Development of Nanoscale Hybrids from Ionic Liquid–Peptide Amphiphile Assemblies as New Functional Materials

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ABSTRACT: Over the years, ionic liquids (ILs) have gained tremendous importance because of their unique properties and plethora of applications. In this work, we have developed a new nanoscale hybrid gel consisting of 1-ethyl-3-methylimidazolium dimethyl phosphate, [C$_{n}$mim][dmp], and self-assembled peptide nanoassemblies. The peptide nanoassemblies were formed by self-assembly of a newly synthesized peptide bolaamphiphile bis(N-$\alpha$-amido-threonine) 1,7 heptane dicarboxylate (ThrC7). Upon mild heating and sonication of the IL and ThrC7 nanoassemblies, ThrC7-IL nanocomposites were formed. We explored the formation of nanohybrids by varying the ratio of IL to ThrC7 assemblies. While at lower IL ratios, a gelatinous matrix was formed, at higher IL ratios, highly ordered multilayered structures were observed by atomic force microscopy (AFM) imaging. The interactions between the ThrC7 nanofibers and [C$_{n}$mim][dmp] IL were probed by Fourier transform infrared spectroscopy, transmission electron microscopy, and AFM imaging. Differential scanning calorimetry and thermogravimetric analysis showed that the nanohybrids illustrated distinct thermal phase changes due to changes in hydrogen bonding interactions and unfolding of the nanoassemblies. The viscoelastic behavior of the nanohybrids indicated that the materials displayed higher storage modulus upon incorporation of the ThrC7 nanoassemblies when compared to the IL. Furthermore, the nanohybrids were found to adhere to and promote proliferation of human dermal fibroblasts, while cytotoxicity was observed toward MCF-7 breast cancer cells. Thus, for the first time, we have developed peptide-based nanohybrids with an imidazolium-based IL with unique structural properties that may open new avenues for exploring potential biological applications.

INTRODUCTION

Ionic liquids (ILs) are organic salts with melting points below 100 °C and may remain in the liquid state at room temperature. Over the years, ILs have been found to have a wide range of applications such as in the processing of spent nuclear fuels, separations, stabilizers, green catalysis, batteries, and fuel cells and for the dissolution of biopolymers such as cellulose and chitin. Through careful selection of the anion and cation, physical properties such as conductivity, thermal stability, viscosity, density, hydrophilicity, or hydrophobicity can be manipulated. Over the past decade, several studies have been carried out in order to develop hybrid materials encompassing ILs as new functional materials with enhanced properties. In particular, taking advantage of their unique properties, several ILs and their composites are being explored for biological applications. For example, stable nanoemulsions containing mixtures of ILs 1-hexyl-3-methylimidazolium chloride [C$_{n}$mim][Cl] and 1-butyl-3-methylimidazolium hexafluorophosphate [C$_{n}$mim]-[PF$_{6}$] with surfactants have been synthesized for encapsulation of the drug piroxicam. Salehi and co-workers synthesized nanoscale drug delivery vehicles consisting of IL-chitosan bound poly(ethylene glycol) (PEG) for targeted multidrug delivery of chemotherapeutic drugs to MCF-7 breast cancer cells. Furthermore, several studies have shown that ILs can significantly improve the stability of proteins, DNA, and enzymes.

In particular, ILs composed of imidazolium cations ([C$_{n}$mim], $n=1–6$) have garnered attention not only because of their plethora of applications in biphasic catalysis and...
separation sciences but also because of their ability to interact with proteins due to the structural presence of the imidazole ring system which can form coordination complexes and display CH−π stacking interactions with histidine and aromatic amino acids of proteins. Imidazolium-based ILs have been found to stabilize proteins such as lysozyme and human serum albumin. Other reports have shown that imidazolium ILs can interact with amyloid aggregates and cause dissolution of those aggregates and thus may have potential applications against neurodegenerative diseases. Studies have also indicated that the hydrophilic imidazolium-based ILs are environmentally benign and relatively biocompatible. For example, it has been reported that 1,3-dimethylimidazolium dimethyl phosphate [C1mim][dmp] had no effect on enzymatic activity of cellulase and cell growth of {Rhodococcus opacus} bacteria. It has also been observed that growth of {Saccharomyces cerevisiae} was not affected in the presence of 1-ethyl-3-methylimidazolium diethylphosphate [C2mim][dep] or 1-ethyl-3-methylimidazolium acetate [C2mim][OAc]. Furthermore, in a recent study, dicatonic imidazolium-based ILs paired with amino acid anions such as phenylalanine or methionine showed high cytocompatibility with host cells and therefore have been touted for use as coatings for titanium dental implants. In another study, hemocyanin-[C2mim][amino acid] complexes showed selectivity toward mammalian cells and exhibited enhanced cytotoxicity toward MCF-7 breast cancer cells and at the same time showed low cytotoxicity toward 3T3 cells. However, some reports have also indicated toxic effects of 1-butyl-3-methylimidazolium hexafluoro phosphate [C2mim]-[PF6], 1-ethyl-3-methylimidazolium, 1-butyl-3-methylimidazolium, 1-hexyl-3-methylimidazolium chloride, and 1-hexadecyl-3-methyl-imidazolium chlorides toward certain bacteria, plants, and mammalian cells. The difference in toxicities has been attributed to the lipophilicity of the ILs as well as the type of the anion involved. In general, studies have shown that increasing lipophilicity of ILs may result in higher toxicity.

