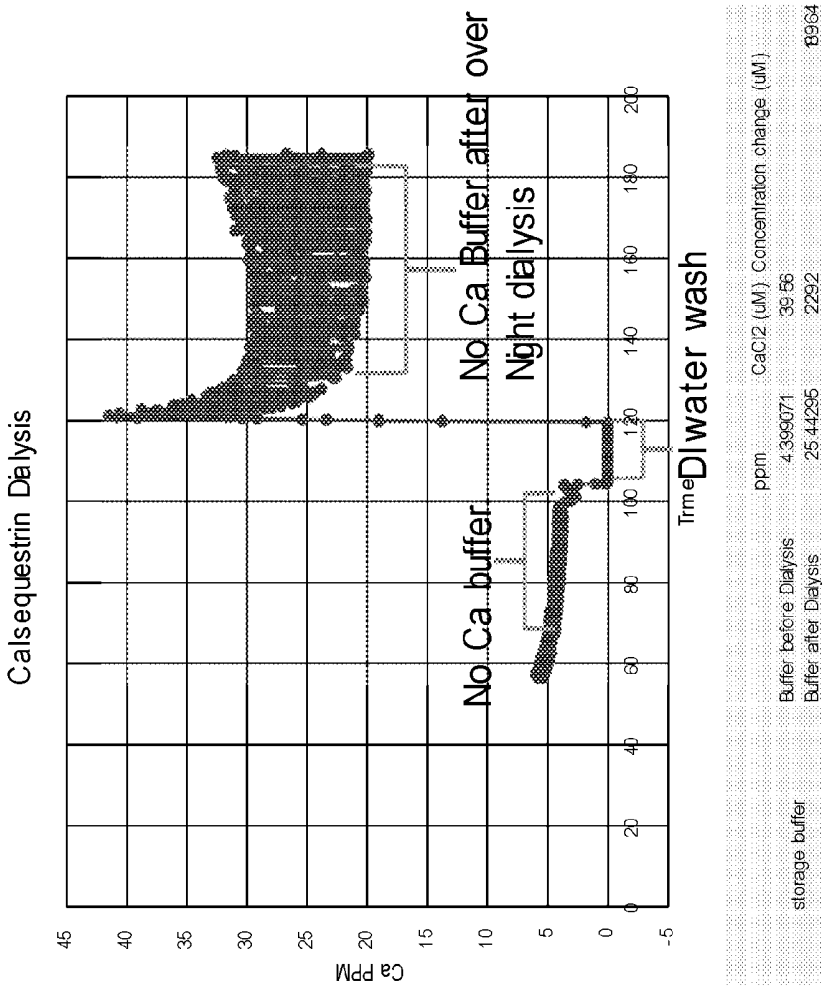
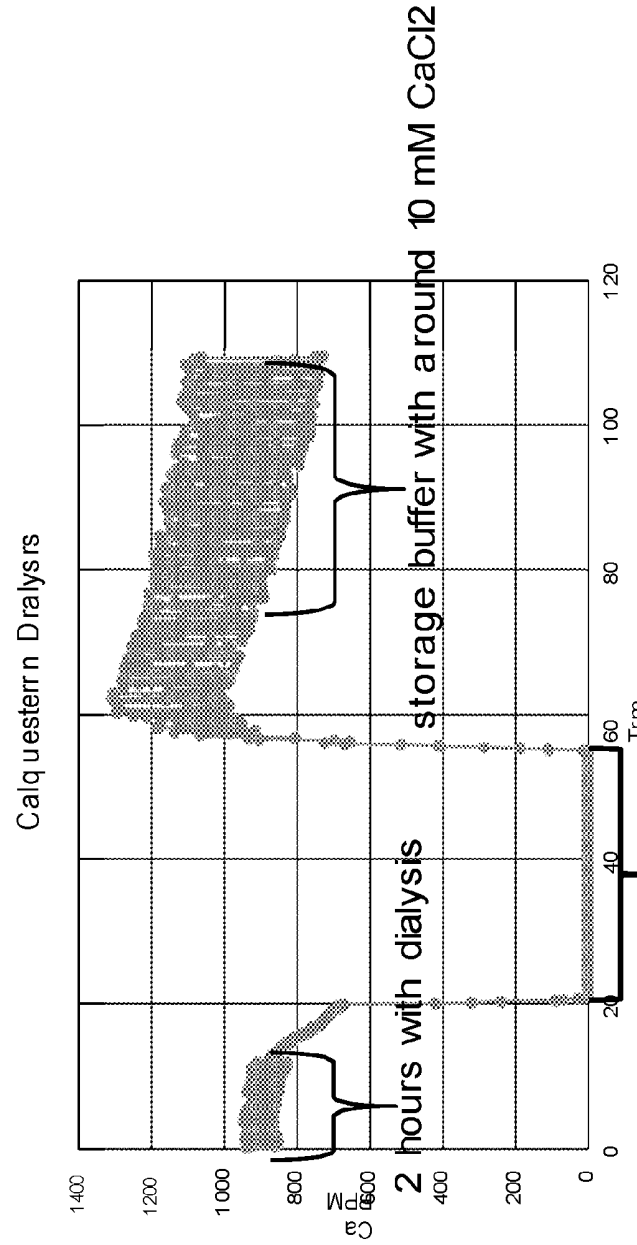

Calsequestrin Dialysis
Ca PPM
Time
DI water wash
No Ca buffer
No Ca Buffer after over Night dialysis
ppm
CaCl2 (uM) Concentration change (uM)
Buffer before Dialysis
4.399071
39.56
storage buffer
Buffer after Dialysis
25.44295
2292
8964

FIG. 5A



	CaCl2 Concentration ppm (uM)	change (uM)
storage buffer +10 mM CaCl2	855.01	
	after 2 hours 99	7705
	891.32	
	before 2 hours 77	8034
		329

