Supplementary information

Hierarchical intrafibrillar mineralization co-assembled with EGaIn nanocapsules through a one-step collagen self-assembly approach

Mwangi H. Kevin[1], Yue Qu[2], Sengpav Tong[1], Xiaodan Sun,[1], Lingyun Zhao[1], XiuMei Wang[1]*

Authors and Affiliations

[1]State Key Laboratory of New Ceramics and Fine Processing, Key Laboratory of Advanced Materials, School of Materials Science and Engineering, Tsinghua University, Beijing 100084, China.

*Mwangi. H. Kevin, Sengpav. Tong, Xiaodan Sun, Lingyun Zhao, XiuMei Wang**

[2]Department of Medicine, School of Life science, Center of Biology, Tsinghua University, Beijing 100084, China.

Yue Qu

Experimental Setup

Materials

Type I collagen (MW = 280 kDa) was purchased from Hebei Collagen Biotechnology Co., Ltd. (Atelocollagen from bovine skin), and used as received. The chemicals used in this study, including; calcium nitrate tetrahydrate (Ca $(NO_3)_2.4H_2O$), diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and polyacrylic acid (PAA, Mv= 450 kDa) were all purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol (200 proof, anhydrous; Sigma Aldrich) was obtained from stock on hand and used as received. Gibco™ HEPES (1M) was purchased from Thermo Fisher Scientific. All the chemicals used in this study were of analytical grade.

Matrix Composition

Bone and other mineralized biological materials have complex hierarchical architectures made up of inorganic and organic phases. These architectures have unique dimensions that range from the nanoscale to the macroscale. With respect to the collagen mass fraction, the apatite nanocrystal content (wt.%) is set at 70%. About 65% of the chemical composition of bone matrix is composed of mineral materials, 25% of organic materials, 10% is water, and free ions (like CO_3^{2-}, Na^+, and Mg^{2+}). The selected values represent the theoretical apatite phase contents necessary for the formation of mineralized collagen constructs, with a 70% nanoapatite content being typical tidemark of subchondral bone and calcified cartilage [1-4].

Mineralization of collagen matrices

Intrafibrillar mineralised collagen apatite (Col-Ap lamellar) constructs were synthesized using a modified procedure based on the existing approaches [5, 6]. In the first step, the adoption of the ethanolic-Collagen system (Ethanolic-Col matrix) and in the second, the fabrication of materials that resemble bone through HPAA-ACP as an intermediate precursor. High-molecular weight Polyacrylic acid (HPAA: M_V= 450,000 g/mol) was used as a biomimetic analog of extracellular matrix proteins involved in biomineralization to stabilize the Ca^{2+} and PO_4^{3-} ion-containing solution in the form of nucleation inhibitor–stabilized CaP solutions (Pa-ACPs).

Briefly; experiment proceeded by the addition of [0.05 M] Ca $(NO_3)_2.4H_2O$ to the already self-assembled ethanolic solution of Collagen (24mg/ml); the solution was kept at 4 °C with continuous stirring for at least 12h. To meet the experimental needs, the collagen stock solution was diluted by incorporating the required volumes of 0.1 M acetic acid and ethanol. The polyacrylic acid (120 mg mL^{-1}PAA) was dissolved in [0.03 M] $(NH_4)_2HPO_4$ counter-ion solution of equal volume and added to the ethanolic Collagen/ Ca^{2+} precursor solution at the same temperature. The HPAA used

here as a biomimetic analog of non-collagenous proteins to stabilize the Ca^{2+} and PO_4^{3-} ion-containing solution in the form of stabilizer of amorphous calcium phosphate (ACP) for collagen biomimetic mineralization. The mineralization was achieved by maintaining the pH at 7.4-7.9 with addition of either 0.1M HEPES or with 0.1 M NaOH and kept for 3 -/ 7d at 37 ^{0}C (Entropic stabilization) and only stoichiometric Ca: P molar ratio near 1.67 similar to that of natural bone were utilised for all subsequent analysis. After the biomineralization process, the composite slurry was washed 2-3 consecutive times in milli-Q water and filtered through micro sieve (25 μm) to eliminate any unreacted product and counterions. Afterwards, the lamellar constructs, were subjected to a 15 min self-compression process and then transferred to a lyophilizer and freeze-dried unidirectionally at −80 °C. The final recovered composite slurry is referred to as ethanolic - HPAA collagen lamellar membranes (Ethanolic-Col-Ap lamellar). The biomineralized collagen membranes without the adoption of the benign aqueous ethanol served as a control specimen (HPAA Col-Ap lamellar mimetic).

Elemental PEG-EGaIn nanocapsules

The fabrication of the elemental PEG-EGaIn nanocapsules was based on a facile approach with end result of achieving an engineered nano-sized PEG-EGaIn nanocapsules of <200 nm via centrifugation. The average sizes of these two material particles were carefully determined by DLS. These nanomaterials were homogenized for 3-5min prior to every use.

