ABSTRACT: The current study introduces two novel, smart polymer three-dimensional (3D)-printable interpenetrating polymer network (IPN) hydrogel biomaterials with favorable chemical, mechanical, and morphological properties for potential applications in traumatic brain injury (TBI) such as potentially assisting in the restoration of neurological function through closure of the wound deficit and neural tissue regeneration. Additionally, removal of injury matter to allow for the appropriate scaffold grafting may assist in providing a TBI treatment. Furthermore, due to the 3D printability of the IPN biomaterials, complex structures can be designed and fabricated to mimic the native shape and structure of the injury sight, which can potentially assist with neural tissue regeneration after TBI. In this study, a peptide-only approach was employed, wherein collagen and elastin in a blend with gelatin methacryloyl were prepared and crosslinked using either Irgacure or Irgacure and Genipin to form either a semi or full IPN hydrogel 3D-printable neuromimicking platform system, respectively. The scaffolds displayed favorable thermal stability and were amorphous in nature with high full width at half-maximum values. Furthermore, no alteration to the peptide secondary structure was noted using Fourier transform infrared spectroscopy. The IPN biomaterials have a stiffness of around 600 Pa and are suitable for softer tissue engineering applications—that is, the brain. Scanning electron micrographs indicated that the IPN biomaterials had a morphological structure with a significant resemblance to the native rat cortex. Both biomaterial scaffolds were shown to support the growth of PC12 cells over a 72 h period. Furthermore, the increased nuclear eccentricity and nuclear area were shown to support the postulation that the IPN biomaterials maintain the cells in a healthy state encouraging cellular mitosis and proliferation. The Genipin component of the full IPN was further shown to exhibit antimicrobial properties and this suggests that Genipin can prevent the growth of pathogens associated with postsurgical brain infections. In addition to these findings, the study presents an anomaly, wherein the full IPN is found to be more brittle than the semi IPN, a finding that is in contradiction with the literature. This research, therefore, contributes to the collection of potential biomaterials for TBI applications coupled with 3D printing and can assist in the progression of neural treatments toward patient-specific scaffolds through the development of custom scaffolds.

1. INTRODUCTION

The homeostasis of the extracellular matrix (ECM) is vital for the biological functioning of any living tissue. For optimal tissue functioning, the interplay of the various ECM components with the cells and surrounding capillaries to maintain the homeostatic balance is, therefore, specific for a given tissue. To achieve this, an important feedback loop exists between the ECM and the resident cells of the tissue. The resident cells secrete and establish a versatile ECM network and the ECM, in turn, influences the biology of the cells by controlling, among others, their migration, survival, differentiation, and proliferation. Through this loop, the ECM plays a crucial role in the regulation of repair and regeneration after physiological ECM degradation and most importantly, after injury. With this in mind, it is no surprise that ECM dysregulation is implicated in several disease and injury states because the ECM is remodeled in response to various stimuli such as changes to tissue stiffness, inflammation, and fibrosis. Due to the uniqueness of the ECM in native biological tissues, developing an “ideal” biomaterial has proven to be difficult in the application of three-dimensional (3D) printing in the tissue
engineering field. Despite the many achievements in this field, a single biomaterial exhibiting properties to be a potential universal biomaterial for any tissue requiring replacement has yet to be identified.

Biomaterial selection for the design of 3D-printed artificial tissues through ECM mimicking is vitally important for all tissue engineering applications. Careful biomaterial selection on a per tissue basis, therefore, allows for the adequate mimicking of the native tissue properties to provide regeneration and host tissue integration at the injury site. As the World Health Organization (WHO) predicted that traumatic brain injury (TBI) will be the third leading cause of death and disability worldwide by 2020, biomaterial selection for adequate native brain mimicry and the minimization of secondary neural damage after TBI is vital.