FIG. 5B

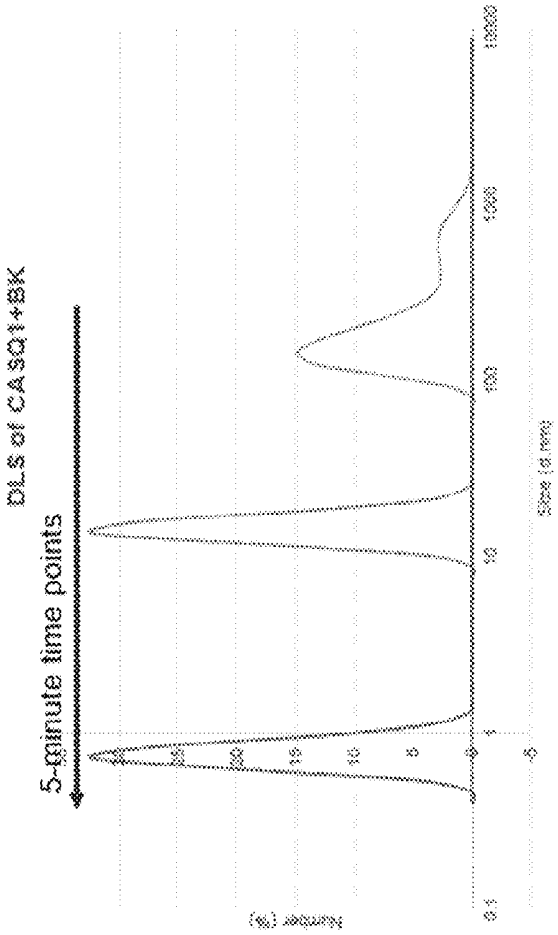


FIG. 6A

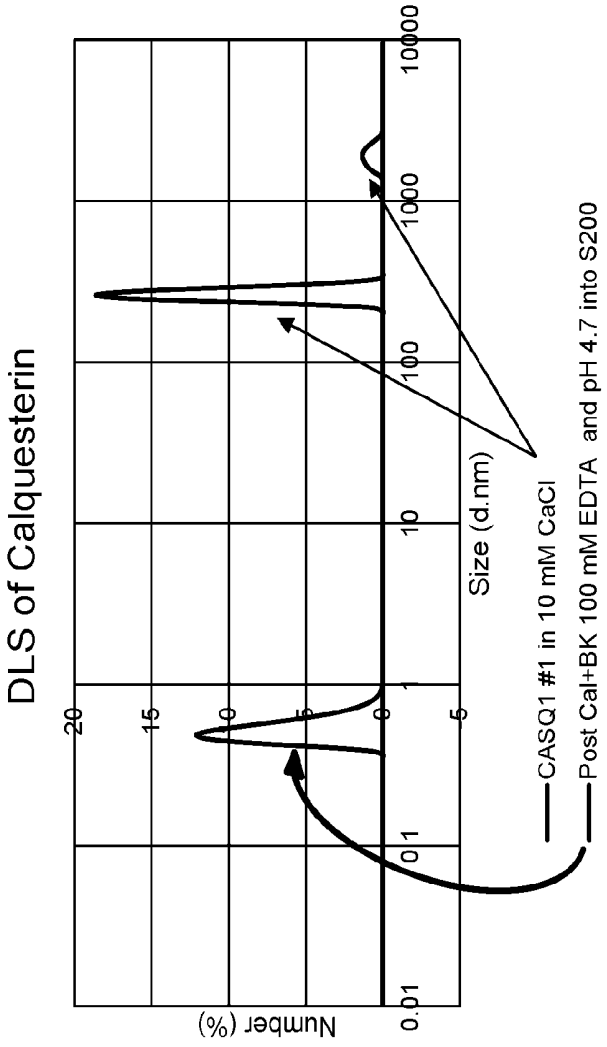

DLS of Calquesterin
Number (%)
20
15
10
5
0
0.01
0.1
1
10
100
1000
10000
Size (d.nm)
CASQ1 #1 in 10 mM CaCl
Post Cal+BK 100 mM EDTA and pH 4.7 into S200

FIG. 6B

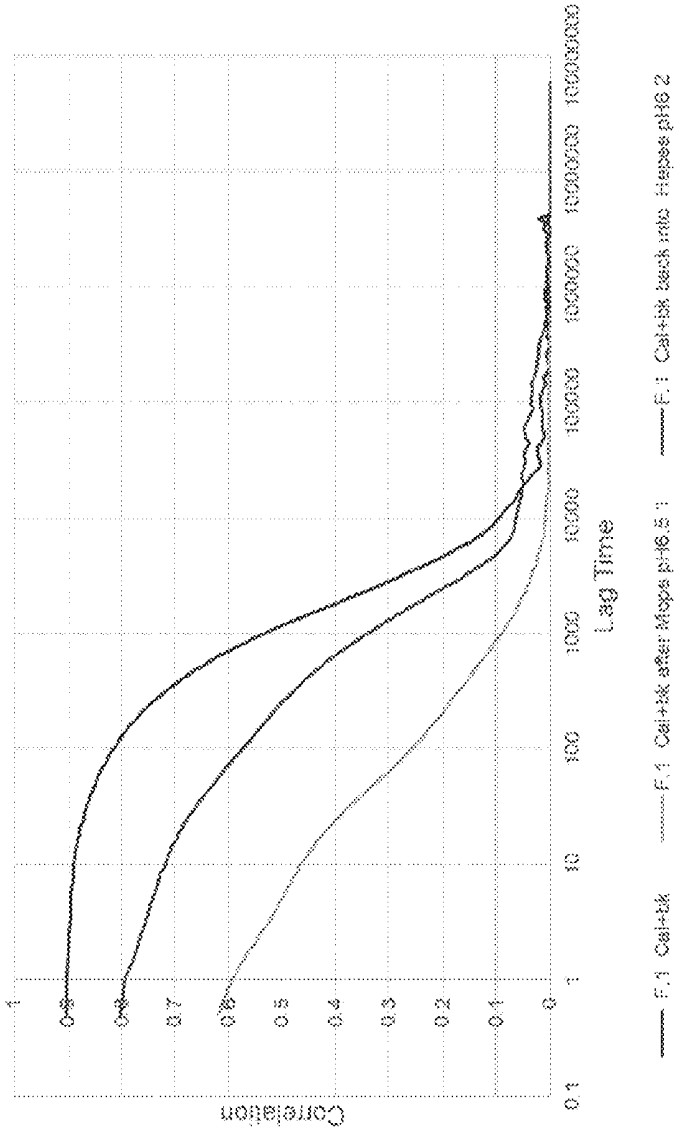

Correlation
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
1
10
100
1000
10000
100000
1000000
10000000
Lag Time
F.1 Cal+Lk
F.1 Cal+Lk after Mops pH6.5 1
F.1 Cal+Lk back into Hepes pH6.2