PEG-EGaIn nanocapsules were fabricated according to previous studies [7-9], with slight modifications. A small quantity of EGaIn (0.6 mL) is introduced into a 20 mL, 28mm OD borosilicate glass vial) with a negligible quantity of halide acid (HCL final concentration of 100 mM) is included in the glass vial to avert the development of an oxide layer. Prior to the start of ultrasonication, 14.4 ml solution containing 64 mM ethanolic thiol (C12SH) (1-dodecanethiol were added to allow ligand attachment during the nanocapsule formation process followed by addition of small amount of Polyethylene glycol (PEG). To produce nanocapsule, the vials were then immersed (approximately half the vial height) into a chilled water bath maintained at 4-10 °C to minimize undesired side effects brought about by the high suspension temperature during ultrasonication. A 3 mm tapered microtip powered by a Sonics and Materials, Inc., VCX 500 ultrasonic processor was then immersed into the open vial (~1 cm from the bottom). The opening between the vial and probe microtip was covered as completely as possible with Parafilm M to aid in solvent retention. Sonication was achieved at a power level of 80% and with a pulse mode (45 s pulses with 15 s delay in between) for < 5 min (the actual total sonicating time is 3 min). After beginning ultrasonication, the liquid in the vial was observed to darken, indicating production of EGaIn nanocapsule. To recover purified particle suspensions following ultrasonication, particle suspensions were centrifuged at 800 rpm for 10 mins followed by redispersion in fresh ethanol. This process was repeated three times to remove excess unbound thiols. Next, 30ml of Polyethylene glycol (PEG) was incorporated and ultrasound pulses under the similar conditions for 1h. Separation of the PEG-EGaIn nanocapsules (diameter < 200 nm) was achieved by centrifugation at 6300 × g for 15 min. This supernatant was mixed with four times

larger volume of deionised water and centrifuged at 7000 × g for 12 min each time. After the final centrifugation step, the nanocapsule dispersions (ethanol) were stored in capped vials until performing further sample preparation or analysis.

Characterization of PEG-EGaIn nanocapsules

We used a high-resolution transmission electron microscopy system (JEOL JEM 2100) at an accelerating voltage of 200 kV was used for imaging to assess structural characteristics of formed nanocapsule. 1−2 drops of the purified final suspensions were scattered over ultrathin (3−4 nm) carbon films on 400-mesh copper grids purchased from Electron Microscopy Sciences (CF400-Cu-UL) held in self-closing, anticapillary tweezers until a single drop fell from the grid. A folded piece of a clean filter paper was then used to wick excess solvent from the grid underside. The morphologies of the PEG-EGaIn nanocapsule were examined using a field emission scanning electron microscopy (FESEM) (Merlin Compact, Zeiss, Germany) operating at an accelerating voltage of 5 kV. Energy Dispersive X-Ray Spectroscopy (EDS) microanalysis was performed at 15 kV. All specimens were coated with 10 nm platinum. The hydrodynamic distribution of PEG-EGaIn nanocapsule was obtained using a zeta-sizer (Zetasizer Nano ZS, Marvern Instrument, USA). The surface potential of the colloidal nanocapsule was measured by a zeta sizer (Malvern Panalytical).

X-Ray photoelectron spectroscopy (XPS) measurements

To investigate gallium oxide formation, XPS was utilized on films made from PEG-EGaIn nanocapsule. These films were produced by spin coating a small amount of PEG-EGaIn nanocapsule suspension onto substrates, which consisted of copper foil adhered to 1 cm × 1 cm glass slides. The nanocapsule or nanocapsules suspension was added incrementally until the film was sufficiently thick to prevent the visibility of the copper foil. To ensure consistency, the suspensions were vortex-mixed immediately before deposition. Films were prepared 30 minutes before XPS analysis, with measurements commencing within 90 minutes.

Immobilization of PEG-EGaIn metal on 2D collagen matrices "PEG-EGaIn-Col matrix"

The 'nano-matrix interface' biomimetic was accomplished using a modified procedure based on the existing approaches from previous studies [10, 11]. Briefly 100mg stock solution of collagen suspensions were produced by blending in 0.5 M acetic acid and incubated at 4 °C for 24-48h. Next, collagen fibril assembly was achieved by increasing the pH to 5.5 (isoelectric point of collagen) with slow dripping of 0.1M NaOH solution and left to blend for O/N to generate a uniform slurry. In order to reduce gel-like precipitation and disintegration events of the triple helix during incorporation of PEG-EGaIn nanocapsules to the already homogenized collagen slurry, the experiment proceeded under the same condition. The PEG-EGaIn nanocapsules was added at various concentrations 5-/10-/20% (v/v) to a final working collagen concentration of 24 or 30mg/mL as required by the experiment at 4 °C. Samples were stored under refrigeration (4 °C) until performing further sample preparation or analysis.

