Video Article

Generation of Scalable, Metallic High-Aspect Ratio Nanocomposites in a Biological Liquid Medium

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Abstract

The goal of this protocol is to describe the synthesis of two novel biocomposites with high-aspect ratio structures. The biocomposites consist of copper and cystine, with either copper nanoparticles (CNPs) or copper sulfate contributing the metallic component. Synthesis is carried out in liquid under biological conditions (37 °C) and the self-assembled composites form after 24 hr. Once formed, these composites are highly stable in both liquid media and in a dried form. The composites scale from the nano- to micro- range in length, and from a few microns to 25 nm in diameter. Field emission scanning electron microscopy with energy dispersive X-ray spectroscopy (EDX) demonstrated that sulfur was present in the NP-derived linear structures, while it was absent from the starting CNP material, thus confirming cystine as the source of sulfur in the final nanocomposites. During synthesis of these linear nano- and micro-composites, a diverse range of lengths of structures is formed in the synthesis vessel. Sonication of the liquid mixture after synthesis was demonstrated to assist in controlling average size of the structures by diminishing the average length with increased time of sonication. Since the formed structures are highly stable, do not agglomerate, and are formed in liquid phase, centrifugation may also be used to assist in concentrating and segregating formed composites.

Video Link

The video component of this article can be found at https://www.jove.com/video/52901/

Introduction

Copper is a highly reactive metal that in the biological world is essential in some enzyme functions [1,2], but in higher concentrations is potently toxic including in the nanoparticulate form [3,4]. Concern over copper toxicity has become more relevant as CNPs and other copper-based nanomaterials are utilized, due to the increased surface area/mass for nanostructures. Thus, even a small mass of copper, in nanoparticle form, could cause local toxicity due to its ability to penetrate the cell and be broken down into reactive forms. Some biological species can complex with and chelate metal ions, and even incorporate them into biological structures as has been described in marine mussels [5]. In studying the potential toxic effects of nanomaterials [4], it was discovered that over time, and under biological conditions used for typical cell culturing (37 °C and 5% CO_2), stable copper biocomposites could be formed with a high-aspect ratio (linear) structure.

By a process of elimination, the initial discovery of these linear biocomposites, which occurred in complete cell culture media, was simplified to a defined protocol of essential elements needed for the biocomposites to self-assemble. Self-assembly of two types of highly linear biocomposites was discovered to be possible with two starting metal components: 1) CNPs and 2) copper sulfate, with the common biological component being cystine. Although more complex, so called “urchin” and “nanoflower” type copper-containing structures with nanoscale and microscale features have been previously reported, these were produced under non-biological conditions, such as temperatures of 100 °C or greater [6-8]. To our knowledge, synthesis of individual, linear copper-containing nanostructures that are scalable in liquid phase under biological conditions has not been previously described.

One of the starting materials utilized for synthesis of nanocomposites, namely CNPs, has been reported previously to be very toxic to cells [4]. It has recently been reported that after the nanocomposites are formed, these structures are less toxic on a per mass basis than the starting NPs [9]. Thus, the synthesis described here may be derived from a biological and biochemical reaction that has utility in stabilizing reactive copper species, both in the sense of transforming the NP form into larger structures and in producing composites less toxic to cells.

In contrast to many other nanomaterial forms which are known to aggregate or clump upon interaction with biological liquid media [10,11], once formed, the highly linear composites described here avoid aggregation, possibly due to a redistribution of charge which has been previously reported [9]. As detailed in the current work, this avoidance of aggregation is convenient for the purposes of working with the structures once

formed for at least 3 reasons: 1) composite structures once formed may be concentrated using centrifugation and then easily dispersed again using vortex mixing; 2) formed structures can be decreased in average size by sonication for different periods of time; and 3) the formed linear structures may provide an additional tool for avoiding the recently described “coffee ring effect” [12] and thus provide a dopant for creating more evenly distributed coatings of materials, especially those containing spherical particulates.

Protocol

1. Planning of Experiments

1. Determine the volume of copper nanocomposites needed for synthesis. On that basis, choose a number of small volume flasks (25 cm^2), or larger flasks as indicated below in preparation of materials.
2. For this synthesis, use a 37 °C incubator with 5% CO_2 and at least 40% humidity. Ensure that such an incubator is available and that it will not be repeatedly disturbed over the period of synthesis (approximately 24 hr).
   CAUTION: Repeated opening and closing of the incubator will certainly cause temperature fluctuations which may result in altered synthesis of the nanocomposite structures.