FIG. 7

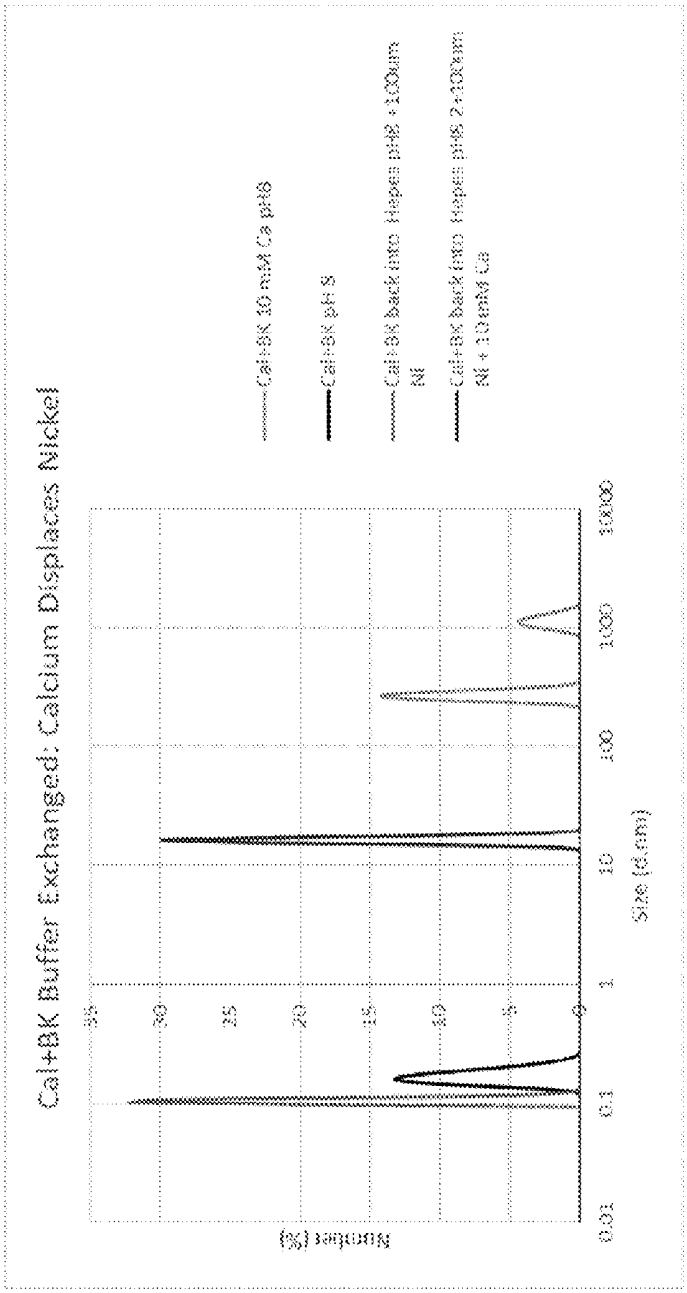
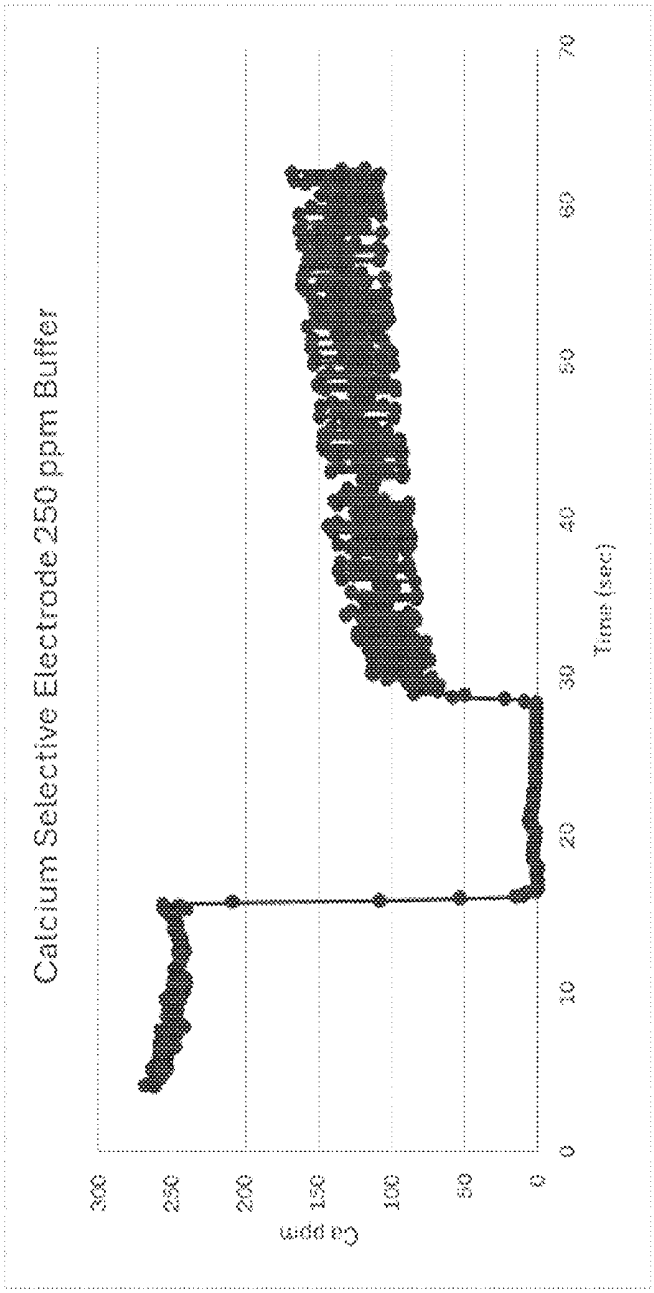
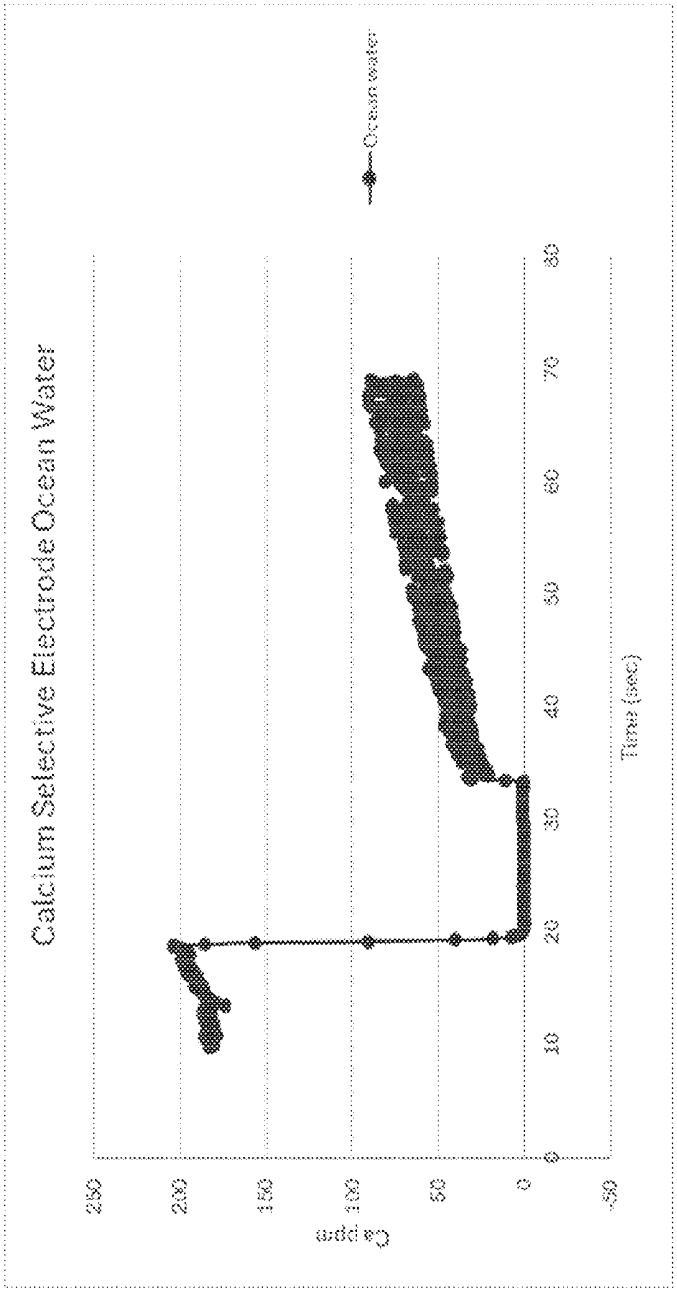

Cal+BK Buffer Exchanged; Calcium Displaces Nickel
Cal+BK 10 mM Ca pH8
Cal+BK pH 8
Cal+BK back into Hepes pH8 +100um Ni
Cal+BK back into Hepes pH8 2+100um Ni + 10mM Ca
Number (%)
Size (d.nm)
0.01
0.1
1
10
100
1000
10000
45
30
25
20
15
10
5
0

FIG. 8



Before Dialysis (ppm)	After Dialysis (ppm)	Change (ppm)
247.32	128.99	118.33

FIG. 9



Before Dialysis (ppm)	After Dialysis (ppm)	Change (ppm)
180.6818	67.66394328	113.0178567