To further explore and mitigate toxicity effects of ILs, researchers have also examined interactions of imidazolium-based ILs with amphiphilic lipids, surfactants, and natural biomolecules. For instance, binding studies between imidazolium-based ILs containing 1-butyl-3-methylimidazolium ([C2mim]) cations with 1-palmityl-2-oleoylphosphatidylcholine lipid bilayers revealed extensive interactions because of insertion of the imidazolium cations into lipid bilayers confirming that hydrophobicity of imidazolium-based ILs plays an important role in binding and toxicity observed toward some biological systems. Other studies have shown that amphiphilic imidazolium ILs containing ω-undecenyl chains form supramolecular complexes with cucurbit[n]urils in water through self-assembly. In a separate study, Smirnova and co-workers have shown that imidazolium-based ILs have the ability to promote self-assembly of surfactants because of hydrophobic interactions.

While several studies have been carried out to examine the interactions of proteins, lipids, and amphiphilic surfactants with imidazolium-based ILs, to our knowledge, relatively few studies have been carried out to explore the interactions between self-assembled peptide bolaamphiphiles and imidazolium-based ILs. Self-assembled peptide bolaamphiphiles are known for their versatility, biocompatibility, and their utility in various biological applications such as tissue engineering and drug delivery. To investigate the interactions of imidazole-based ILs with self-assembled peptide bolaamphiphiles and examine the impact of peptide bolaamphiphile-imidazolium-based IL hybrid nanocomposites on mammalian cells, in this study, we have designed and synthesized a new nanoscale hybrid gel by combining the IL 1-ethyl-3-methylimidazolium dimethyl phosphate, [C2mim][dmp], with self-assembled peptide bolaamphiphile bis(N-α-amidethreonine) 1,7 heptane dicarboxylate bis([ThrC7]). We specifically chose [C2mim][dmp] because of its relatively short ethyl group attached to the imidazolium cation and the presence of dmp anion which enhances its hydrophilicity, ability to form complexes and therefore may be relatively less toxic to mammalian cells.

We explored the interactions and the formation of the hybrid materials at three different ratios (1:1, 1:2, and 2:1 IL to ThrC7 nanoassemblies) and studied the rheological properties as well as performed atomic force microscopy (AFM) and transmission electron microscopy (TEM) imaging to confirm the formation of the hybrids. The interactions between the ThrC7 and [C2mim][dmp] was also probed by Fourier transform infrared (FTIR) spectroscopy. We then examined if the incorporation of ThrC7 into the [C2mim][dmp] IL had an impact in lowering toxicity of IL toward mammalian cells. Upon testing the cytotoxicity with adult human dermal fibroblasts, our results indicated that the cells continued to proliferate over time, and the materials were relatively biocompatible. For the MCF-7 breast cancer cells, relative cytotoxicity was observed indicating that the nanohybrids may display selectivity toward specific lines. Thus, in this work, we have developed a new class of nanohybrid materials comprised of self-assembled peptide nanoassemblies dispersed in [C2mim][dmp] IL that may have promising potential in biological applications.

## RESULTS AND DISCUSSION

Peptide amphiphiles are facile bioorganic materials that can efficiently form nano and microscale assemblies depending upon growth conditions. Based on the functional groups, the formation of such assemblies is promoted by various inter- and intramolecular interactions such as hydrogen bonding, hydrophobic−hydrophilic interactions, π−π stacking interactions or through cation−π interactions, van der Waals forces, and electrostatic interactions between the peptide moieties. The synthesized bolaamphiphile, HOOC−Thr−NH−CO−(CH2)n−NH−CO−Thr−COOH contains two carboxylic acid groups and two −OH groups (side chains of Thr) as hydrophilic head groups between the alkyl spacer tail consisting of a hydrophobic seven carbon chain making it amphiphilic. The presence of the carboxyl groups renders it pH sensitive. In the previous work, it has been shown that in general at pH < 5, the terminal carboxyl groups of peptide bolaamphiphiles remain largely protonated and hence can participate in hydrogen bonding interactions. The hydroxyl groups of threonine can also participate in hydrogen bonding interactions.

After self-assembly at pH 4, as shown in Figure 1, we observed the formation of short, thick oval-shaped nanostructures attached together in the size range of 20−50 nm in diameter (Figure 1a). In a recent study, it was shown that amphiphilic dipeptides derived from d-or L-threonine are capable of self-assembly into helical nanofibers. The formation of the nanostructures in the case of ThrC7 assemblies is likely due to the presence of the seven carbon

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chain alkyl spacer which contributes to hydrophobic interactions and facilitates interactions between the ThrC7 assemblies in addition to hydrogen bonding interactions between the carboxyl groups and the hydroxyl groups of threonine and hydrogen bonding between the amide $-\text{NH}$ and $\text{O}=$ groups.\textsuperscript{40–42} Upon formation of the nanohybrids when the IL was incorporated, we observed the formation of a gel. The corresponding morphology changes are shown in Figure 1b–d. For the 2:1 ThrC7 to IL nanohybrids, a gelatinous mesh was observed where the ThrC7 appeared to be entangled with the IL. For the 1:1 ThrC7 to IL nanohybrids, multilayered structures with the IL centered around the ThrC7 nanostructures which are projecting outward are formed. For the 1:2 ThrC7 to IL nanohybrids, the ThrC7 appears to be entrapped in trapezoidal clusters of ILs. Such clusters are formed because of self-assembly of the IL itself at higher amounts of IL used in the case of 1:2 ratio.\textsuperscript{43} Furthermore, the morphology of the ThrC7 assemblies is lost, and small globular structures are seen instead.