Fibril formation studies

The effect on the process of self-association of collagen in the presence of ethanolic PEG-EGaIn nanocapsules, was observed using a modified procedure based on the existing approach [11]. Picrosirius red stained samples of the optimized fibrin-collagen was further analyzed. In light microscopy, collagen is visible as clusters of pink to crimson fibers that become disrupted during abnormal states. The fibrils formed were applied to glass slides, air dried, and assessed for birefringence by use of an optical polarized microscope (Olympus, BX63).

Intrafibrillar mineralization of nano EGaIn Collagen matrices

The mineralization of the PEG-EGaIn -Col-Ap constructs was synthesized using a modified procedure based on the existing approaches [10, 12]. Having constructed "nano-matrix interface" biomimetic, our experiment proceeded by following the procedure similar to the ethanolic Col-Ap lamellar with the final recovered composite slurry is referred to as mineralized PEG-EGaIn collagen mimetic (PEG-EGaIn -Col-Ap).

Transmission Electron Microscope (TEM)

The morphologies of all collagen matrices were evaluated on carbon-and-formvar-coated Cu grids with 200 mesh size through electron transmission microscopy (TEM) using a (H-7650B, Hitachi, Japan) microscope operated at 200 kV or in high-resolution electron transmission microscopy (HRTEM) images were obtained on a JEOL 2100 STEM using a LaB6 electron source at an accelerating voltage of 200 kV was used for imaging. All the samples were analysed in bright-field, High-angle annular dark-field, fast Fourier, high-resolution and crystalline phase of the mineralised mimetics were examined using selected area electron diffraction (SAED) modes. Spectrum acquisition and elemental mapping (STEM-EDX) were conducted using an Oxford Instruments INCA x-sight detector. Images were collected with a Gatan 1K x 1K CCD camera. Elemental mappings were acquired with the FEI TIA software using a spot dwell time of 300 msec. Drift correction was performed after the acquisition of every 30 images.

High-Resolution Scanning Electron Microscopy (FE-SEM)

The morphology of the hierarchical interfibrillar mineralisation of all collagen matrices were examined using a SEM (Merlin Compact, Zeiss, Germany) operating at an accelerating voltage of 5 kV. Energy Dispersive X-Ray Spectroscopy (EDS) microanalysis was performed at 15 kV. All specimens were coated with 10 nm platinum.

Attenuated total reflection–Fourier transform infrared spectroscopy

Infrared spectra of all mineralized collagen matrices, nano PEG-EGaIn Col matrix and bare collagen matrices were collected using a Nicolet 6700 Fourier transform infrared (FTIR) spectrophotometer (Bruker, German) equipped with an attenuated total reflection (ATR) setup. Infrared spectra between 4,000-400 cm^{-1} at 4 cm^{-1} resolution were collected using 32 scans. The spectrum of collagen matrices was normalized against the spectrum of bare collagen sponge along the collagen amide A peak (~3300 cm^{-1}) and superimposed for comparison.

Powder X-ray diffraction

X-ray diffraction (XRD; D8 Discover, Bruker, Germany) of all mineralized collagen matrices with were performed using Ni-filtered Cu Kα radiation (40 kV, 200 mA) was used in the 2θ range of 10-60° at a step scanning rate of 2° min^{-1}. Phase composition was assigned by comparing the acquired spectra with peaks identified from the International Center for Diffraction Data database.

Thermogravimetric and derivative thermogravimetric analysis (TG/DTG) of the Mineralized Collagen sponges

The mineral content in the collagen lamellar matrices were determined by TG/DTG (Q5000IR, TA INSTRUMENTS, China). The analysis was carried out by from room temperature to 1000 °C in a nitrogen atmosphere at elevated temperatures of 10°C/min. The lyophilized mineralized collagen sponges from day 7 were cut into small pieces of 10mg of each sample were used for the TG/DTG measurements.

Cell culture

IMR-90 human lung fibroblasts cells (ATCC®; CCL-186 ECACC; 85020204) were obtained from Procell (CL-0538) were kept at 37°C in a normal humidified atmosphere supplemented with 5% CO_2. The growth medium was DMEM/F12 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific). The FBS supplemented in our IMR-90 cells culture medium was pre-filtered through 0.22-μm membranes to eliminate most of the FBS-originated MVs in the analysis.

Twenty-four hours after cell seeding (5×10^3 cells/well), the IMR-90 cells were nurtured in the presence of varying concentrations of PEG-EGaIn nanocapsules (0.15–1.0 g/L) in a 96-well microtitre plate, a protocol modified from [13] was used for 48 and 72 h at 37 °C for the study of following parameters unless otherwise stated.