FIG. 10

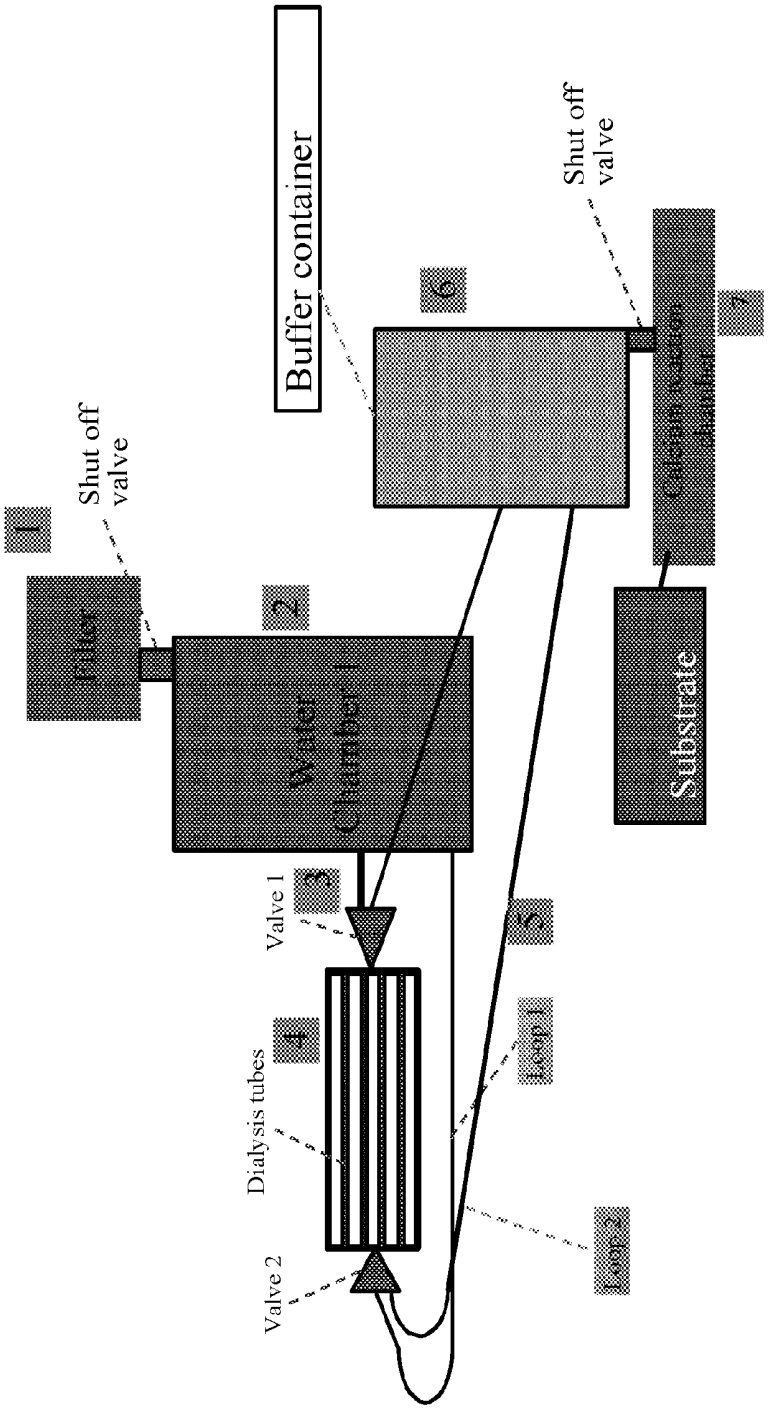

Buffer container
Shut off valve
Shut off valve
1
Filter
2
Water Chamber 1
6
7
Collagen reaction chamber
Substrate
Valve 1
3
5
Dialysis tubes
4
Loop 1
Valve 2
Loop 2