To further examine the structures of the nanohybrids, we conducted AFM imaging (Figure 2). The AFM image of neat ThrC7 nanoassemblies shows individual short thick oval-shaped nanostructures in the diameter range of 20–50 nm (Figure 2a). Upon formation of the 2:1 ratio ThrC7 to IL nanohybrids, the corresponding AFM image shows a gelatinous matrix, with the ThrC7 nanoassemblies embedded throughout the surface of the matrix (Figure 2b). For the 1:1 ThrC7 to IL nanohybrids, thick globular structures aggregated together and covered by the IL film were observed (Figure 2c). For the 1:2 ratio ThrC7 to IL nanohybrids, highly ordered multilayered structures were observed (Figure 2d). These ordered structures are attributed to the interactions of the mica surface with the imidazolium component of the IL bound ThrC7 assemblies. Similar structures were also seen in the case of 1-butyl-3-methylimidazolium hexafluorophosphate, [C$_3$mim][PF$_6$] IL\textsuperscript{44} at higher concentrations of IL because of the "drop-on-the-layer" phenomenon as a result of contact with the mica surface, as explained by de Gennes’ theory.\textsuperscript{45} The individual ThrC7 nanostructures are no longer seen under those conditions possibly because at high IL concentrations, the nanofibers are completely entrapped within the IL which was demonstrated in the TEM image, and the morphology of the ThrC7 nanostructures are altered. Additionally, it is conceivable that at a higher IL ratio, the IL anionic moiety may disrupt the hydrogen bond network,\textsuperscript{46} between the ThrC7 assemblies, and the IL mostly self-assembles on its own on the surface with the ThrC7 entrapped within the self-assembled IL. The overall changes in the morphology seen at the various ratios of ThrC7 to IL is likely because [C$_3$mim][dmp] is capable of strong intra- and intermolecular hydrogen bonding interactions with ThrC7 assemblies.\textsuperscript{47,48} Furthermore, the hydroxyl groups of Thr are involved in strong hydrogen bonding interactions with the cationic imidazolium group of the IL along with side chain carboxyl groups of the ThrC7 nanoassemblies. These interactions between the imidazolium groups and ThrC7 assemblies allow for the formation of three-dimensional cross-linked networks that lead to the formation of the gel. The proposed scheme for the formation of the nanohybrids and interactions of the ThrC7 nanoassemblies with the [C$_3$mim][dmp] is shown in Figure 3. As shown in the figure, the formation of the ThrC7 nanoassemblies is promoted by extensive H-bonding interactions. Upon incorporation of [C$_3$mim][dmp], intra- and intermolecular interactions are observed between the imidazolium groups and...
dmp and the threonine assemblies that form three-dimensional networks.

We also examined the changes in surface roughness of the ThrC7 assemblies upon formation of the nanohybrids. For the neat ThrC7 nanoassemblies, the average roughness ($R_a$) was found to be 11.6 nm, while the maximum roughness ($R_{max}$) was found to be 65.3 nm. For the nanohybrids comprising 2:1 ThrC7 to IL, we observed a slight increase in surface roughness. The average roughness ($R_a$) was found to be 13.5 nm, while the $R_{max}$ was found to be 72.3 nm. For the 1:1 ThrC7 to IL nanohybrids, the average roughness ($R_a$) was found to be 18.4 nm, while the maximum roughness ($R_{max}$) was found to be 273.3 nm. This increase is attributed to the aggregation of the ThrC7 nanoassemblies upon interacting with the IL. The increase in surface roughness further confirms the formation of gelatinous matrices. The previous reports have also indicated an increase in surface roughness upon gelation.\textsuperscript{49,50} For the 1:2 ThrC7 to IL, we observed a decrease in surface roughness. The average roughness ($R_a$) was found to be 9.3 nm, while the maximum roughness ($R_{max}$) was found to be 65.8 nm which is lower than the other nanohybrids. This can be explained by the fact that there is relatively less aggregation at 1:2 ratios of ThrC7 to IL nanohybrids. These results further confirm the incorporation of the IL into the ThrC7 nanoassemblies. In a recent study, it was shown that [C\textsubscript{2}mim][dmp] is capable of strong intra- and intermolecular hydrogen bonding interactions.\textsuperscript{46} Furthermore, it is likely that the hydroxyl groups of Thr are involved in strong hydrogen bonding interactions with the cationic imidazolium group of the IL along with side chain carboxyl groups of the ThrC7 nanoassemblies.

**FTIR Spectroscopy.** We further probed the interactions between the IL and the Thr\textsuperscript{7} nanoassemblies by FTIR spectroscopy. The results obtained are shown in Figure 4. The neat IL (Figure 4a) shows a broad peak at 3403 cm\textsuperscript{-1} because of $\nu$OH stretching vibrations with shoulders at 3153 and 3103 cm\textsuperscript{-1}. The hydroxyl peak is indicative of the presence of water because of moisture from air. The $\nu$CH stretching peaks are observed at 2953 cm\textsuperscript{-1} and at 2843 cm\textsuperscript{-1}. The C=C stretch because of the imidazolium group is observed at 1663 cm\textsuperscript{-1}, and the C=C stretching peak is observed at 1579 cm\textsuperscript{-1}. The C–N stretching peak is seen at 1472 cm\textsuperscript{-1}, while the peaks at 1234 cm\textsuperscript{-1} and 1174 cm\textsuperscript{-1} are attributed to the N–H bending and C–H in-plane bending peaks, respectively.\textsuperscript{51,52} The neat ThrC7 assemblies (Figure 4b) showed a sharp peak at 1737 cm\textsuperscript{-1} because of the hydrogen bonded carboxyl groups, while the amide I and amide II peaks were observed at 1655 and 1539 cm\textsuperscript{-1} along with a shoulder at 1575 cm\textsuperscript{-1}. A sharp peak was observed at 1418 cm\textsuperscript{-1} because of the $\nu$C–H bending. Short peaks at 1306 and 1236 cm\textsuperscript{-1} and strong peaks at 1115 cm\textsuperscript{-1} and 1145 cm\textsuperscript{-1} are attributed to the C–O stretching vibrations.