Live imaging by Operetta high content imaging system Confocal

After 48 and 72 h of exposure to the different concentration of PEG-EGaIn nanocapsules, IMR-90 cells were stained in a cocktail composed of SYTO™ Deep Red (Thermo Fisher Scientific) and BioTracker 488 Green Mitochondia Dye (Sigma Aldrich) covered in aluminium foil and performed using Operetta CLS™ High Content Imaging system (PerkinElmer, Skovlunde, Denmark). To visualize the DNA within IMR-90 cells, a 1 μM of SYTO™ Deep Red was employed (according to the manufacturer's instructions); conversely, the demonstration of metabolic activity was delineated using 100 nM BioTracker 488 Green Mitochondia Dye (according to the manufacturer's instructions). Subsequently, cells were washed three times with Hank's Balanced salt solution (1 x HBSS ca^{2+} and Mg^{2+} negative) and Live Cell Imaging Solution (Thermo Fisher Scientific) in the presence of Pro-Long Live Antifade Reagent (Thermo Fisher Scientific). A sequential excitation of fluorophores was utilized, with 488 nm or FITC/GFP channel for BioTracker™ 488 Green Mitochondia Dye and CY5/deep red filter set, or 647 nm laser line. Fluorescent images were analyzed at × 10 magnification using Harmony High Content Imaging and Analysis Software (PerkinElmer, Denmark). The amount of DNA was quantitatively determined by using DNA content threshold. All pictures were acquired with the same parameters.

Cytotoxicity

Lactate dehydrogenase (LDH), typically intra-cytosolic, leaks from compromised cells, hence elevated levels in the supernatant signal cellular damage [14].

The toxicity effect of PEG-EGaIn nanocapsules on IMR-90 cells were determined via the Cytotoxicity Detection Kit according to the manufacturer's instructions (Beyotime, China). Since most cellular intake of PEG-EGaIn nanocapsules is through endocytosis, the stability of the IMR-90 cell membrane needed to be examined. The LDH activity was used as an index of cell death and lysis of IMR-90 damaged cells in the culture media (cytosol leakage). After 24 h of exposure to the different concentration of PEG-EGaIn nanocapsules, the LDH release in the supernatant and the intracellular LDH content of the corresponding pure cell culture medium with cells was used for maximum LDH release. Finally, the fluorescence was measured at A_{490} nm using a microplate reader (PerkinElmer EnSpire™ Multimode Plate Reader) at OD value of each well. The percentage LDH activity was calculated by = amount of activity/ total activity (cell lysates + medium) x 100%.

Statistical analysis

Data are expressed as the mean ± SEM one-way analysis of variance (ANOVA) was used for statistical testing involving more than two groups, followed by pair-wise multiple comparison procedure (Tukey's HSD test). Data were statistically computed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA). A P value < 0.05 was considered to be

significant. *n* = number of independent experiment and each experiment was repeated as least 3 times. The level of significance was expressed as one to four stars ***p* <0.0001 (****)**, ***p* <0.001 (***)**, ***p* <0.01 (**)**, **or *p* < 0.05 (*)**.

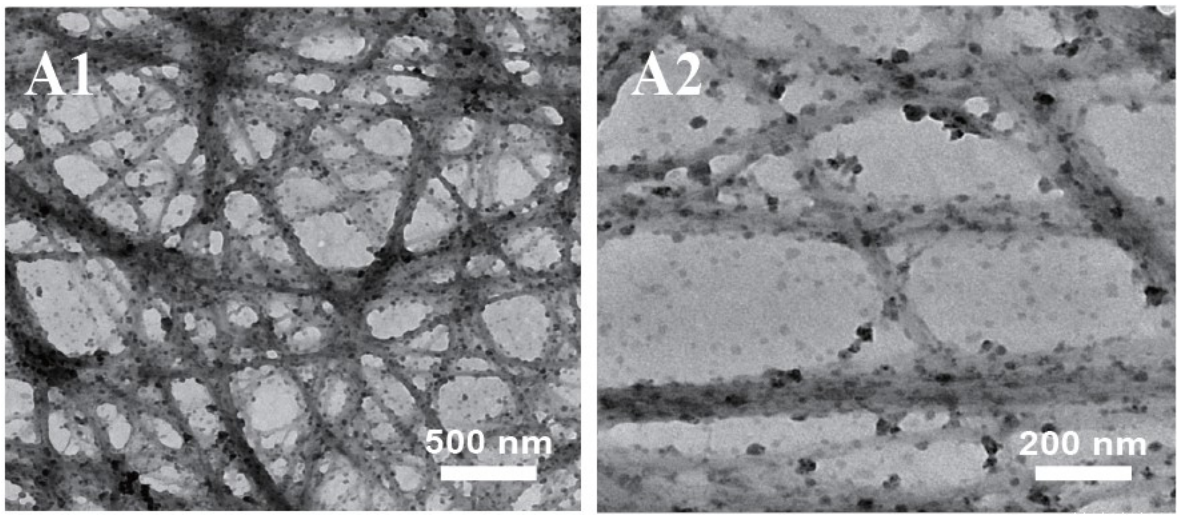

Figure S1. Characterization of early biomineralization phase on HPAA Col-Ap matrices after 48h incubation. (**A1-2**) Representative EM images of the hierarchical bundle-like col-fibrils that appear to have remnant PILP globules adsorbed to its fibers. Notably, collagen fibrils displayed their normal morphology as no imbibition could be discerned. Scale bar: representative 500nm (left) and Scale bar: representative 200nm (right).