As tissue engineering explores the usage of materials to produce 3D scaffolds to mimic the native microenvironment, it is no surprise that the formation of artificial ECM (aECM) hydrogel systems, formed by aqueous networked polymeric chains, incorporating native ECM material constituents has generated a lot of interest. Hydrogel systems have been shown to exhibit similar tuneable characteristics to the native in vivo ECM microenvironments. The rational design of polymer hydrogel scaffolds for soft tissue engineering is a promising strategy because hydrogels have the ability to absorb large volumes of water/bodily fluid without dissolving. Furthermore, in their swollen state, hydrogels are considered to be soft polymer systems, making them ideal candidates for the engineering of softer tissues such as the brain. Finally, once swelling equilibrium has been reached, the cohesive forces within the hydrogel systems resist further expansion. This property in TBI applications is extremely important so to prevent further damage to the lesion site or surrounding tissue. In the fabrication of these hydrogel systems, natural materials are generally a first-choice selection due to their favorable advantages over their synthetic counterparts. Most of the native ECM materials utilized in the fabrication of aECM hydrogel scaffolds are manufactured from connective tissue ECMs, making the materials deformable and fiber-reinforcing matrix composites, ideal for tissue engineering approaches. A list of selected ECM materials, specifically for neural tissue engineering, is summarized in Table S1. Additionally, natural materials are advantageous for biomaterial incorporation because they are able to be degraded by tissue-specific enzymes, providing a foreseeable “guarantee” that the fabricated implant will be metabolized/degraded through physiological mechanisms. This property ensures the implant is able to provide the necessary function for the required time before being naturally removed. In spite of the benefits, natural materials exhibit drawbacks (poor printability, batch variation, immunogenicity, etc.) that have hindered the progression of material development for TBI. In response, biomaterial blends of natural—natural, synthetic—natural, or synthetic—synthetic polymers, and material functionalization have assisted in the reversal of such shortcomings to produce materials with cell-binding domains and ECM motifs as well as tissue-specific mechanical strength, degradation, pore size, and architecture among others.

Further advancements in 3D-printable biomaterials are the design and fabrication of interpenetrating polymer network (IPN) systems. IPN systems are described as an elastomer-type polymer, where two or more polymers (natural or synthetic) with individual characteristics coexist and are crosslinked in the presence of each other. As such, IPN systems are generating much interest as IPNs can be used to control, including but not limited to: swelling, elasticity, porosity, and stimuli-responsive behavior by modulating the proportion of crosslinking agents and polymers utilized. As a broad distinction, IPNs can be classified as semi IPNs or full IPNs depending on whether one or both polymers are crosslinked in the presence of the other, respectively. IPN systems are favored over single-hydrogel systems because they provide enhanced mechanical strength as well as superior drug release, drug targeting, and drug entrapment. Therefore, it is no surprise that IPN systems are emerging as potential novel solutions due to their vast properties and benefits.

In the fabrication of IPN systems, several crosslinking agents, for example, Genipin, Irgacure 2959/819, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, and vi- initiated (Ru(II)bpy) are available to tailor and control the property of the designed IPN. In some cases, crosslinkers are reported to possess other beneficial properties, such as antimicrobial properties, as is the case with Genipin and as such is widely utilized in various biomedical fields. In a previous study, Genipin was shown to reduce sepsis, and therefore was proposed to be a potential therapeutic agent for this life-threatening condition. As such, the inclusion of Genipin in prospective TBI treatments may assist in reducing the risk of patients developing nosocomial infections after a sustained TBI—especially penetrating wounds—due to its beneficial dual properties (crosslinking and antimicrobial properties).