2. Preparation of Materials

1. Prepare all materials fresh before the start of an experiment, by adding solid materials to solvents right before synthesis is to begin. Keeping stock solutions of cystine and copper starting materials in liquid for long times before the experiment is not recommended and may lead to variable results. Once opened from the vendor, keep starting materials dry by wrapping the top of the container with Parafilm.
   Note: The following protocol is used as an example for reaction in a 25 cm^2 cell culture flask using 7 µl of cystine, 6,643 µl of sterile water, and 350 µl of CNPs.
2. Prepare a 2 mg/ml solution of copper nanoparticles by weighing out at least 2 mg of CNPs. Wear disposable gloves during this step to prevent possible contact of CNPs with skin. Place the nanoparticles in an empty sterile 16 ml glass vial.
   1. To the vial containing CNPs, add sterile deionized water in the appropriate volume to make a 2 mg/ml solution and vortex the solution for 20 sec to provide dispersion of the nanoparticles before synthesis starts (at least 1 ml total volume is recommended). Do not fill the vial more than half way with water as this will inhibit mixing by vortexing. CNPs will quickly settle to the bottom of the vial and will appear dark in color (grey to black).
   2. Sonicate the CNP solution for 17 min at RT to provide maximal dispersion of CNPs before start of synthesis. Periodically check to make sure that CNPs are mixing due to sonication. After a successful sonication, CNPs remain suspended in solution for at least 30 min and the solution will be dark in color.
3. Weigh out sufficient mass of cystine to make a 72.9 mg/ml solution for the synthesis. Since cystine is not directly soluble in water, place the weighed cystine in an antistatic weighing vessel.
   1. To the weighing vessel containing cystine, add sufficient volume of sterile, 1 M NaOH, so that the cystine completely dissolves. For example, dissolve 7.29 mg of cystine completely in 100 µl of 1 M NaOH, to make a 72.9 mg/ml solution.
   2. To maintain sterile conditions, carry out this step in a sterile flow tissue culture hood.
      CAUTION: NaOH at 1 M concentration is caustic, so wear disposable gloves during this step to prevent contact of concentrated NaOH with skin
4. Working in a sterile tissue culture hood, add 7 µl of cystine with 6,643 µl of sterile water to the sterile synthesis flask first, and let incubate for 30 min in the incubator at 37 °C with the flask cap vented (loose) to provide effective mixing. Resuspend the 2 mg/ml CNP solution by vortexing for 30 sec, since CNPs will have settled after the sonication step.
   1. Add sufficient CNP solution to the synthesis flask (using sterile technique) to maintain the following component ratios: combine 1 parts cystine, 50 parts CNPs, and 949 parts sterile water in a 25 cm^2 cell culture flask to start the synthesis. For example, for a 7 ml synthesis volume, combine 7 µl of cystine stock solution, 350 µl of CNPs, and 6,643 µl of sterile water. Replace the cap on the flask and tighten so that it is secure.
   2. After combining all components for the synthesis, gently mix in the flask by swirling 4-5 times. Place flask in the CO_2 incubator and vent the flask by loosening the cap so that there will be gas exchange in and out of the flask during synthesis.
5. Allow synthesis to run in the incubator for approximately 24 hr. During synthesis, one can observe, with microscopy and by eye, formation of highly linear composites.
   Note: The process of formation of the structures may happen suddenly in the sense that structures are initially hard to detect, then appearance proceeds quickly to an increasing density. Formation may therefore occur before 24 hr. The process can also be observed by eye once structures become larger and their density increases. While generation of the structures can be observed over time under the microscope and by eye at later time points, continuously interrupting the synthesis conditions and temperature will lead to poor synthesis results.
6. Terminate synthesis of biocomposites by tightly capping the synthesis flask and storing the vessel in a refrigerator (4 °C). Structures, once generated, remain stable in this form for at least a year. Label the flask with synthesis conditions, including components utilized, date of the synthesis, and incubation time of the synthesis before termination.

3. Synthesis Using Copper Sulfate

1. Carry out self-assembly synthesis by replacing CNPs with copper sulfate salt. Using sterile technique, dissolve at least 2 mg of copper sulfate in sufficient volume of sterile deionized water to make a 2 mg/ml solution. The copper sulfate crystals easily go into solution at this concentration, but vortex the vial if needed, and inspect by eye to ensure all crystals are dissolved.

2. After preparation of the copper sulfate, carry out synthesis as described previously, but replacing CNPs with the copper sulfate.
   Note: Self-assembled nanocomposites using copper sulfate as a starting material were found to be much more consistent in final shape than for structures synthesized from CNPs.
3. Terminate the synthesis of copper sulfate biocomposites as for CNP composites (step 2.6) and store them long-term at 4 °C.