FIG. 11A

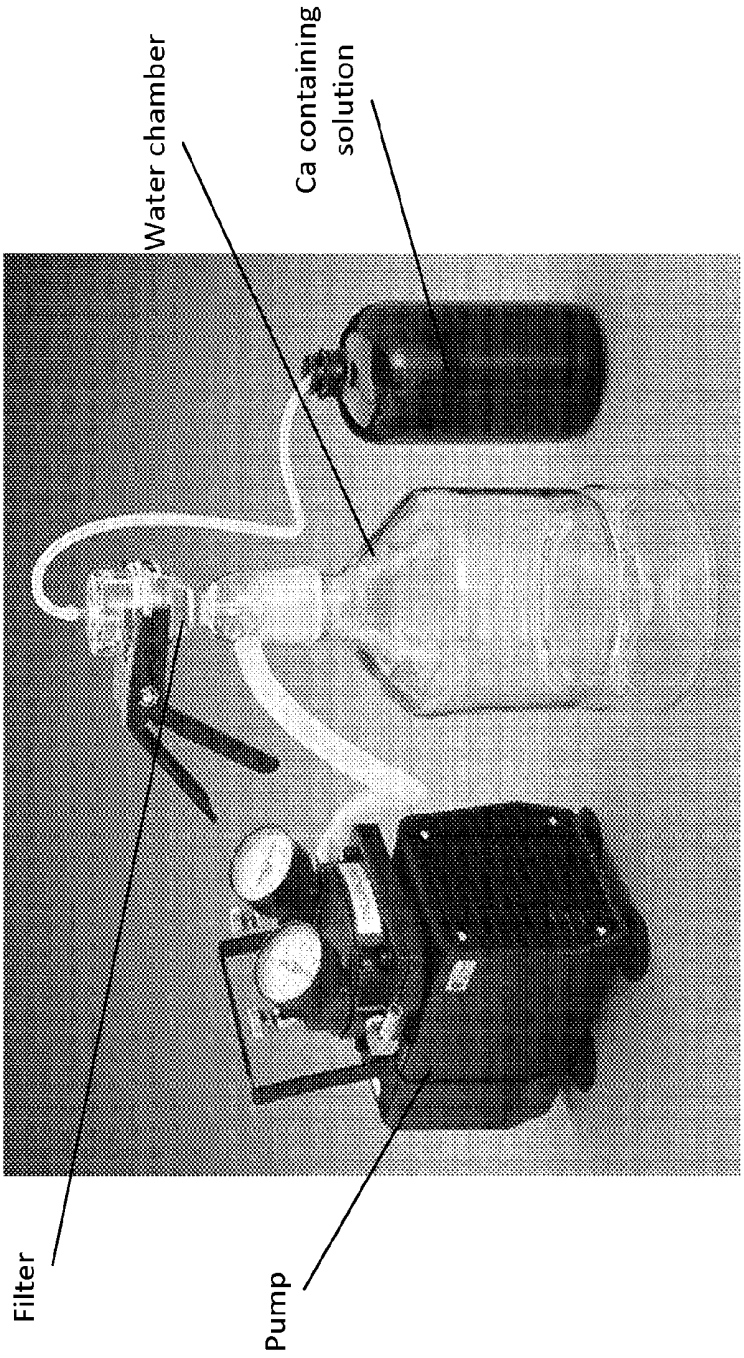


FIG. 11B

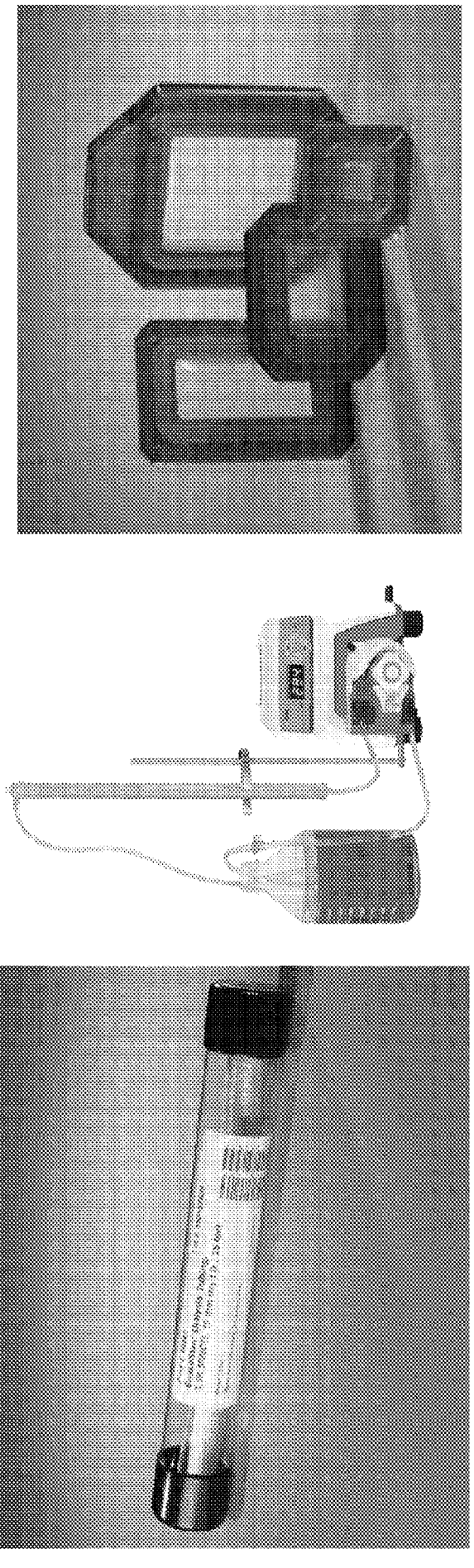


FIG. 11C

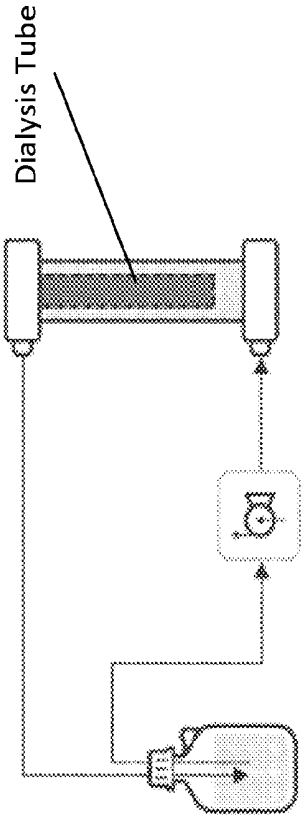


FIG. 11D

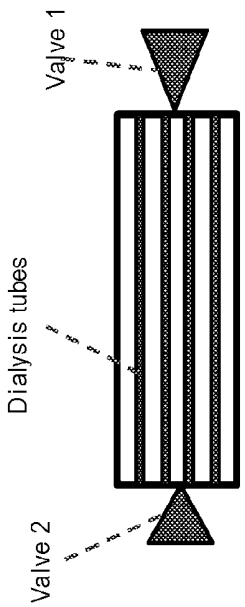


FIG. 11E

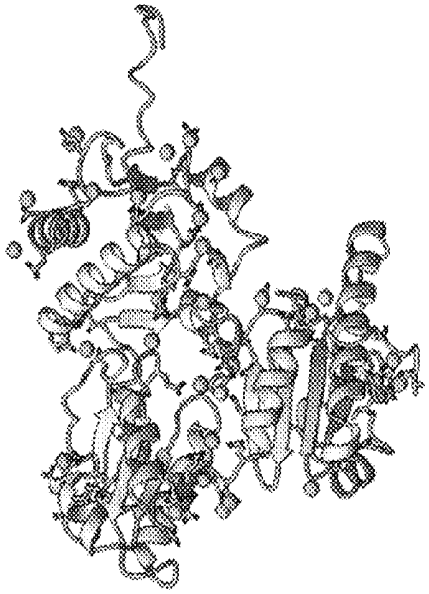


FIG. 12B

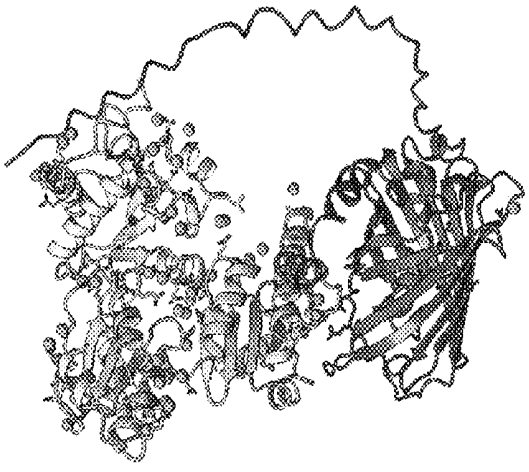


FIG. 12A

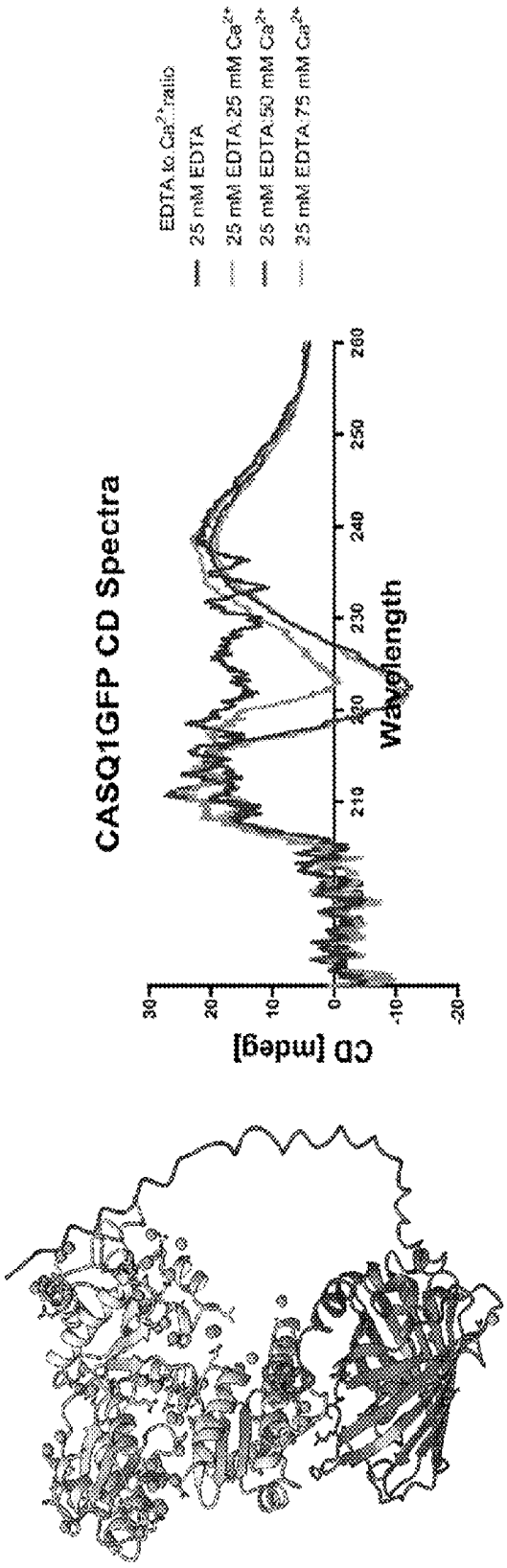

CASQ1GFP CD Spectra
CD [mdeg]
Wavelength
EDTA to Ca2+ ratio
25 mM EDTA
25 mM EDTA:25 mM Ca2+
25 mM EDTA:50 mM Ca2+
25 mM EDTA:75 mM Ca2+