Because a tissue engineering scaffold for TBI is required to adequately mimic various tissue properties including brain region strength, cellular function, tissue integration, and tissue morphology, several tissue engineering approaches combining different engineering applications, stem cells, and materials have the potential to assist in developing novel biomaterials for the replacement of damaged tissues and organs in several tissue engineering applications. Of the several approaches, 3D printing has evolved into an indispensable tool to fabricate intricate 3D scaffold shapes to mimic the native tissue in terms of cell placement, bioactive cues, and biomaterial selection for the required application. In addition to biomaterial selection, the physicochemical properties when using a respective 3D printing technique for the aECM scaffold fabrication provide another level of material selection complexity as not all 3D printing techniques are capable of printing hydrogels due to their harsh printing conditions and viscosity requirements. The 3D printing techniques capable of successfully 3D printing hydrogels include: laser-based, extrusion-based, and inkjet printing. Of these techniques, extrusion-based 3D plotting is the most suitable for hydrogel scaffold fabrication due to the variety of hydrogel biomaterials that are able to be printed. The biomaterials selected for extrusion-based printing are required to be viscous as well as undergo shear thinning and yield stress. Furthermore, the biomaterial must exhibit quick gelation or solidification of the layers postprinting to ensure build-up structural fidelity of the scaffold. Because extrusion-based printing is a pneumatic driven technique, biomaterials are required to flow under modest pressure.
the incorporation of the patient’s cells, in comparison to conventional tissue engineering approaches. In addition, for adequate tissue mimicry, scaffold archetype resolution must be exceptional, and therefore 3D printing resolution for organ replacement is also starting to improve.

This study, therefore, formulated two protein fibrous novel IPN systems that were optimized for 3D printing. To the best of our knowledge, this study provides one of the first completely protein-based IPN aECM scaffolds for potential tissue engineering applications. The material properties were assessed for their biofunctionality using Fourier-transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), X-ray diffraction (XRD), and scanning electron microscopy (SEM) as well as viscoelastic and real-time mechanical analysis in an artificial biological fluid. In addition, the study assessed the biocompatibility of the scaffolds through in vitro cytocompatibility studies and through the monitoring of cell cycle characteristics. This study may provide a new perspective for tissue engineering through the 3D printing of IPN systems as well as assist in improving patient outcomes post TBI due to the antimicrobial nature of Genipin.

2. RESULTS AND DISCUSSION

Two GelMA, collagen, and elastin IPN hydrogels, summarized in Table 1, were successfully 3D printed. Geometrically macroporous, equiangular equilateral quadrilateral scaffolds were obtained, which maintained their shape after UV crosslinking and lyophilization (Figure 1A). After rehydration, the scaffolds maintained their 3D fabricated internal structure; however, care was taken during handling due to the soft nature of the biomaterial.

Table 1. Summary of the Analytical Data of the IPN Biomaterial Components

|               | semi IPN | full IPN |
|---------------|----------|----------|
| definition    | only one of the polymers is crosslinked into a network, in the presence of another | both polymers are crosslinked into independent networks, in the presence of the other |
| IPN class     | hydrogel IPNs | noncovalent IPNs |
| IPN classification | Irgacure 2959 | Irgacure 2959 and Genipin |
| crosslinked polymer | GelMA | GelMA (Irgacure 2959) |
| sterilization | ultraviolet light (254 nm) | extraction 3D printing |
| method of fabrication | | |
| 3D-printed scaffold colour | white | blue |

2.1. Molecular Vibrational Transitions and Thermo-dynamic Analysis Confirming the Formation of Different 3D-Printable IPN Biomaterial Hydrogel Scaffolds

The FTIR spectrum of GelMA showed characteristic wavenumber bands at 3289 cm⁻¹ (amide A), 2936 cm⁻¹ (amide B), 1638 cm⁻¹ (amide I), and 1235 cm⁻¹ (amide III). The full IPN (the presence of Irgacure 2959 and Genipin) had characteristic wavenumber bands at 3297 cm⁻¹ (amide A), 2936 cm⁻¹ (amide B), 1630 cm⁻¹ (amide I), 1535 cm⁻¹ (amide II), and 1235 cm⁻¹ (amide III) (Figure 1B). For both IPN biomaterials in comparison to the blank formulation, particularly the amide B, amide II and amide III bands had small shifts in their wavenumber possibly due to the addition of the crosslinkers (the presence of –OH functional groups in both crosslinkers), a stronger molecular water association could be present. Furthermore, it is evident that the 2 min of UV exposure required for polymer photocrosslinking does not affect the main amide peaks of the biomaterial IPN systems as the amide bands are still intact with similar transmittance intensities. Therefore, no alteration to the peptide primary structure was noted. Due to the similarities in the profiles, it was further established that the presence of the crosslinking agents did not impede the peptides from assuming a self-assembled, native interlocking mesh-like ECM network conformation, as seen in native tissues.