4. Characterization and Handling of Biocomposites Post-synthesis

1. Characterize biocomposites derived from CNPs and from copper sulfate by white light microscopy[9] and by electron microscopy[9].
   1. For characterization and inspection of biocomposites post-synthesis by white light microscopy, use an inverted microscope as composites will settle to the bottom surface of the flask within a few minutes of laying the flask flat, and can then be brought into focus. Use the bright field setting on the microscope to maximize contrast between biocomposites and the liquid medium. Composites derived from CNPs and copper sulfate will both appear clear to opaque in color, but unreacted CNP aggregates will appear very dark in color.
      1. Use a digital camera connected to the microscope to capture images of the composites. A range of lengths for the individual structures will be observed.
   2. For characterization and inspection of biocomposites post-synthesis and after storage at 4 °C, allow flasks to come to RT for at least 15 min as flasks will form condensation initially upon removal from refrigerator, which will obscure effective focusing while carrying out microscopy imaging. After allowing equilibration to RT, wipe the top and bottom surfaces of the flask with a clean paper towel to maximize microscopy imaging quality.
   3. When working with or imaging composites that have been stored long-term, vortex the flask for 30 sec to dissociate clumps of composites that form while in the refrigerator. After vortexing, inspect the structures with an inverted microscope to ensure that aggregates have dissociated, and repeat vortexing as necessary.
   4. Use inverted white light microscopy to assess the efficacy of the synthesis for a given experiment using CNPs. For example, document the presence or absence of unreacted CNPs in synthesis flasks used for CNP-derived biocomposites from flasks with different parameters such as time of synthesis.
      Note: Individual CNPs are too small to observe with a light microscope, but unreacted CNP aggregates will appear as round-shape and dark objects, in contrast to the successfully synthesized CNP-composites which will have a high-aspect ratio, linear form, and will have a range of different lengths. Avoid carrying out synthesis for too long of a period of time before termination, as this will result in highly branched “urchin” type structures, which are difficult to disperse into individual structures once formed.
   5. Use inverted white light microscopy to assess the efficacy of the synthesis for a given experiment using copper sulfate. Since copper sulfate goes fully into solution using this protocol, the solution will appear less dark than the solution from synthesis using CNPs. Document the size and extent of copper sulfate composites by comparing flasks with different synthesis conditions such as time of synthesis before termination.
      Note: Successfully synthesized composites will show a range of different lengths. Avoid carrying out synthesis for too long of a period of time before termination, as this will result in highly branched aggregates of composites, some of which will be “urchin-like” in structure, and which are difficult to disperse into individual structures once formed.

2. To concentrate biocomposites post-synthesis, centrifuge solutions of composites in a centrifuge tube. Add 6 ml of either CNP-derived structures or copper sulfate-derived structures to a 15 ml centrifuge tube. Centrifuge for 10 min at 500 x g at RT to form a pellet. For smaller volumes, add 500 µl of structures in solution to 0.6 ml sized tubes. Centrifuge at 2,000 x g at RT for at least 10 min to form a pellet.
   1. After centrifuging for sufficient time (at least 10 min for microfuges), save the observable pellet at the bottom of the tube where the structures are concentrated by carefully removing the supernatant liquid above the pellet. Biocomposite structures derived from copper sulfate appear blue in color and structures derived from CNPs are darker (grey to black).
   2. Add more composites to this tube and repeat the process in the same tube to concentrate structures if desired. To disperse the concentrated pellets, add the desired volume of solution to the tube, and vortex for 10-30 sec.

3. Sonicate structures once formed, to move the average population size (lengths) of the structures to lower values. Place structures in sterile deionized water and sonicate for at least 10 min. Using this process, over time, structures become fragmented and smaller in average length (see **Figure 6** of the text). Document changes in composite sizes with different sonication times using an inverted white light microscope and digital camera.

Representative Results

Figure 1 shows a flow-chart schematic of the synthesis steps to form the linear biocomposites described in this work. CNPs or copper sulfate as starting materials are combined with sterile water to form a 2 mg/ml solution, this solution is mixed and sonicated to provide an even mixture, and this copper solution is then mixed in the following ratio for synthesis: 949 parts sterile water: 50 parts copper mixture: 1 part cystine stock solution. The actual volumes may be increased or decreased according to these ratios to scale up or scale down the final synthesis yield. After incubation for at least 2 hr as indicated, linear biocomposite structures are formed which over time can be observed under normal white light microscopy or by eye as the liquid solution changes in appearance.

Figure 2 shows a representative result from the initial discovery of these linear structures in complete cell culture media over a period of 82 hr. Our laboratory was carrying out evaluation of the potential toxicity of different nanomaterials, including CNPs on normal cells and cancer cells, and the cells shown in **Figure 2** are a fast-growing brain tumor cell line from ATCC (CRL-2020). A key supplement to the complete media used for these cells is cystine, which turned out to be the essential component to the discovery of why these linear structures were forming in the cultures (see below and discussion section). From an initial even dispersion of CNPs which quickly aggregate into microstructures (**Figures 2A** and **2B**), over time the smaller particles are cleared and larger aggregates are formed (**Figures 2C** and **2D**). Finally, larger aggregates with fine, linear structures appear in the same wells (**Figures 2E-H**), forming the “urchin” type structures previously reported in the literature using non-

biological methods [6]. Comparison of the final two time points, at 69 and 82 hr (**Figures 2G** and **2H**, respectively), shows that development of the large urchin type structures remains quite stable, as indicated by imaging the same exact field.

To explain why these urchin-like structures under these conditions in the cell cultures were observed, we started eliminating media components to determine if the essential elements could be isolated. We discovered that a key component and supplement to the cell culture media was cystine, which by the process of elimination was ultimately identified as an essential component for the self-assembly process. By simplifying the synthesis components (see **Figure 1**), we could ultimately form high-aspect ratio (linear) structures in liquid, that could be shown over time to transform from nanoparticle form to linear form, without the need of cells, or any of the other cell culture components (**Figures 3A-C**).

Figure 4 shows characterization of the discovered novel structures using electron microscopy, including a transmission electron microscopy (TEM) image capturing nanoparticle starting material and forming linear nanostructures (**Figure 4A**). Representative scanning electron micrograph (SEM) images are shown for starting material CNPs, linear structures formed from the NPs, and linear structures formed from copper sulfate starting material (**Figures 4B-F**, respectively).