Upon formation of the nanohybrids, because of incorporation of the ThrC7 nanoassemblies, we observed shifts in the bands. For the 2:1 ThrC7 to IL nanohybrid (Figure 4c), the $\nu$OH vibrations were observed at 3382 cm\textsuperscript{-1} with a short shoulder at 3292 cm\textsuperscript{-1}. A peak at 1750 cm\textsuperscript{-1} was observed because of H-bonded carboxyl groups of Thr\textsuperscript{7} assemblies. The amide I peak was seen at 1650 cm\textsuperscript{-1}, and the amide II bands appeared at 1598 and 1506 cm\textsuperscript{-1}. A peak was also seen at 1457 cm\textsuperscript{-1}, correlated to shift because of changes in the C–N stretching. The peaks at 1349 cm\textsuperscript{-1} and at 1196 cm\textsuperscript{-1} are because of shifts in C–H bending and C–O stretching vibrations, respectively. For the 1:1 ThrC7 to IL nanohybrid (Figure 4d), the hydroxyl peak was shifted to 3452 cm\textsuperscript{-1} the $\nu$CH alkyl stretch is observed at 2924 and 2852 cm\textsuperscript{-1}. A broad split peak was observed in the 1600 region with peaks at 1712 cm\textsuperscript{-1} and 1685 cm\textsuperscript{-1} because of the carboxyl group and the amide I bond from the ThrC7 assemblies, and the peak because of the imidazolium cation C=C bond was shifted to 1652 cm\textsuperscript{-1}. Additional peaks were observed at 1572, 1403, and 1212 cm\textsuperscript{-1} indicating shifts because of changes in the C–N stretching as well as the C=C stretching because of interactions with the ThrC7 assemblies. The $\nu$NH and $\nu$CH bending peaks were also shifted to 1172 cm\textsuperscript{-1} and 1064 cm\textsuperscript{-1}. Similar shifts have been observed upon the formation of polymer gel electrolytes when 1-ethyl-3-methyl imidazolium bis (trifluoromethylsulfonyl)imide ([C\textsubscript{2}mim]-[NTf\textsubscript{2}]) IL was incorporated into poly (1-vinylpyrrolidone-co-vinyl acetate) copolymers.\textsuperscript{53} For the 1:2 (Figure 4e) ThrC7 to IL nanohybrids, further shifts were observed confirming the formation of the nanohybrid. Broad peaks were observed at 1709 and 1656 cm\textsuperscript{-1} because of the carboxyl peak and the amide I groups of the ThrC7 assemblies, and the C=C peak of the imidazole was seen at 1647 cm\textsuperscript{-1}. The amide II peak shifted to 1573 cm\textsuperscript{-1}, and the C–C stretch peak shifted to 1524 cm\textsuperscript{-1}. Split peaks were observed at 1046 cm\textsuperscript{-1} and 1057 cm\textsuperscript{-1} along with a shoulder at 1087 cm\textsuperscript{-1} indicative of changes in the C–O stretching and C–H and N–H bending because of interactions between the IL and ThrC7 assemblies. In general, the biggest changes observed were in the 1600–1700 cm\textsuperscript{-1} region upon incorporation of the IL into the ThrC7 assemblies. In the case of nanohybrids, we observed peak broadening compared to the neat ThrC7 assemblies. This is attributed to extensive inter- and intramolecular hydrogen...
bonding interactions between the ILs and the ThrC7 assemblies particularly between the −NH and C=O groups and the −NC−HN− of the imidazolium group of the IL.

The proton on the carbon between the two nitrogens of the imidazolium group is acidic. Thus, further H-bonding may be facilitated between the −OH group and −NH groups of the ThrC7 nanoassemblies.55,56 Shifts in the hydroxyl region are indicative of the role of hydrogen bonding interaction with the −OH groups of the Thr moiety.

**Differential Scanning Calorimetry Analysis.** To determine the thermal phase changes upon formation of the nanohybrids, we conducted differential scanning calorimetry (DSC) analysis. As shown in Figure 5, the glass transition temperature of the neat IL (Figure 5a) was found to be at −78.0 °C, followed by broad peaks at 4.6 °C and at 35.4 °C because of loss of frozen water and loosely bound water, and the decomposition peak was found to be at 282.7 °C. No crystallization behavior was observed. This result is similar to phase changes observed for imidazolium-based ILs such as 1-(2-hydroxyethyl)-3-methylimidazolium tetrafluoroborate, [HeMIm][BF$_4$]$^-$, and 1-methyl-3-(3-triethoxysilylpropyl) imidazolium hexafluorophosphate, [spmim][PF$_6$].57,58 Upon formation of the nanohybrids, for the 1:1 ThrC7 to IL nanohybrids (Figure 5b), the glass transition temperature was found to be at −78.2 °C; a short endothermic peak was observed at 8.3 °C because of loss of frozen water followed by another strong endothermic peak at 85.1 °C with shoulders at 78.4 and 83.9 °C. These peaks may be attributed to the unfolding of ThrC7 assemblies because of sequential changes in hydrogen bonding interactions upon heating. Similar changes have been observed in protein and peptide-based materials.59,60 A short endothermic peak is also observed at 97.6 °C because of loss of tightly bound water, followed by two short, broad endothermic peaks at 234.6 and 273.1 °C because of thermal decomposition of the composite. The decomposition peak of the IL component was found to be at a lower temperature (273.1 °C) when compared to the neat IL (282.7 °C). Such lowering of the temperature of decomposition is further confirmation of the formation of the nanohybrid composite. For the 1:2 ThrC7 to IL nanohybrid (Figure 5c), the glass transition temperature was found to be slightly higher (−74.7 °C). A sharp endothermic peak was seen at 1.9 °C because of loss of frozen water. The broad endothermic peak at 62.2 °C is indicative of gradual unfolding of the ThrC7 assemblies and rearrangement of the intermolecular interactions. Endothermic peaks are also seen at 122.9, 232.8, and at 252.6 °C because of loss of tightly bound water and decomposition of the composite subsequently. The decomposition temperature for the IL component is further lowered to 252.6 °C for the 1:2 ThrC7-IL nanohybrid assemblies. For the 2:1 ThrC7 to IL (Figure 5d) nanohybrids, the Tg was found to be at −74.9 °C followed by short endothermic peaks at −14.6 °C and 94.9 °C. A broad endothermic peak is observed at 222.5 °C. For the neat ThrC7 assemblies (Figure 5e), no Tg was observed below 0 °C because of the absence of IL. However, a sharp endothermic peak was observed at 0.1 °C because of loss of loosely bound water, followed by endothermic peaks at 92.2 °C and 170.2 °C because of loss of tightly bound water and unfolding and disorganization of the peptide assemblies subsequently.