All starting materials exhibited a broad peak during the first thermogram from 0.00 to 125.00 °C (Figure 1C) signifying the release of molecular water due to the rupturing of hydrogen bonds between the water molecules and the proteins around
100.50, 100.20, and 90.60 °C for collagen, elastin, and GelMA, respectively.\textsuperscript{53,54} Considering the second DSC thermograms in Figure 1D, collagen has two endothermic peaks at 224.08 °C (B) and 272.07 °C (C) signifying the conformational change and glass transition temperature from the triple helix structure to random coil and the bulk loss of mechanical integrity (degradation), respectively.\textsuperscript{53,55,56} GelMA has an endothermic peak at 225.14 °C (D) signifying the degradation of the gelatin matrix.\textsuperscript{57} Elastin has a thermal transition at 223.32 °C (A) signifying the transition from a glassy to a rubberier state (glass transition temperature: $T_g$).\textsuperscript{56} All starting materials have overlapping peaks around $\sim$220.00 °C attributed to the peptide nature of all materials.

Considering the thermograms of the GCE (no crosslinkers present) and IPN systems, all systems had a broad peak around 94.00, 93.80, and 95.10 °C for GCE, semi IPN, and full IPN hydrogels, respectively during the first DSC thermogram signifying the release of molecular water (Figure 1E). This finding was expected as this feature is found in all thermograms of the starting materials. More crucial information was generated during the second thermogram, where both IPN systems as well as the blend with no crosslinkers had a single endothermic

Figure 1. (A) Visual representation and relative size of the white, semi IPN (left), and the blue, full IPN (right). (B) FTIR spectra of the comparison between the starting material and the two IPN biomaterials. From the spectra, it is evident that, despite the small wavenumber band shifts, the addition of the crosslinking agents had no effect on the appearance of the spectra. (C) DSC thermograms for all materials during the first run from 0.00 to 125.00 °C. (D) Indication of the thermal transitions of the starting materials and (E) indication of the thermal transitions of the semi and full IPN biomaterials as well as the biomaterial without any crosslinker (GCE). (F) TGA thermograms depicting the weight changes as a function of temperature for GelMA, the semi IPN, and full IPN hydrogels. (G) XRD diffractograms of the starting materials and the IPN biomaterials. “A” indicates the peak considered for analysis.
peak at different temperatures. The endotherm was attributed to the denaturation of the peptide matrices. The endotherm peak of the GCE system, semi IPN, and full IPN occurred at 183.60 °C (H), 140.59 °C (G), and 168.18 °C (F), respectively (Figure 1E). The GCE finding could potentially indicate that mixing the peptides with the polymer causes a decrease in the endothermic peak likely due to the penetration of the peptides into the GelMA network.57 Considering the IPN biomaterials, the full IPN system required more energy to break the bonds due to the presence of a double crosslinking mechanism. This melting point increase was attributed to increased thermal stability of the system,58,59 which was used to confirm the formation of a full IPN.

The TGA thermograms (Figure 1F) were then correlated with the thermal events obtained from the DSC thermograms. All TGA thermograms experience minimal loss of weight, occurring around 100.00 °C. This step correlates with the dehydration process and loss of the residual molecular water entrapped in the GelMA and peptide backbones in both the GelMA polymer and IPN scaffolds. Following this, the greatest loss of weight in the IPN scaffolds is observed, which was attributed to the degradation of the GelMA and peptide matrix in all samples. For the 3D-printed IPN hydrogels, ~64 and ~67% loss of weight is observed for the semi IPN and full IPN, respectively. This loss of weight is observed between approximately 175.00–475.00 °C and approximately 200.00–500.00 °C for the semi IPN and full IPN, respectively. Because the DSC melting point endotherms of the IPN biomaterials were within 30.00 °C of each other, it is no surprise that the TGA thermograms present with a similar appearance between the two IPN hydrogels, with spectra that are close together (Figure 1F). Despite this, there is a temperature shift between the two IPN systems. Furthermore, the DSC and TGA data correlate because there is a slight temperature shift toward a lower temperature for the semi IPN in comparison to the full IPN. This shift indicates that the protein stability in the semi IPN is less than that of the full IPN and, therefore, further confirms the formation of two IPN hydrogel systems.