FIG. 13

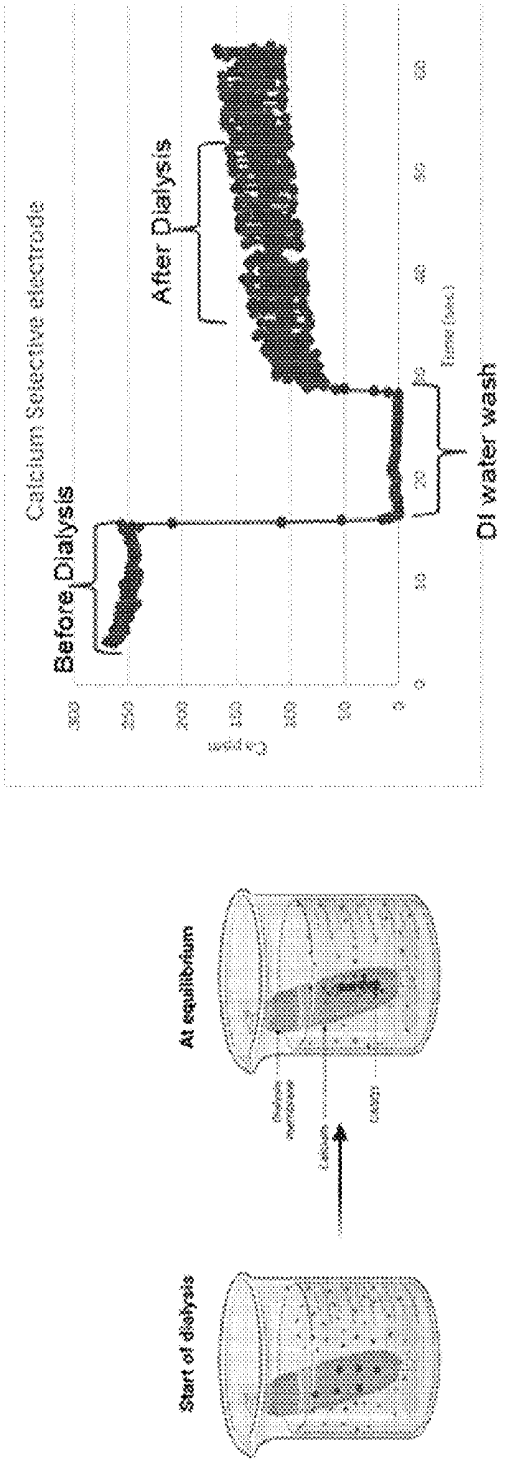

Calcium Selective electrode
Before Dialysis
After Dialysis
DI water wash
At equilibrium
Start of dialysis

FIG. 14A

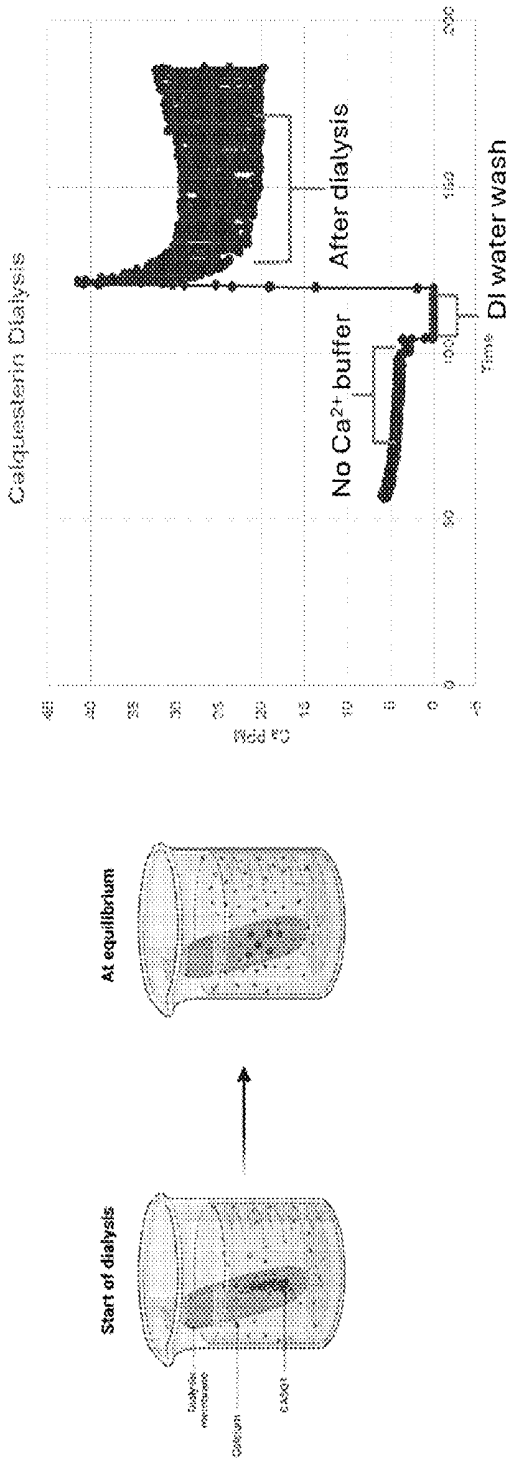
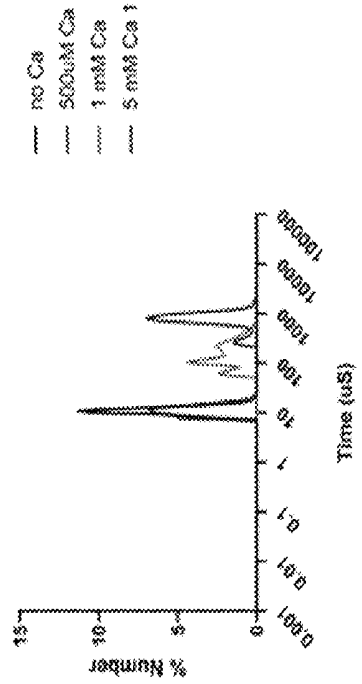

Calquestrin Dialysis
After dialysis
No Ca^{2+} buffer
DI water wash
Time
Ca PPM
At equilibrium
Start of dialysis
Dialysis membrane
Calcium
Calcein

FIG. 14B


no Ca
500uM Ca
1 mM Ca
5 mM Ca
% Number
Time (uS)

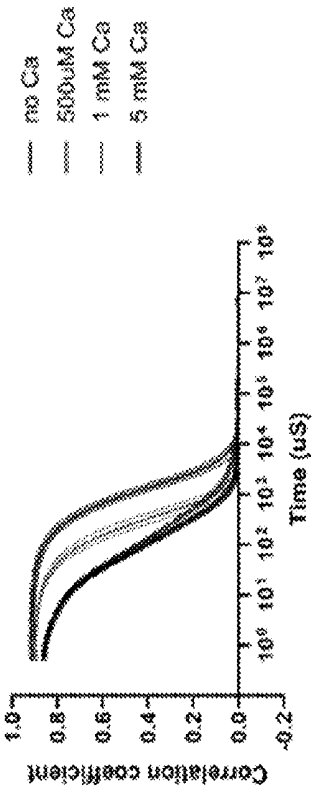

no Ca
500uM Ca
1 mM Ca
5 mM Ca
Correlation coefficient
Time (uS)