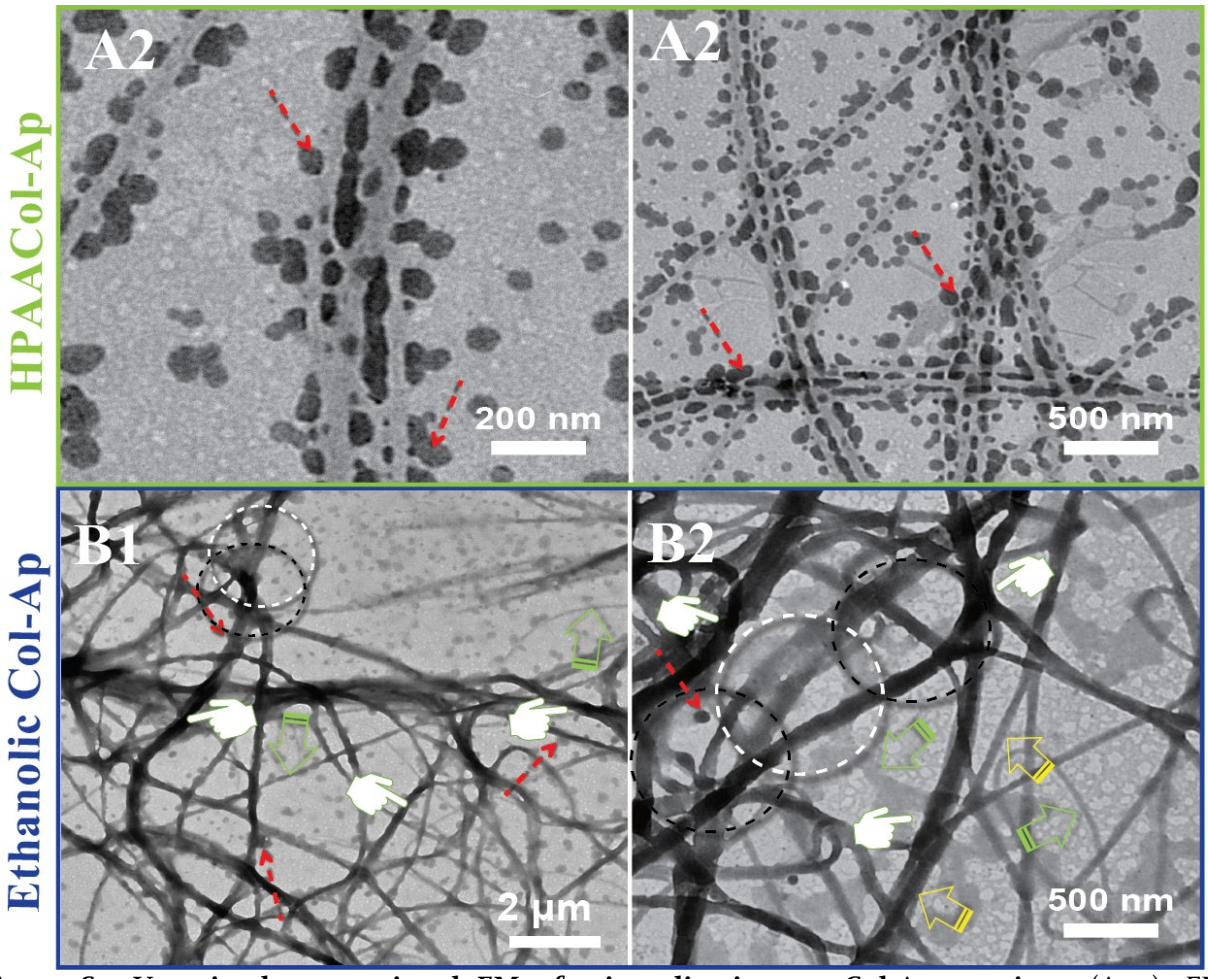

Figure S2. Unstained conventional EM of mineralization on Col-Ap matrices. (**A1-2**). EM tomography shows hierarchical bundled-like arrangements of collagen within the HPAA Col-Ap matrices following 24-48h incubation. PILP globules could be easily discerned (denoted by red dashed arrows). Scale bar: 200nm and 500nm. (**B1-2**). Representative EM image depicts ethanolic-Col-Ap matrices following 24-72 hr incubation. Remnant PILP droplets can be seen adsorbing to the fibers (denoted by red dashed arrows), collagen fibril was already entrenched with intrafibrillar minerals (pointer). Moreover, collagen fibrils after the mineralization reaction shows distinct striated texture that runs parallel to the fibers (indicated by yellow open arrows). At low contrast, silhouettes of a continuous braided-microfibrils and vaguely discerned unmineralized portions of collagen fibrils that were not infiltrated by the prenucleated ACP droplets (denoted by green open arrows). Distinct regions between well organised is partly filled with intrafibrillar minerals and amorphous ACP precursors (black dotted circles), and unmineralized part and poorly organised were partially infiltrated by the fluidic ACP and with randomly oriented acicular nanoplatelets (white dotted circles) of the same fibril (left). Scale bar 1µm and 500nm.