### 2.2. Confirmation of the Amorphous Nature of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds

The X-ray diffractogram of the GelMA polymer exhibits a spectral profile very similar to that of gelatin, which was expected because the conjugated group (methacrylate group) is small in comparison to the gelatin backbone.60,61 Despite the crystallinity of gelatin, during functionalization and formulation, the resulting GelMA polymer exhibits an amorphous structure (Figure 1G). In terms of the X-ray diffractograms of collagen and elastin, both peptides are known to produce broad, amorphous peak patterns,24,62,63 which was confirmed in this study (Figure 1G). From the GCE diffractogram, it is evident that the amorphous structure of the GelMA polymer is maintained over the 2θ angle range (Figure 1G). This trend was further noted for the semi and full IPN diffractogram patterns. This indicates that the addition of the peptides to the GelMA polymer does not affect the profile.

For analysis, only peaks around 20.00° were considered as this peak is largely indicative of the GelMA amorphous nature.64 The amorphous nature of the samples was confirmed by the high full width at half-maximum (fwhm) values of 12.30, 9.30, 7.82, and 7.67 obtained for the GelMA polymer, GCE, full IPN, and semi IPN, respectively. A similar high fwhm was observed for both peptides. The fwhm is understood to indicate that the higher the value the broader the peak, the more amorphous/disordered the structure. Interestingly, over the 2θ angle range, all samples exhibited an amorphous halo, confirming the fwhm values as an indicator of a decrease in crystallinity. Despite all samples being amorphous, GCE, as well as both IPN biomaterials are more crystalline than the GelMA polymer due to the increase in the observed intensity. Furthermore, there was a notable difference in the intensities between the semi and full IPN biomaterial systems. This was attributed to the increase in crosslinking in the full IPN relative to the semi IPN, which induces an increase in the amorphous properties of the material.65 Also worthy of noting, the structure of the crosslinking agents is different, and hence could explain the intensity differences between the two IPN structures because the crystal structures between the crosslinkers differ.66 Furthermore, the crosslinking ratio is increased in the full IPN in comparison to the semi IPN and may further contribute to the decrease in the observed crystallinity.66 These results are in agreement with the DSC and TGA findings and further confirm the formation of both a semi and full IPN system.

### 2.3. Real-Time Viscoelastic Properties, Material Stiffness, and Hydration of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds Using a Constant Frequency