FIG. 15A

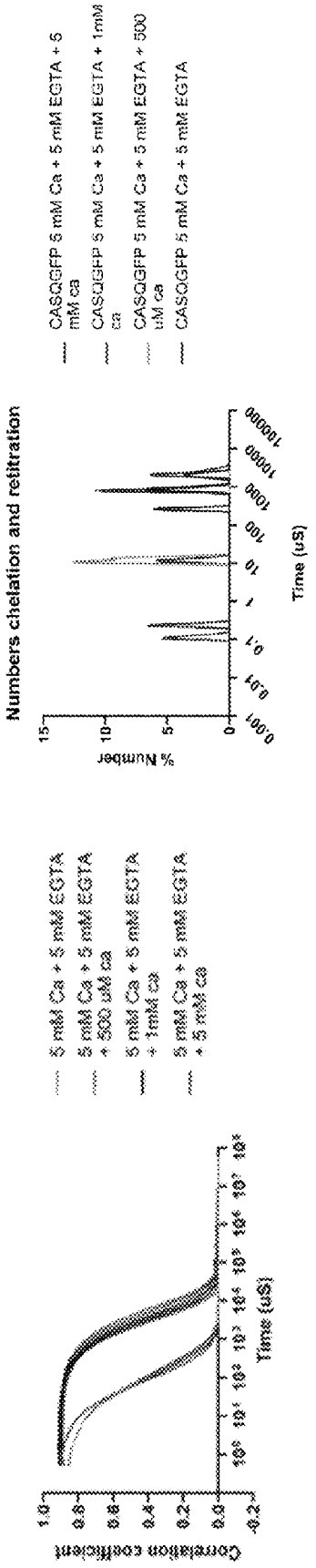

Correlation coefficient
Time (uS)
5 mM Ca + 5 mM EGTA
5 mM Ca + 5 mM EGTA + 500 uM ca
5 mM Ca + 5 mM EGTA + 1mM ca
5 mM Ca + 5 mM EGTA + 5 mM ca
Numbers chelation and retitration
% Number
Time (uS)
CASQGFP 5 mM Ca + 5 mM EGTA + 5 mM ca
CASQGFP 5 mM Ca + 5 mM EGTA + 1mM ca
CASQGFP 5 mM Ca + 5 mM EGTA + 500 uM ca
CASQGFP 5 mM Ca + 5 mM EGTA

FIG. 15B

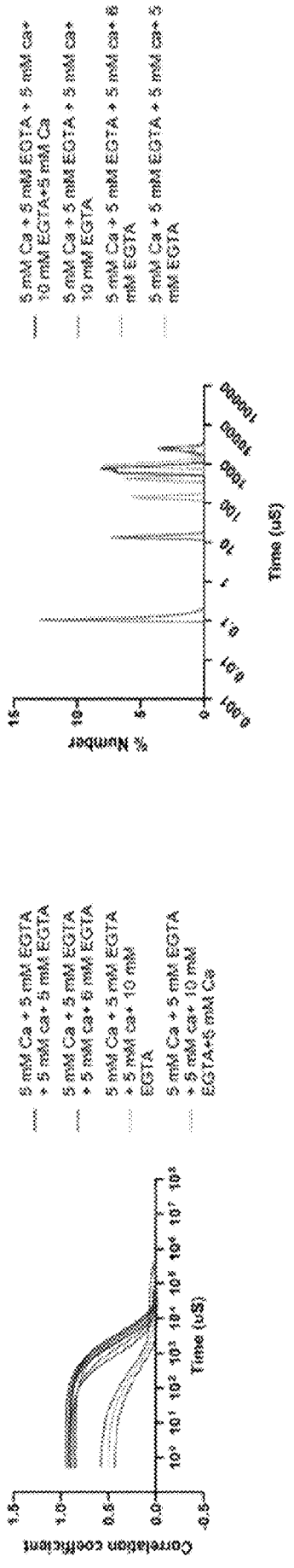

5 mM Ca + 5 mM EGTA + 5 mM ca+ 10 mM EGTA+5 mM Ca
5 mM Ca + 5 mM EGTA + 5 mM ca+ 10 mM EGTA
5 mM Ca + 5 mM EGTA + 5 mM ca+ mM EGTA
5 mM Ca + 5 mM EGTA + 5 mM ca+ mM EGTA
% Number
Time (uS)
5 mM Ca + 5 mM EGTA + 5 mM ca+ 5 mM EGTA
5 mM Ca + 5 mM EGTA + 5 mM ca+ 8 mM EGTA
5 mM Ca + 5 mM EGTA + 5 mM ca+ 10 mM EGTA
5 mM Ca + 5 mM EGTA + 5 mM ca+ 10 mM EGTA+5 mM Ca
Correlation coefficient
Time (uS)

FIG. 15C

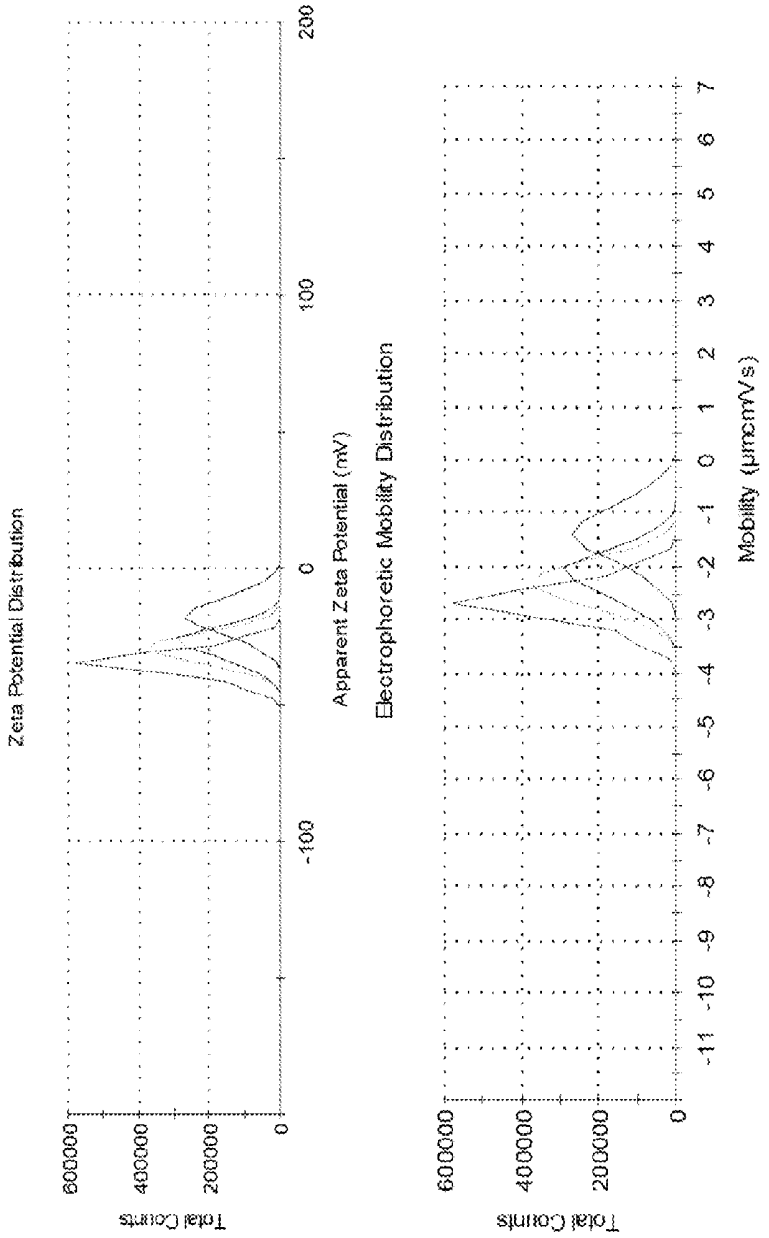
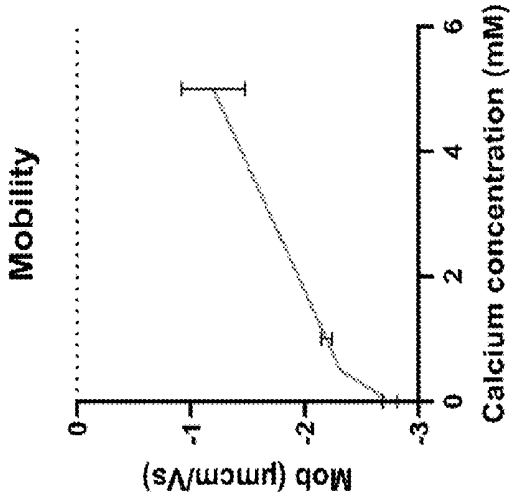