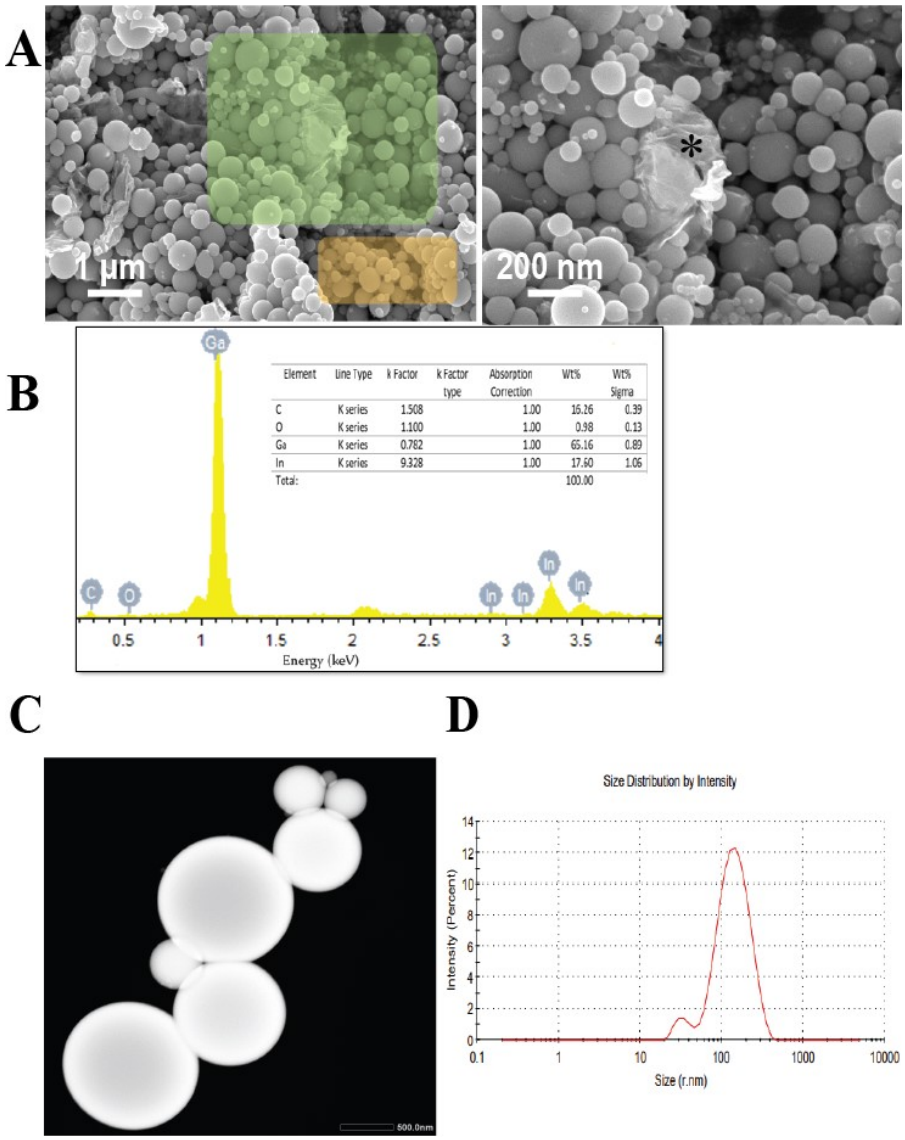

Figure S3. Representative SEM and STEM micrographs of PEG-EGaIn nanocapsules.
A) SEM micrograph of the produced PEG-EGaIn nanocapsules after treatment (with a 5 min sonicating time) with temperature control. Large, free, and continuous sheets are accented (Asterix) to aid visualization. Scale bar 1µm. High magnification SEM micrograph from ((***A***)) revealing large sheets of native oxide (Ga_2O_3) enshrouding multiple particles (Asterix). Scale bars 1µm and 200 nm. **B)** FESEM-EDS spectrum of freshly prepared functionalized- EGaIn nanocapsules showing the presence of both Ga and In signals which compare favorably with an estimated expected Ga (78.98 wt %) and indium (20.59 wt %), concentration of the original material composition. Deconvolution and integration of the EDS peaks provide concentrations of 0.98 wt % O. Although EDS spectrum could not reveal the presence of Sulfhydryl signal even when the peaks are deconvoluted