Viscoelastic elastic gelation studies were carried out on both the IPN systems without the radical polymer crosslinker to assess whether the physical crosslinking and the shear storage modulus G′ of the GelMA would be affected by the addition of the peptides. As previously noted,67 in the GelMA polymer, the methacrylamide content of modified gelatin chains obstructs the coil–helix transition (fewer helices are present), which results in the formation of weaker hydrogels.67 Despite forming weaker hydrogels, it is shown that the addition of the peptides to the GelMA polymer did not affect the ability of the coil–helix transition to occur, and therefore the hydrogel IPN systems were able to physically crosslink at 10 °C (Figure 2A). Therefore, both IPN systems can be considered “smart” polymer systems as they respond to changes in temperature.68 The semi IPN was shown to have the highest shear storage modulus G′ (∼9195.50 Pa), followed by the GelMA polymer (∼7797.60 Pa) and the full IPN (∼6317.60 Pa) (Figure 2A). In the case of the semi IPN, it is proposed that the protein combinations could be forming native bodily protein networks, and hence could explain the increase in the G′ values, which could support the development of an eECM hydrogel scaffold. The lower G′ values in the full IPN could be explained by the higher degree of cross-linking, which can create brittle structures resulting in less elasticity.24 Viscoelastic studies for all samples indicate that G′ > G″, and therefore indicate that the gels are more “solid” in nature. From Figure 2B, it is evident that the semi IPN had the highest shear loss modulus G″ (∼533.30 Pa), followed by the GelMA polymer (∼304.10 Pa) and the full IPN (∼234.90 Pa) had the lowest shear loss modulus G″. Following gelation, the IPN hydrogel disks were removed and photographed. From Figure 2C, it is evident that the semi IPN has a higher elasticity, and it can retain its structure even after being deformed several times (n > 5), thereby confirming the elasticity (G″) results, whereas the full IPN is less elastic, and therefore does not hold its shape (Figure 2C). No deformation of the full IPN system was possible validating the lower G″ values observed during the viscoelastic studies.
full IPN was 577.00 and 544.00 Pa, respectively. This indicates that prior hydration of the samples had no effect on the final material stiffness of the samples (Figure S1). Because brain tissue is known to be around the order of 1 kPa69,70 and is known to soften with age,71 slight biomaterial stiffness enhancements may need to occur to accurately mimic the stiffness of the brain at the patient’s age.

2.4. Validation of the Formation of a Morphological and Physiological Neuromimicking Platform. The SEM image results indicated that both the semi and full IPN systems have an ordered, porous microstructure consistent with the structure of the GelMA polymer. This indicates that cross-linking, lyophilization, and the 3D printing process did not alter the material porosity as there was no difference in the pore distribution between the samples. Although both IPN systems are crosslinked using the same photoinitiator, the full IPN system has a smaller pore structure than the semi IPN72 as well as an increased pore density. This was proposed to be because of the increased stability of the proteins after Genipin crosslinking. Furthermore, the pores in both systems form “pockets” that do not penetrate through the system but show and interconnected pattern around the “pockets” that are separated by thin walls.73 Both systems have a fibrous-like network evident over the pores, which could be attributed to the elastin and collagen protein fibers (Figure 3A,B).

If an electron micrograph of the rat cortex is considered (Figure 3C), it is evident that the cortex contains the neural cells surrounded by the extracellular space (ECS), the space where the ECM is found (red channels).74 Upon close examination, it is evident that the morphological features of both biomaterial IPN scaffolds share a significant similarity to the rat cortex. Just as the cells are surrounded by the channels of the ECS, so too are the “pockets” of the IPN biomaterials surrounded by channels. This finding confirms the formation of a neuromimicking platform with respect to the scaffold morphology. Despite the morphological similarities between the biomaterials and the rat cortex, there are several physiological similarities between the brain and the IPN biomaterials. To adequately mimic the brain, the IPN biomaterials are a soft material interwoven with elastin and collagen chains. This is similar to the brain as the brain is a soft, networked organ comprised of intertwining neurons and glial cells.74 Despite the neural cells possessing individual characteristics and shapes, they remain distinctive entities that are separated by the ECS.74 This is similar to the IPN biomaterials as the polymers in the networks retain their individual characteristics, which has contributed to the slight differences in pore structures shown in Figure 3C.

2.5. Degradation, Fluid Uptake, and Swelling Profiles of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. Because GelMA is a derivative of gelatin, GelMA is a hydrophilic polymer that forms a hydrogel of hydrophilic networks, which allows for the biodegradation of the network by the uptake of the surrounding fluid.75 Folded collagen has been reported to have a largely hydrophobic side opposite a side with multiple hydrophilic lysine residues.76 Similar to this, elastin contains alternating hydrophilic and hydrophobic domains (crosslinkable lysine sites).77 In this way, both peptides are important considerations in aECM biomaterials as they exhibit good aqueous chemical compatibility and controlled biodegradation.