Zeta Potential Distribution
Total Counts
600000
400000
200000
0
-100
0
100
200
Apparent Zeta Potential (mV)
Electrophoretic Mobility Distribution
Total Counts
600000
400000
200000
0
-11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7
Mobility (µmcm/Vs)

FIG. 16A


Mobility
Mob (μmcm/Vs)
0
-1
-2
-3
0
2
4
6
Calcium concentration (mM)

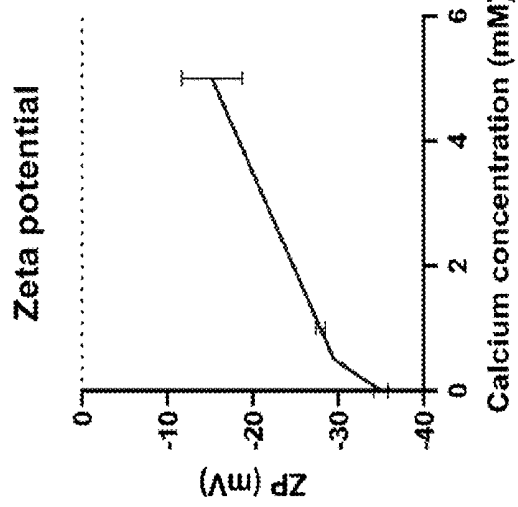

Zeta potential
ZP (mV)
0
-10
-20
-30
-40
0
2
4
6
Calcium concentration (mM)

FIG. 16B

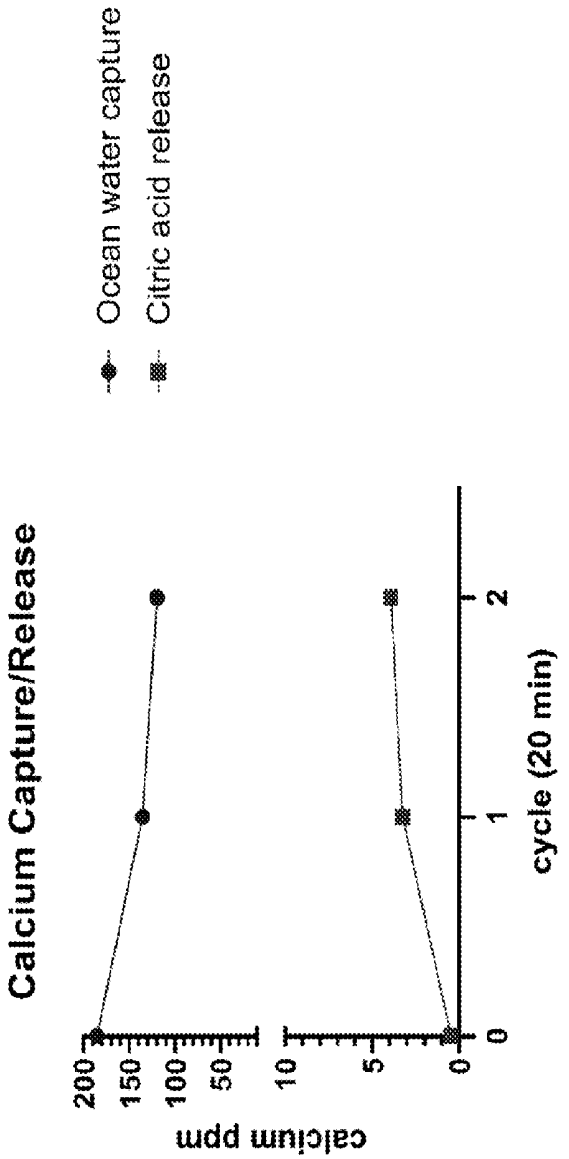

Calcium Capture/Release
Ocean water capture
Citric acid release
calcium ppm
200
150
100
50
10
5
0
0
1
2
cycle (20 min)

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2026/021042

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***G01N 33/84*** (2026.01); ***C07K 14/47*** (2026.01); *G01N 33/53* (2026.01)

CPC: ***G01N 33/84; C07K 14/4728; C07K 14/47;*** *G01N 33/53*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KR 20100089443 A (Gwangju Institute of Science and Technology, Kookmin University Industry-Academic Cooperation Foundation) 12 August 2010 (12.08.2010) abstract, pg 2 para 10, pg 3 para 2, pg 3 para 6, pg 3 para 7	16-18
A	US 2022/0235106 A1 (KOREA INSTITUTE OF CERAMIC ENGINEERING AND TECHNOLOGY) 28 July 2022 (28.07.2022) entire document	1-3, 16-18
A	US 2021/0382044 A1 (Immundiagnostik AG) 09 December 2021 (09.12.2021) entire document	1-3, 16-18
X	Chakravarty et al., "First Insight on Small Molecules as Cardiac Calsequestrin Stabilizers", 02 July 2019, ACS Omega 2019, 4, 11508-11514, DOI: 10.1021/acsomega.9b01113 pg 11508 col 1 para 1, pg 11508 col 2 para 2, pg 11512 col 1 para 5, pg 11512 col 1 para 5	1-3
Y	pg 11508 col 1 para 1, pg 11508 col 2 para 2, pg 11512 col 1 para 5, pg 11512 col 1 para 5	16-18

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 2026 (13.05.2026)	**09 July 2026 (09.07.2026)**

Name and mailing address of the ISA/US	Authorized officer
COMMISSIONER FOR PATENTS **MAIL STOP PCT, ATTN: ISA/US** **P.O. Box 1450** **Alexandria, VA 22313-1450** **UNITED STATES OF AMERICA**	**HARRY KIM**
Facsimile No. **571-273-8300**	Telephone No. **PCT Help Desk: 571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2026/021042

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Deshwal et al., "Influence of pH, temperature and concentration of calcium sequestering salts on the solubilisation of individual caseins and minerals from rennet casein", 20 July 2023, International Dairy Journal 146 (2023) 105761, DOI: 10.1016/j.idairyj.2023.105761 entire document	1-3, 16-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2026/021042

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2026/021042

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☑ Claims Nos.: **4-12, 19-26, 30, 36-44**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: Claims 1-3, 16-18, directed to a method of capturing calcium, the method comprising: contacting at least one calcium-sequestering protein to a first calcium-containing solution for a first predetermined amount of time.

Group II: Claims 13-15, directed to an extraction device for capturing calcium from a calcium-containing solution.

Group III: Claims 27-29, directed to a variant of a calsequestrin protein, wherein the variant exhibits calcium-sequestering activity that is at least 10% greater than the calsequestrin protein.

Group IV: Claims 31, directed to a method for producing a variant of a calsequestrin protein.

Group V: Claims 32-35, directed to a system for producing a variant of a calsequestrin protein.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2026/021042

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-3, 16-18**

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.