To verify that the biocomposites contained cystine or cystine-derived material as an essential biological component of the composites, the formed structures were analyzed using EDX with SEM microscopy. Representative screen shots from analyzed materials are shown in **Figure 5**. Importantly, when comparing CNPs and biocomposites from the CNPs, a prominent sulfur peak appears (**Figure 5B**), which is not present in the CNP starting material (**Figure 5A**). For the biocomposites using copper sulfate as starting material (**Figure 5C**), carbon and nitrogen peaks appear (**Figure 5D**), which is consistent with the presence of cystine for this biocomposite.

At this time, a method for controlling length and size of composites during synthesis has not been identified. However, to investigate if average size of structures could be controlled after synthesis, linear biocomposites were sonicated for different periods of time as shown in **Figure 6**. With increased time of sonication, it was shown that the average size of the linear biocomposites decreased, as shown by bright field microscopy (**Figures 6A-D**). As a method for concentrating and segregating formed composites, centrifugation can be used, and as shown in **Figures 6E-G**, a visible pellet can be developed depending upon the volumes and centrifugation forces used.

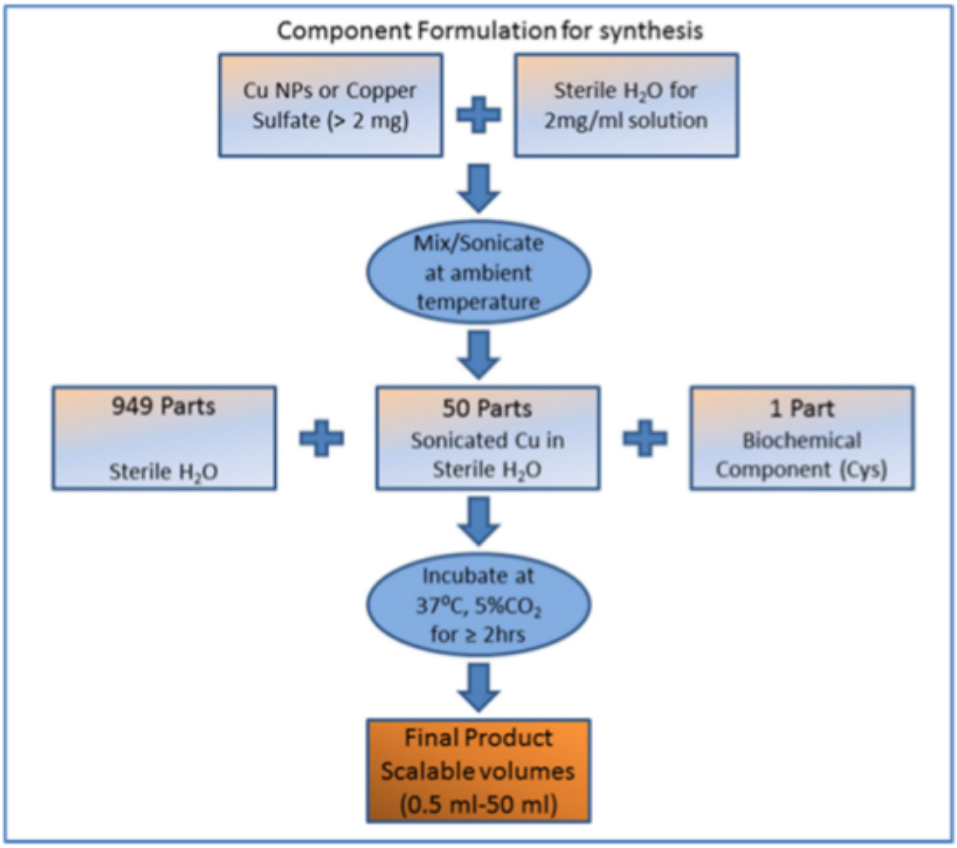

Figure 1. Representative flow chart of synthesis design. Cu NPs = copper nanoparticles; Cys = cystine. Please click here to view a larger version of this figure.

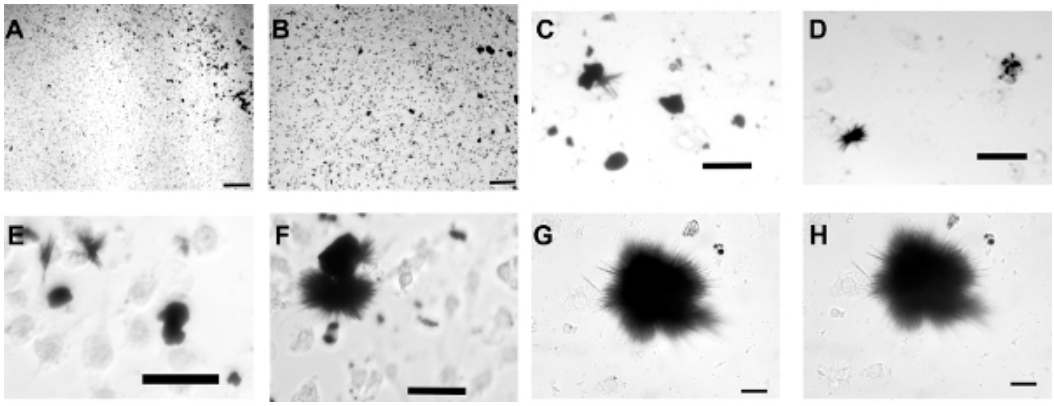

Figure 2. Representative formation of copper biocomposite structures in brain tumor cell cultures with complete cell culture media. A representative experiment tracked over a total of 82 hr is shown in cell culture containing brain tumor cells and CNPs (50 µg/ml). Panels A and B show the culture wells at time 0, after CNPs have settled to the bottom of the well. Panels C-H show subsequent time points at 17, 24, 36, 49, 69, and 82 hr, respectively. Panels G and H represent the same field at 69 and 82 hr. All images were obtained using brightfield microscopy to enhance contrast of copper material. Scale bars = 100 microns for A+B, 50 microns for C-E, and 25 microns for F+G. Please click here to view a larger version of this figure.