and quantified compared to TEM-EDX of PEG-EGaIn nanocapsules. **C)** HAADF-STEM image showcasing PEG-EGaIn nanocapsules ensemble revealing light-grey core (is assumed to be composed of EGaIn (25 at. % In, 75 at. % Ga). Also. it is evident that the gallium oxide shell is not totally continuous. **D)** DLS corresponding to particle size distribution (the average particle size is ≤200 nm).

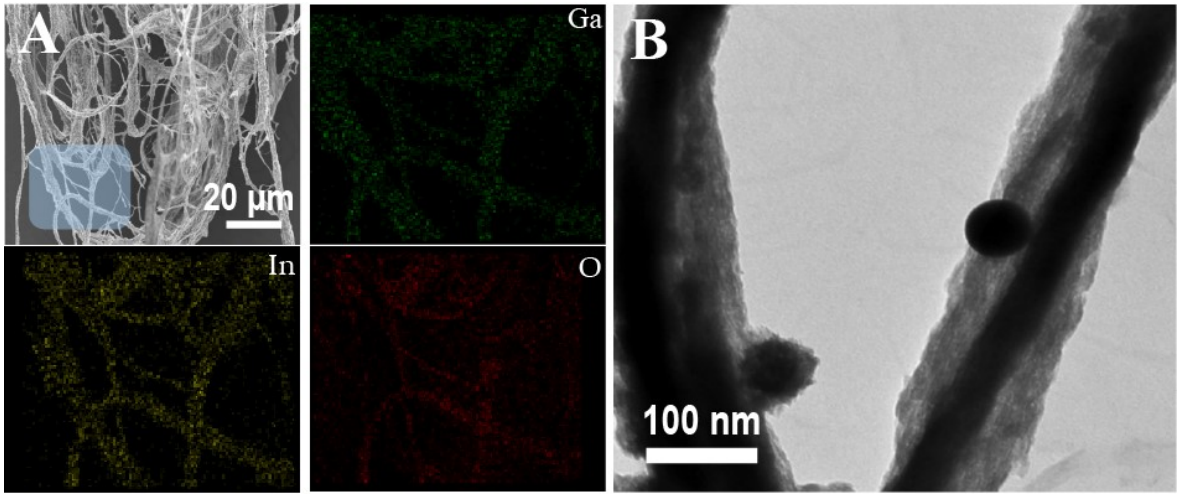

Figure S4. Characterization on the effects of collagen template immobilized with PEG-EGaIn nanocapsules. A) FE-SEM EDX analysis of the fibrillar collagen template co-assembled with PEG-EGaIn nanocapsules which further confirmed a uniform distribution of the PEG-EGaIn metal at the spatial of braided collagen fibrils. Scale bar 20µm. **B)** A representative high-magnification TEM image showcasing intrafibrillar mineralized PEG-EGaIn-Col-Ap fibrils. Note the PEG-EGaIn nanocapsules interacting with collagen fibrils. Scale bar 100 nm.