Because biodegradation influences the repair and regeneration of the native tissue, both systems exhibited a consistent

For tissue engineering applications, the hydrogel material must be stiff enough to promote and mimic the native microenvironment but not too strong that a secondary injury is caused. Material stiffness results indicated that the material stiffness of both the IPN systems were within a small margin of each other. The average material stiffness of the nonhydrated and hydrated semi IPN was 575.00 and 597.00 Pa, respectively. The average material stiffness of the nonhydrated and hydrated

Figure 2. (A) Kinetic gelation, shown as the shear storage modulus ($G'$), of the semi and full IPN (without Irgacure 2959) relative to the GelMA polymer alone. (B) Shear loss modulus of the semi and full IPN relative to the GelMA polymer alone. In all spectra, the semi IPN is shown in green, the full IPN is shown in red, and the GelMA polymer is shown in black. (C) Indication of the elastic ability of the disks after 4 h of hydration in aCSF. The semi IPN is shown at the top and the full IPN is shown at the bottom.
degradation rate over 3 and 6 days for the full and semi IPN (Figure 3D), respectively, after which the degradation increases. It has been reported in the literature that the higher the degree of substitution (DoS) of the GelMA polymer, the slower the degradation rate.\textsuperscript{78} Furthermore, 10% GelMA polymer alone has a degradation of 65 \(\pm\) 3\% over 7 days.\textsuperscript{79} Despite the GelMA DoS being high in this work, the semi and full IPN exhibited an increased biodegradation rate of 8 and 6 days, respectively. However, the semi IPN degrades slightly slower than the full IPN. The increased rate of degradation of the two IPN systems in comparison to the GelMA polymer alone is attributed to the presence of the two peptides as they are easily degraded by the artificial cerebral spinal fluid (aCSF).\textsuperscript{79,80} Furthermore, the full IPN performed worse than the semi IPN—a finding that presents an anomaly because higher crosslinking systems have a higher stability and are expected to degrade at a slower rate, which in this case was not observed. This finding may be because increasing the crosslinking density could have embrittled the full IPN biomaterial.\textsuperscript{25} Therefore, when the network structure began to degrade, the brittle structure collapses and an increased degradation rate was observed. Furthermore, although unlikely based on the results generated through DSC, XRD, and TGA, incomplete UV crosslinking due to the intense royal blue color of the Genipin could have occurred resulting in the poor performance of the biomaterial.

In terms of the fluid uptake, the full IPN exhibits almost 1.50 times greater fluid uptake ability than the semi IPN. After 8 h, the semi IPN and full IPN had a fluid uptake of 483.00 and 725.60\%, respectively, in comparison to their initial dry masses (Figure S2). In terms of the swelling properties as seen in Figure 3E, the full IPN swells to its maximum swelling ratio after 8 h, whereas the semi IPN swells to its maximum swelling ratio after 1 day. Furthermore, over the course of the experiment, both the IPN systems experience minor fluctuations around this maximum until fully degraded. This is a vital finding as the IPN biomaterials will not cause secondary damage if implanted in the swollen state as no further material swelling will occur. A similar trend to the fluid uptake was noted as the full IPN exhibited around 1.34 times more swelling than the semi IPN (Figure 3E). If the structure of Genipin is considered, it is noted that a couple of \(\cdots\)OH functional groups are present, therefore increasing the hydrophilicity of the system increased fluid uptake, and swelling is possible.\textsuperscript{81} Furthermore, the increased porosity as seen from the SEM micrographs could further improve the fluid uptake and swelling properties of the full IPN.

2.6. Determination of Neurocompatibility and Neuronal Cell Proliferation. The MTT proliferation studies indicated that the 3D-printed IPN scaffolds could support the growth of PC12 cells compared to the control over a 72 h period. This confirmed the neurocompatibility of the 3D-printed IPN scaffolds as displayed in Figure 4A. The full IPN scaffold exhibited an increased cell viability, attributed to the neuroprotective effects of Genipin.\textsuperscript{82} Furthermore, both IPN systems indicated a higher proliferation to GelMA alone. This was accredited to the morphological similarities between the IPN scaffolds and the rat cortex and ECM. The MTT results indicate that both 3D-printed IPN scaffolds would be safe for in vivo implantation.