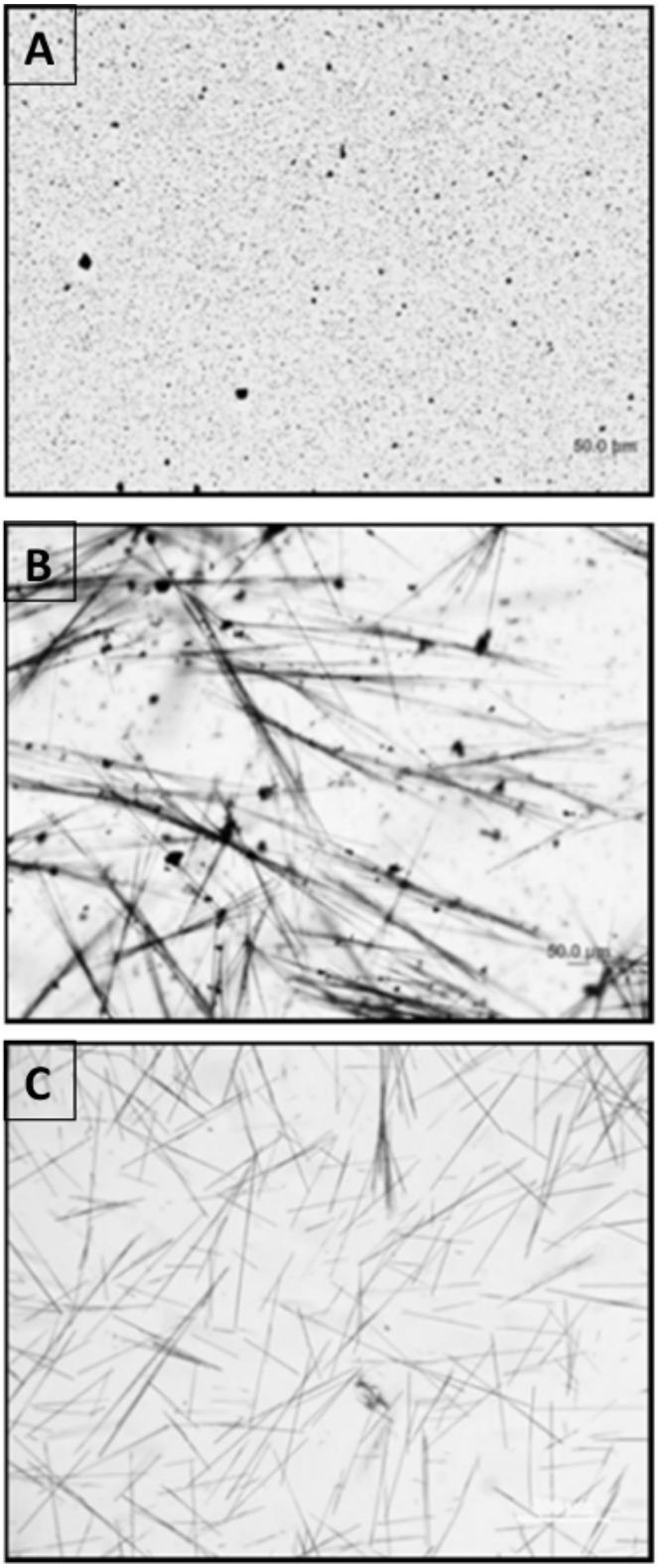

Figure 3. **Transformation of CNPs to linear biocomposites.** CNPs were combined with cystine and water as indicated in **Figure 1** and in protocol section with a total volume of 7 ml. Panel A shows the synthesis vessel at time 0, panel B shows 3 hr, and panel C shows 6 hr. Images were obtained using brightfield microscopy with scale bar indicated (50 microns). Please click here to view a larger version of this figure.