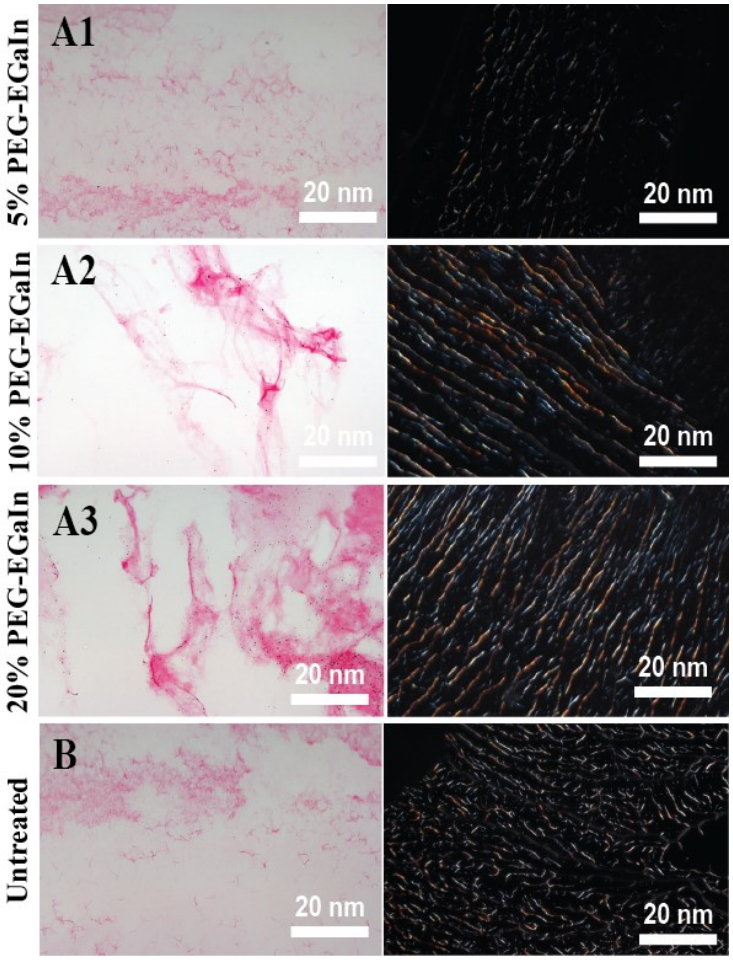

Figure S5. Images captured through polarized optical microscopy depict nano PEG-EGaIn-collagen matrices formed at varying concentrations of PEG-EGaIn nanocapsules *(A1-3)*. The picrosirius red stain, also known as Sirius red stain, is a well-known histochemical technique that highlights collagen networks by utilizing the birefringent properties of collagen matrices. Although the picrosirius red stain alone does not selectively attach to collagen networks, it becomes more specific when combined with polarized light detection. The assembly of collagen in this study suggests that regardless of the conditions, increasing the concentration of ethanol expedites collagen fibril formation. Moreover, collagen matrices remain in solution at various ethanol concentrations. Scale bar representations 20 nm.

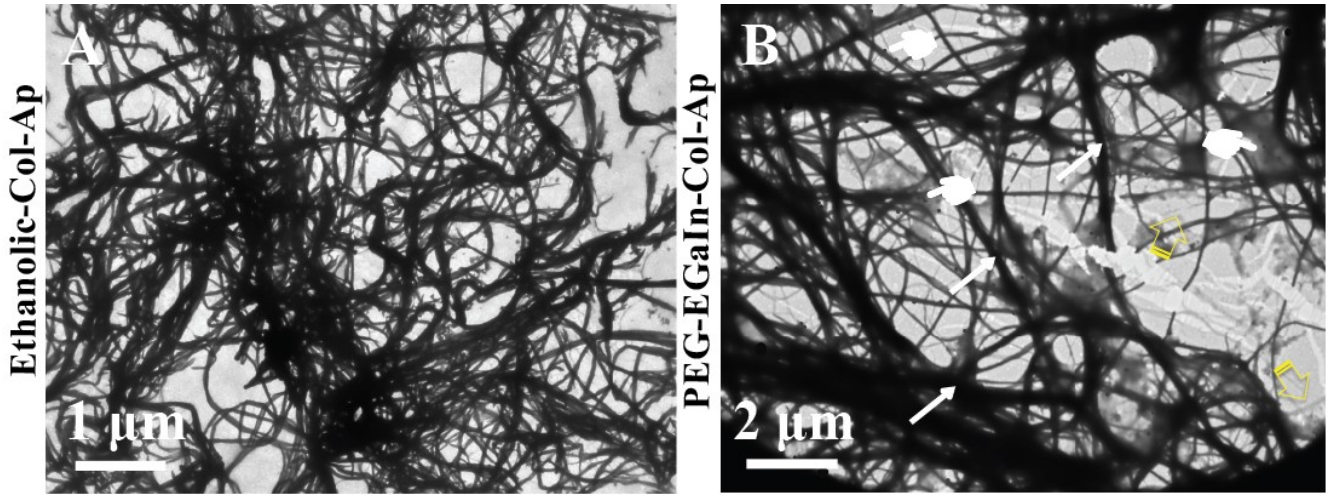

Figure S6. Unstained conventional STEM tomography revealing spatiotemporal effects of intrafibrillar mineralization of both ethanolic-Col-Ap-lamellar and nano PEG-EGaIn-ColAp-lamellar matrices. ***a)*** Representative EM topography showcasing dense hierarchical ribbon-like arrangement of intrafibrillar minerals in the ethanolic-Col-Ap-lamellar mimetic group. Scale bar 1µm. ***b)*** Recorded from an isolated dense region, unstained TEM cross-section of the mineralized nano PEG-EGaIn -Col-Ap-lamellar mimetic construct showcases a heavily intrafibrillar mineralization with hierarchical arrangement of discrete acicular apatite incorporated with PEG-EGaIn nanocapsules that were homogeneously distributed along the braided mineralized collagen microfibrils as denoted by pointer. Scale bar: 2µm.

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