**Thermogravimetric Analysis.** We also compared the thermal properties of the nanohybrids with that of the neat IL and ThrC7 assemblies using thermogravimetric analysis (TGA). The results obtained are shown in Figure 6. The onset of decomposition temperature for neat ThrC7 assemblies was found to be at 185.2 °C, and complete decomposition was found to occur at 262 °C (96% weight loss) because of decomposition of the assemblies. For the [C$_2$mim][dmp] IL alone, a weight loss of 3.8% was observed below 105 °C likely due to evaporation of the water content of the IL,61 followed by decomposition on set temperature at 264 °C. A weight loss of 62% was observed at 363 °C, and a 95.25% weight loss was seen at 800 °C. Previous thermal stability studies carried out for [C$_2$mim][dmp] revealed similar results.62 This is indicative that the neat IL is more thermally stable when compared to neat ThrC7 assemblies. Upon formation of the nanohybrids, distinct changes were observed. For the 2:1 ThrC7 to IL nanohybrids, the onset temperature was found to be lower than that of ThrC7 assemblies (148.3 °C) and the neat IL because of changes in H-bonding interactions, overall the nanohybrid did not show complete decomposition even at 800 °C. At 210.2 °C, a 42.2% weight loss was observed, and at 502 °C, a 48.2% weight loss was observed. The sample remained relatively stable and at 800
°C, a weight loss of 52.3% was seen. This is indicative of the fact that incorporation of the IL into the ThrC7 nanoassemblies at this ratio probably causes thermally driven chemical changes that lead to the formation of complex polymorphs that remain stable at high temperatures. The formation of such polymorphs has been seen widely in amphiphilic assemblies, as well as amphiphilic co-polymers. As a result of interactions with solvents which bring about changes in intermolecular interactions with the self-assembled amphiphiles. Because ThrC7 is at a higher ratio, it is likely that the ThrC7 group entraps the IL leading to the formation of complex polymorphs because of electrostatic interactions between the phosphate groups and amide and carboxyl groups of the assemblies.

For the 1:1 IL to ThrC7 nanohybrids, the onset temperature was found to be 195.8 °C, while at 307.1 °C, we observed a transition and weight loss of 58.1% and at 500 °C, a weight loss of 70.6% was seen. At 800 °C, the weight loss was found to be 75%. This clearly indicates that at the 1:1 ratio, stepwise thermally induced changes occur, but the polymorphic structures formed are less stable at higher temperatures when compared to those observed for the 2:1 ThrC7 to IL assemblies. This could be because of the higher amount of IL that leads to a change in aggregation. For the 1:2 ThrC7 to IL nanohybrids, the onset temperature was found to be 227 °C while at 280 °C, a weight loss of 42.96% was observed. Further stepwise weight loss was observed at 482 °C (68% weight loss) and at 522 °C (75.1% weight loss). At 800 °C, the % weight loss was found to be 80.1%. This indicates that for hybrids containing higher amounts of ILs are more stable at the lower temperature range as the onset temperature is higher and less stable at the higher temperature range as the sample appears to have a weight loss of over 80% at 800 °C. Overall, the nanohybrids showed multistep thermal changes, which is correlated to the changes in the interactions between the ILs and the ThrC7 assemblies. As the ratio of the IL is increased, the interactions are drastically different as the IL component dominates most likely causing the major changes in interactions of the ThrC7 assemblies resulting in structural and chemical changes in the final structures formed. Other reports have also suggested changes in the thermal properties of ILs such as 1-ethyl-3-methylimidazolium dicyanamide ([C2mim][DCA]) when incorporated into glucose–albumin-based carbogels because of changes in the anion–π interaction between the IL anions and the surfaces of flexible support materials. A summary table comparing the thermal stabilities of the ThrC7-IL nanocomposites with other nanocomposites of ILs is given in the Supporting Information (Table S1). Overall, as the amount of IL was increased in the nanohybrids, higher thermal decomposition temperature was observed although the decomposition was relatively less when compared to pure IL. Because different thermal changes were observed at varying ratios of the nanohybrids, this is indicative of the fact that the ratio of ThrC7 and the IL plays a key role in the thermal-driven chemical changes observed for the nanohybrids.