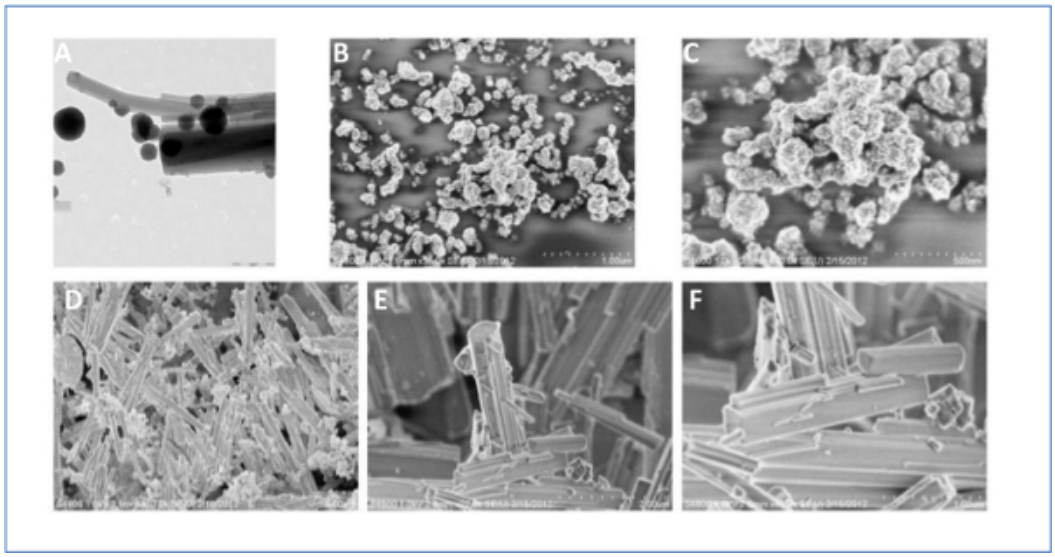

Figure 4. Electron microscopy characterization of synthesized biocomposites. Panel (**A**): TEM of CNP starting material (round) with the forming linear composites. Panels (**B**) and (**C**) show characterization of the starting CNPs using SEM. Panel (**C**) is a zoomed image of (**B**). Panel (**D**) shows SEM of composites formed from CNPs and cystine. Panels (**E**) and (**F**) show SEMs of the copper sulfate biocomposites. Panel (**F**) is a zoomed image of (**E**). Scale bars are indicated in all images and = 200 nm in (**A**), 1 micron in (**B**), 500 nm in (**C**), 5 microns in (**D**), 2 microns in (**E**), and 1 micron in (**F**). Please click here to view a larger version of this figure.