Rheology. In order to examine the viscoelastic behavior, the nanohybrids, IL, and the ThrC7 nanoassemblies were subjected to dynamic oscillatory frequency sweep at room temperature (25 °C) at an angular frequency range of 0.01 to 200 rad/s at 4% strain. The results obtained are shown in Figure 7. The ThrC7 nanoassemblies displayed higher G’ when compared to the nanohybrids and the neat IL (Figure 7a). At a very low angular frequency, there is an initial decrease; however, once the angular frequency attains a critical value, the storage modulus increases indicating a shear-stiffening effect. This is likely because ThrC7 assemblies have higher rigidity and mechanical strength because of close packing of the nanoassemblies. In comparison, the neat IL has the lowest G’ and remains independent of the frequency up to an angular frequency of 100 rad/s and even at the critical value, the increase is not substantial. This behavior is typical of nonassociated materials as the IL is a viscous liquid at room temperature. As the quantity of ThrC7 was increased in the composite, the overall storage modulus was found to increase. This behavior indicates that the IL molecules integrate into the ThrC7 assemblies and are not forming the phase separated, heterogeneous mixture as seen in the case of 2:1 ThrC7:IL. There is very little difference in the behavior of the 1:1 ThrC7 to IL and 1:2 ThrC7 assemblies to IL ratios, indicating that for those two hybrids, the networks formed between the ThrC7 assemblies and the ILs are different when compared to those formed at the higher ratio of the ThrC7 assemblies. Particularly at the 2:1 ratio of IL to ThrC7 assemblies, where there is an excess of IL, the IL molecules are likely to self-assemble, entrapping the ThrC7 assemblies (as confirmed by the TEM and AFM images as well). Figure 7b shows the stress–strain curves of the various composites as well as neat ThrC7 assemblies and the IL. As shown in the figure, ThrC7 assemblies shows a higher initial slope of the curve when compared to the neat IL indicating higher elasticity of ThrC7 assemblies. Upon incorporation of IL into the ThrC7 assemblies at the 2:1 ratio of ThrC7 to IL, we observed further increase in the initial slope indicating increase in elastic.
behavior because of formation of the gelatinous matrix. Further increase in the IL content at the 1:1 ratio of IL to ThrC7 decreases the initial slope of the curve comparatively but is still higher than the neat IL. This behavior is likely because of interactions with the ThrC7 nanoassemblies resulting in a more ordered structure. The degree of elongation of the modulus however reduces for the 1:2 ThrC7 to IL. This is likely because a higher IL content seems to work as a plasticizer.

**Cell Studies.** In order to improve biological applications of ILs, several studies have been carried out. It has been shown that reducing the chain length of the cation side chains and incorporation of polar groups may reduce toxicity of ILs. A new class of materials called Bio-ionic gels are being explored where in polymeric hydrogels derived from gelatin methacryloyl and PEG diacrylate have been cross-linked with choline-based ILs. The resulting bio-ILs were found to be biodegradable and supported cell adhesion and proliferation of cardiomyocytes and were found to have low immunogenicity. In another study, gelatin-based ionogel films with antioxidant and antimicrobial properties have been constructed by combining gelatin with choline salicylate. Carbohydrate-based pyridinium ILs have also been prepared which have shown low cytotoxicity toward mammalian fibroblasts. In order to examine the effects of the nanohybrids on mammalian cells, we conducted *in vitro* cytotoxicity studies. We tested two separate cell lines. (a) Human dermal fibroblasts and (b) MCF-7 breast cancer cells. We selected these two cell lines because fibroblasts are one of the most common connective tissue cells present in the human body. We hypothesized that incorporation of ThrC7 nanoassemblies with the [C2mim]-[dmp] may potentially reduce toxicity of mammalian cells. MCF-7 breast cancer cell lines are estrogen receptor positive [dmp] may potentially reduce toxicity of mammalian cells. MCF-7 breast cancer cell lines are estrogen receptor positive [dmp] may potentially reduce toxicity of mammalian cells. MCF-7 breast cancer cell lines are estrogen receptor positive [dmp] may potentially reduce toxicity of mammalian cells. MCF-7 breast cancer cell lines are estrogen receptor positive [dmp] may potentially reduce toxicity of mammalian cells. MCF-7 breast cancer cell lines are estrogen receptor positive. [dmp] may potentially reduce toxicity of mammalian cells. MCF-7 breast cancer cell lines are estrogen receptor positive [dmp] may potentially reduce toxicity of mammalian cells. MCF-7 breast cancer cell lines are estrogen receptor positive. In the previous work, it has been shown that gel stiffness plays an important role in cell adhesion, growth, and morphology. In the case of the nanohybrids, these results indicate that the cell adhesion and spreading are higher at 2:1 ThrC7to IL as the gels are relatively more stiff when compared to the 1:2 ThrC7 to IL nanohybrids.

In the case of MCF-7 cells (Figure 10), our results indicated that when compared to control cells, cells grown in the presence of the nanohybrids as well as IL displayed cytotoxicity (Figure 10a). Cells grown in the presence of nanohybrids at 1:1 IL to ThrC7 showed 53% viability, 1:2 IL to ThrC7 showed 58% viability, while 2:1 IL to ThrC7 showed 51% viability. Comparatively in the presence of neat ThrC7 nanoassemblies, 60% viability was seen, while IL alone showed 65% viability. The decrease in viability in the presence of the ThrC7 nanoassemblies, as well as nanohybrids may be attributed to the C7 (azelaic acid amide chain) within the nanoassemblies. In the previous work, it has been shown that azelaic acid has displayed antiproliferative effects against melanoma cells as azelaic acid inhibits nucleic acid metabolism as well as DNA and RNA metabolism in tumor cells. Additionally, imidazolium-based ILs have been found to be cytotoxic against several tumor cell lines. It is likely that combination of IL with the ThrC7 nanoassemblies further increases the cytotoxic effects on MCF-7 cells. These results are further confirmed by the examining the interactions of the cells with the nanohybrids (Figure 11), which show blebbing and disruption of the cell structures when compared to untreated cells in all cases after treatment with nanohybrids and the ThrC7 nanoassemblies. In a recent study, it was shown that ferrocene-tethered imidazolium-based ILs displayed antiproliferative activity toward MCF-7 breast cancer cells because of inhibition of the lysosomal peptidase enzyme cathepsin B. In another study, it was shown that IL extracts of graviola induced apoptosis in MCF-7 breast cancer cells and therefore may have applications in targeting cancer cells. Furthermore, some of the hydrophobic imidazolium-based ILs containing (C8mim−C8mim) cations have been known to bind to cell MDA-MB231 breast cancer membranes and impact the cellular morphology. Herein, we have used a relatively hydrophilic imidazolium-based IL containing C2mim cation, with a hydrophilic anion like dmp with a peptide bolaamphile to target tumor cells. Although the exact mechanism of inhibition of the nanohybrids designed in this work is not known and will be reported as a separate study, our results indicate that the nanohybrids may be applicable in potential biological applications.

### CONCLUSIONS

In this work, new nanohybrid gels were prepared by incorporating the IL [C2mim][dmp] into self-assembled threonine-based peptide nanoassemblies. Our results indicated that the peptide nanoassemblies interacted extensively with the ILs and nanohybrids of varying morphologies were formed depending upon the ratio of the ILs and the peptide assembly.
used. Imaging analyses showed the formation of highly gelatinous networks. Additionally, when higher amounts of ILs were used, highly ordered structures were formed. The surface roughness values varied depending upon the ratio of the ILs to peptide nanoassemblies used to form the nanohybrids. The interactions between the IL and the peptide nanoassemblies were confirmed by FTIR spectroscopy. Thermal phase changes at higher temperatures (>60 °C) indicated unfolding of the nanoassemblies bound to IL. Furthermore, the nanohybrids showed selective toxicity toward MCF-7 breast cancer cells indicating that such materials may have potential biological applications.

**EXPERIMENTAL SECTION**

**Materials.** 1-Ethylimidazole (95±%, Lot # 078976K09H) was obtained from Oakwood Chemical. Trimethyl phosphate (97%, Lot # MKBZ7687V) was obtained from Millipore Sigma. Azelaic acid, N-(3-diphenylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDAC), N-hydroxy succinimide (NHS), dimethyl formaldehyde (DMF), triethylamine, trypan blue, and threonine were all purchased from Sigma-Aldrich. All chemicals were used as received. Primary dermal fibroblasts (ATCC PCS-201-021 Lot # 80124171), MCF-7 breast adenocarcinoma (ATTC HTB-22 Lot # 70019550), fibroblast basal medium (ATCC PCS-201-030), fibroblast growth kit−low serum (ATCC PCS-201-041), Eagles minimum essential medium (EMEM) with l-glutamine (ATCC 30−2003) were all ordered from ATCC. An WST-8 assay including electron mediator solution (item no. 10010354) and WST-8 developer reagent (item no. 600487) were purchased from Cayman Chemicals.

**Methods.** Synthesis of Bis(N-α-amido-threonine) 1,7 Heptane Dicarboxylate (ThrC7). The peptide amphiphile was synthesized by modification of previously established peptide coupling methods.33 Briefly, azelic acid (0.2 M) was dissolved in DMF followed by the addition of NHS (0.073 M) and EDAC (0.026 M) in order to activate the free carboxylic groups. The solution was stirred at 4 °C for an hour followed by addition of two drops of triethylamine and threonine (0.56 M). The solution was allowed to shake at 4 °C for 24 h. The solvent was rotary evaporated, and the resulting off-white product was recrystallized from acetone. The product was then air dried and weighed. The formation of the product was confirmed by 1H NMR spectroscopy carried in DMSO-d6 using a Bruker 400 MHz NMR spectrometer. Peaks obtained are δ 1.2 (d, 6 H); δ 2.2 (t, 4 H); δ 1.5 (m, 4 H); δ 1.4 (m, 4 H); δ 3.9 (d, 2 H); δ 5.9 (s, 2 H); and 8.1 (s, 2 H).

Self-Assembly of ThrC7. The formed product (1.1539 g) was first dissolved in sodium bicarbonate (0.1 M), and then, the pH was adjusted to pH 4 by addition of citric acid (0.026 M) in order to activate the free carboxylic groups. The solution was stirred at 4 °C for an hour followed by addition of two drops of triethylamine and threonine (0.56 M). The solution was allowed to shake at 4 °C for 24 h. The solvent was rotary evaporated, and the resulting off-white product was recrystallized from acetone. The product was then air dried and weighed. The formation of the product was confirmed by 1H NMR spectroscopy carried in DMSO-d6 using a Bruker 400 MHz NMR spectrometer. Peaks obtained are δ 1.2 (d, 6 H); δ 2.2 (t, 4 H); δ 1.5 (m, 4 H); δ 1.4 (m, 4 H); δ 3.9 (d, 2 H); δ 5.9 (s, 2 H); and 8.1 (s, 2 H).

Synthesis of 1-Ethyl-3-methylimidazolium Dimethyl Phosphate [C3mim][dmp]. The procedure for the synthesis of [C3mim][dmp] is based on established protocols.34 Briefly, 1-ethylimidazolium was added to a molar equivalent of trimethyl phosphate. The reaction mixture was refluxed for 24 h at 80 °C. The resulting pale yellow oil was dried for five days under
vacuum. The liquid solidified under vacuum. The structure was verified using $^1$H NMR spectroscopy. $^1$H NMR (400 MHz, D$_2$O): δ 8.59 (s, 1 H), 7.36−7.29 (m, 2 H), 4.11 (q, 2H), 3.77 (s, 3H), 3.48 (s, 3 H), 3.45 (s, 3 H), and 1.38 (tr, 3 H).