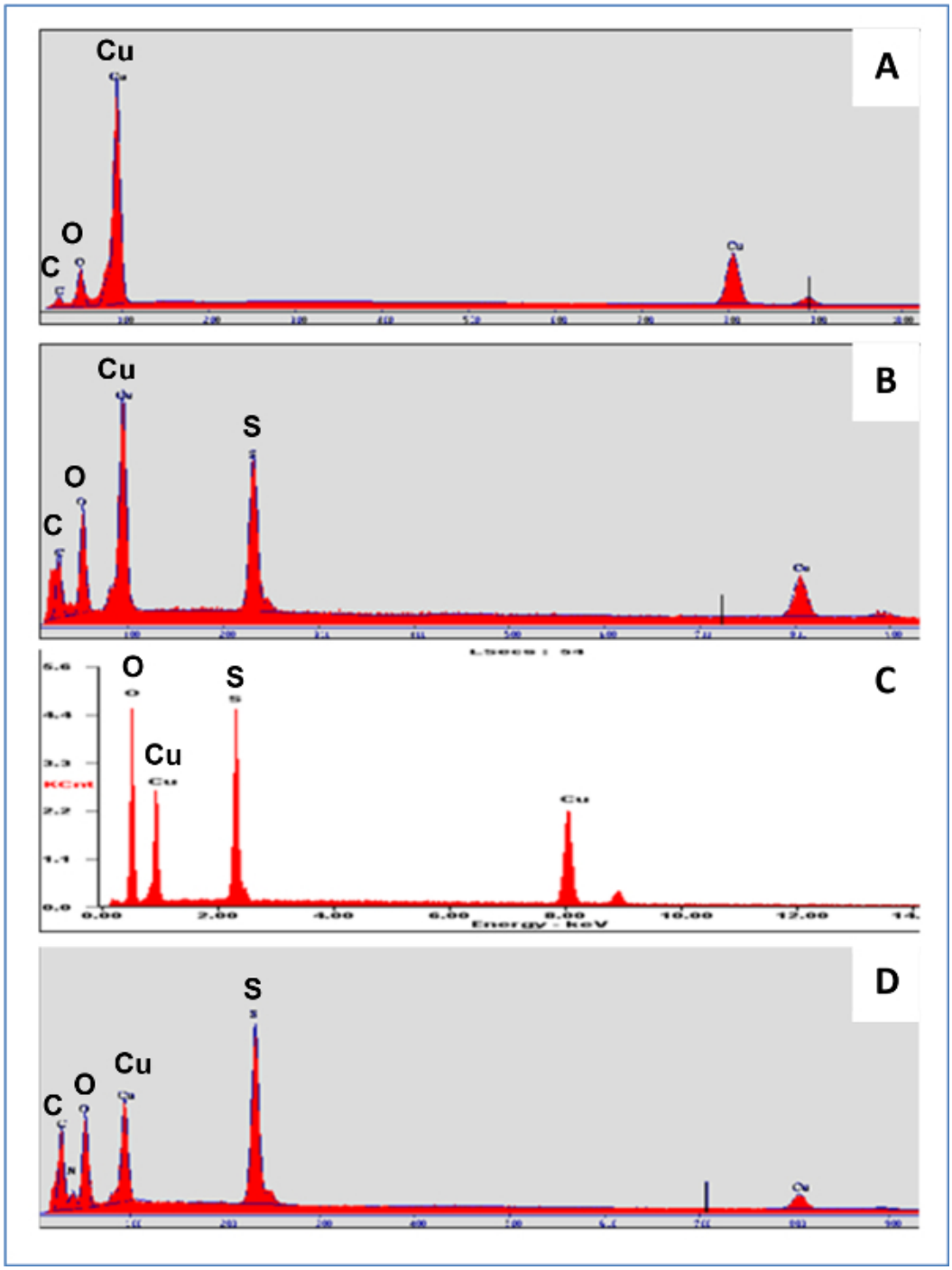

Figure 5. **EDX (SEM) analysis of starting materials and synthesized linear composites.** Screen snapshots of SEM scanned samples using EDX analysis for elemental content. Panel (**A**) = starting CNPs; Panel (**B**) = biocomposites from CNPs and cystine; Panel (**C**) = copper sulfate starting material, and Panel (**D**) = composites from copper sulfate and cystine. For peak labeling, C = carbon, O = oxygen, Cu = copper, S = sulfur, and N = nitrogen. Larger labels for elemental identity have been placed above key peaks for ease of viewing. Please click here to view a larger version of this figure.

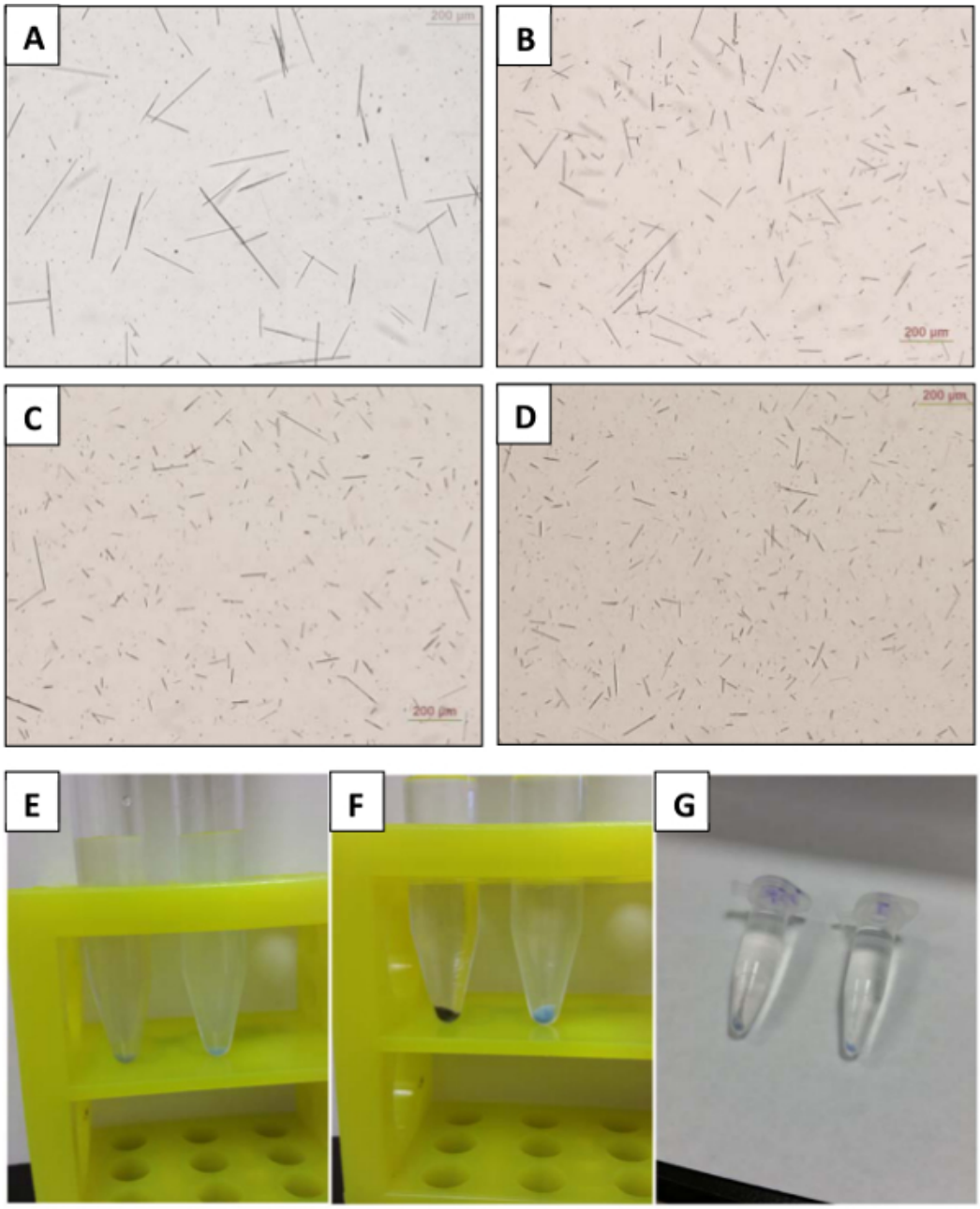

Figure 6. **Modification of linear composite size and concentration post synthesis.** Linear structures synthesized from copper sulfate were sonicated for 0, 15, 30, or 60 min, respectively, as shown in panels (**A-D**). Images were obtained using brightfield microscopy with scale bar of 200 microns indicated in all images. Panels (**E-G**), concentration of biocomposites using centrifugation: 6 ml of linear structures derived from CNPs (**E**, left) and copper sulfate (**E**, right) are shown settling under gravity after 10 min. With 10 min of centrifugation at 500 x g, a compacted pellet is formed (panel **F**). A smaller volume (500 µl) of the same material was concentrated as shown in (panel **G**) (CNP-derived structures are shown in the left tube and copper sulfate-derived structures in the right tube). Please click here to view a larger version of this figure.