**Formation of ThrC7–[C$_2$mim][dmp] IL Nanohybrids.** To prepare the nanohybrids, we examined three different ratios (by mass) of IL to ThrC7 assemblies: (1:2 IL-ThrC7; 1:1 IL-ThrC7; and 2:1 IL-ThrC7 nanoassemblies). Depending upon the ratio, for preparation of the samples, 2 mg of IL was mixed with either 4 mg of ThrC7; 2 mg of ThrC7 or with 1 mg of ThrC7 assemblies. The IL utilized was vacuum dried immediately prior to addition. The mixtures were then sonicated for 30 min and then incubated at 34 °C and shaken slowly in an incubator for 24 h and then vacuum dried before further analysis.

**Characterization.** TEM. To probe the morphologies of the assemblies and nanohybrids, TEM was conducted using a JEOL 120EX TEM operated at 80 kV. The samples were air dried on to 200 mesh carbon-coated grids for analysis, and images were taken at various magnifications.

**FTIR.** The binding interactions between the ThrC7 peptide nanoassemblies and the [C$_2$mim][dmp] IL were analyzed by FTIR spectroscopy. Spectra were recorded using a Thermo Scientific Nicolet iS50 FTIR in the range of 400−3800 cm$^{-1}$.

**DSC.** To examine the phase changes of the IL before and after embedding of the nanoassemblies, DSC analyses were conducted. The samples were sealed in aluminum pans, and data were recorded using a TA instrument Q-200 DSC at a temperature range of −80−300 °C at the rate of 5 °C per minute.

**TGA.** TGA of the nanohybrids and the individual components (IL and ThrC7) were carried out using TA Instruments Q500 TGA (TA Instruments, New Castle, DE, USA). The samples were dried under vacuum prior to analysis. In general, studies were carried out under nitrogen at a heating rate of 10 °C per minute. Studies were carried out in the range of 20−800 °C. Each study was carried out thrice.

**AFM.** The samples were dried onto Muscovite mica sheets for AFM analysis. To image the nanoassemblies and the ThrC7 nanohybrids, AFM was conducted in the ScanAsyst mode on a Bruker Multimode 8HR AFM. The tip used was a Bruker model RTESPA-52S made of 0.01−0.025 Ω cm antimony (n) doped Si with a resonant frequency of 25 kHz and a spring constant of 200 N/m.

**Rheological Analysis.** Rheology measurements of the formed nanohybrids, ThrC7 nanoassemblies, and the ILs were carried out using a Discovery Hybrid HR2 Rheometer (TA instruments, New Castle, DE, USA). Measurements were carried out at 25 °C on a Peltier plate using an 8 mm Peltier cone geometry. Dynamic oscillatory sweeps were carried out between angular frequencies $\omega$ of 0.01 and 100 rad/s. The samples were vacuum dried before analysis. Measurements were carried out in triplicate in air.

**Cell Studies.** We examined the interactions of the nanohybrids with two cell lines, namely, adult human dermal fibroblast cells and MCF-7 breast cancer cells. The fibroblasts were cultured for 48 h in fibroblast basal medium (ATCC PCS-201-030) containing 5 ng/mL rh fibroblast growth factor b, 7.5 mM l-glutamine, 50 μg/mL ascorbic acid, 1 μg/mL hydrocortisone hemisuccinate, 5 μg/mL rh insulin, and 2% fetal bovine serum (ATCC Fibroblast Growth Kit—Low Serum PCS-201-041) and 2% antibiotic-antimycotic 100× solution (Gibco 15240−096 Lot # 2058929). Cells were incubated in an atmosphere of 5% CO$_2$ at 37 °C. Media was changed every two days and cells were split twice a week to maintain cultures. After cells were grown to confluence, to carry out in vitro cytotoxicity studies, cells were seeded at a density of 1 × 10$^5$ cells/mL into 96 well plates and allowed to spread for 3 h. This was followed by addition of 50 μg/mL of 1:1, 1:2, and 2:1 ThrC7−IL nanohybrids or ThrC7 nanoassemblies or IL. Equivalent amount of water was added to control cells. Cells were allowed to grow for a period of 48 h. To determine the cell viability, the adherent and any unattached cells were rinsed with phosphate-buffered saline after removal of media, collected from each well using a cell-scrapere, and immediately mixed with 300 μL of media and centrifuged. Cells were then counted using the trypsin blue assay. To document the cell morphology, cells were plated on 6-well plates with or without each of the constructs, and images were taken using an AmScope inverted phase contrast microscope with digital camera (MU130) after 48 h.

MCF-7 cells (ATTC HTB-22 Lot # 70019550) were cultured in EMEM solution with L-glutamine (ATCC 30−2003) containing 10% Fetalagro (RMBIO) and 1% antibiotic-antimycotic 100× solution (Gibco 15240−096 Lot # 2058929) by volume. Cells were incubated in an atmosphere of 5% CO$_2$ at 37 °C, media was changed every two days, and...
cells were split twice a week to maintain cultures. After growing to confluence, the cells were seeded into a 24-well plate at a density of $1 \times 10^5$ cells/mL and allowed to incubate for 3 h before adding 20 $\mu$g/mL of 1:1, 1:2, and 2:1 ThrC7 to IL nanohybrids, ThrC7 nanoassemblies or ILs, and water control. The cells were allowed to incubate for 24 h before imaging. To test for cytotoxicity in vitro, 10 $\mu$L of the WST-8 mixture, which contains equal parts of electron mediator solution (item no. 10001354) and WST-8 developer reagent (item no. 600487, Cayman Chemicals), was added to each cell. The plate was shaken gently for 1 min and then allowed to incubate for 3 h. Then, after briefly shaking, the plate was read at 37 °C using a Biotek microplate reader at a wavelength of 450 nm.

**Statistical Analysis.** We used two-tailed Student’s t tests for carrying out statistical analysis. Studies were carried out in triplicate ($n = 3$). Data are presented as the mean value ± standard deviation for each sample group. $p < 0.05$ was considered to be statistically significant.

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*DISCLOSURE* 

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