Discussion

While evaluating potential toxic effects of nanomaterials including CNPs, it was observed that over the long-term, CNPs were transformed from an initially more dispersed particulate distribution to a larger, aggregated form (**Figure 2**). In some cases, these highly aggregated formations which were produced in the cell culture dish, under biological conditions, formed highly linear projections from the central aggregate, reminiscent of previously described copper- containing "urchins" [6]. It should be pointed out that under the conditions shown here, the concentration of CNPs added to the cells was sub-maximal, thus not killing all of the cells in culture (see **Figure 2**). From these initial observations, experiments were then continued by eliminating successively more components of the cell culture conditions (including ultimately the cells themselves) to find the remaining key components needed for synthesis of linear copper biocomposites.

We discovered that cystine, a supplemented component of the original cell cultures, when combined with CNPs under the correct biological conditions, could result in transformation of the nanoparticulate form into a highly linear biocomposite that contained both the metal and biochemical components as indicated by EDX analysis by scanning electron microscopy of the prepared samples (**Figure 5**). Thus, sulfur, which is not present in the starting CNP material, shows a prominent peak in the synthesized biocomposites, indicating that both copper and components of the biochemical material cystine are essential for the formed linear structures.

It was further shown that by using similar synthesis conditions, but replacing CNPs with copper sulfate, highly linear biocomposites could also be formed. In fact, synthesis using copper sulfate and cystine tended to result in "cleaner" end products, in that no unreacted CNPs were ever present, since copper sulfate is fully soluble in water, which was used as a solvent in all of the syntheses reported here. Further, copper-sulfate biocomposites distinguished themselves from CNP-derived composites in retaining a blue color which was apparent upon centrifugation of the material.

Other groups have previously studied copper-cystine complexes and defined some key chemical properties. For example, Kahler *et al.* demonstrated the formation of copper-cystine complexes in the form of fine fibers, which were evident upon drying but unstable in solution [13]. In other examples, complexation studies with L-cystine and different metal cations confirms copper and cystine complex formation in an aqueous medium [14] and formed complexes may be mononuclear or polynuclear, with the sulfur from cystine contributing to the complex formation [15]. A number of studies have reported interactions between cystine and copper in biological systems. For example, at physiological pH, copper (II) ions coordinate with histidine and cystine in simulated plasma to form complexes [16], and sulfur containing amino acids were shown to be protective against copper toxicity in chicks [17]. These findings thus support the essential role of cystine in complexing with copper starting material in our synthesis methods for forming linear biocomposites.

At this time a synthesis strategy has not yet been identified that can directly control the length of individual linear biocomposites shown here. However, the critical steps identified in the described synthesis include: 1) good dispersion by sonication of starting materials in the case of synthesis incorporating CNPs; 2) use of freshly prepared CNPs, copper sulfate, and cystine for effective synthesis of composites; 3) allowing synthesis in the flask to remain undisturbed in the incubator for at least 6 hours; and 4) avoiding "over-reaction" conditions in which branched "urchin"-type, aggregated composites form.

After synthesis is completed, it was shown that sonication of the structures can be utilized effectively to decrease the average size (length) of the structures. Sonicating to make smaller structures may aid in applications such as cell uptake or other biocomposite-cellular interactions. As has been previously reported, charge stabilization of the CNPs during this synthesis by combining with cystine altered the measured zeta potential from positive to a less charged (negative) form [9]. This change in charge may help explain why the formed composites show very little aggregation in the dried form or liquid media, which makes handling of the structures much more convenient.

Since the reported synthesis of these CNP-derived and copper sulfate-derived structures is carried out in liquid media, it is anticipated that the process may be highly scalable, meaning that with the correct ratio of components and synthesis conditions, the milliliter synthesis recipe used here could be scaled up or down to include many hundreds of milliliters or more, and would thus be expected to yield more final product, as well. Due to the metal (copper) component of these biocomposites, it is quite straightforward to concentrate synthesized product by centrifugation (**Figure 6**). Biocomposites formed from CNP starting material retain a darker color once concentrated into a pellet, possibly due to unreacted copper oxide nanoparticles (**Figure 6**). In comparison, copper sulfate biocomposites when concentrated into a pellet have a blue color, consistent with properties of copper sulfate with various levels of hydration [18].

The novel synthesis reported here is also scalable in the sense that the self-assembled linear structures scale from the nano-scale to the micro-scale as shown using electron microscopy (**Figure 4**) and traditional white light microscopy (**Figures 3** and **6**). It is interesting to note that recently, engineered coiled-coil protein microfibers were reported that could incorporate curcumin which allowed for the formed fibers to be observed under fluorescence illumination [19]. Similar strategies may be possible to incorporate spacers or tagging agents into the linear composites reported here to provide production of larger structures and/or enhancements for imaging.

Disclosures

Authors have nothing to disclose.

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