2.7. Analysis of the Nuclear Morphology and Migration in Response to the 3D-Printed IPN Scaffolds. From the MTT results above, it is evident that the 3D-printed IPN scaffolds promote an increased PC12 cell viability indicating that a higher number of healthy PC12 cells are present than in the GelMA polymer alone. Furthermore, a
higher cell viability is indicative of an increased cellular activity and from this, it is postulated that the cells are in a proliferative state. This finding is supported by the nuclear area. Because the data were captured as the number of pixels in each region, in this case the nucleus, the greater the number of pixels the greater the area of the nucleus. Because the nuclear volume is known to increase in cells undergoing mitosis up until division, a change in the area will directly impact the nuclear volume. If Figure 4B is considered, both IPN 3D-printed scaffolds promote a greater nuclear area in the PC12 cells, and therefore by default a greater nuclear volume with the increase in leachable time points. This indicates that the components leaching out of the scaffolds are nontoxic as the PC12 cells can survive and flourish in the absence of any physical cues (attachment to the physical scaffold). This could further indicate that the tissue surrounding the scaffold once implanted will not be affected by any chemical leaching and in fact, taken with the viability data, could assist with tissue integration between the scaffolds and the surrounding brain tissue.

Considering the eccentricity of the nucleus, eccentricity is defined as the measure of the circularity or flatness of an object. A sphere has an eccentricity of zero, while a pancake shape would have an eccentricity of one. In the cell cycle, eccentricity of the nucleus is known to increase after cell division has taken place. Figure 4C demonstrates the eccentricity is closer to 1 in all leaching time points after the 24 h incubation. This could indicate that the PC12 cells have completed the cell cycle and further supports the idea of the PC12 cells being in a proliferative state. Because there is an insufficient difference, a 99% confidence level between the eccentricity of the 1 and 24 h leaching periods, the slight difference in eccentricity could be explained by the neuro-related effects, both neuroprotective and neurotrophic, of Genipin. After a 1 h leach, it is likely that the neuroprotective effects of Genipin are observable whereby proliferation is slightly arrested but a high cell viability is maintained. Following this, it is possible that the 24 and 72 h leach then results in the neurotrophic effects of Genipin, wherein proliferation is stimulated. These results, taken with the high cell viability, indicate that the full IPN could be a beneficial treatment for TBI because the Genipin constituent could assist in initially preventing further secondary brain damage (neuroprotective effects reducing apoptosis), while promoting proliferation to initiate the regenerative process.

Figure 5A,B demonstrates that the nuclei stained with DAPI are not rounded but are more elliptical in shape (eccentricity closer to 1), thereby further supporting the idea of proliferating cells. Furthermore, Figure 5C demonstrates the difference between immature and mature PC12 cells. It is evident that as the cells mature, there is a formational change in the neuroplasm. If the cells in Figure 5C are considered, it is evident that the cells have undergone maturation as the cells have transitioned from a circular morphology to an irregular morphology due to the changes in the neuroplasm. This indicates that the cells were able to mature in the presence of the IPN biomaterials.

Figure 5D demonstrates that PC12 cellular migration was promoted in both 3D-printed IPN scaffolds after 24 h (cell cluster populations indicated by the yellow arrows). For this analysis, PC12 cells were stained with DAPI and MitoTracker Orange to monitor the nuclei and mitochondria, respectively. Cellular migration is promoted when cells interact with the ECM. Therefore, with evidence of migration, it is plausible that the fabrication of an aECM was successful. This is because biophysical, topographical, and chemical cues were provided by the aECM to the PC12 cells. Furthermore, the morphological similarities between the IPN scaffolds and the rat cortex could further promote cellular migration, which could positively influence neural tissue regeneration.

2.8. Antimicrobial Activity of Genipin. Table 2 summarizes the MBC and MFC values obtained for the nine
pathogens in the study. All MBC and MFC values obtained were 10.00 mg/mL or below demonstrating notable activity. These results indicate that the concentration of Genipin (10.00 mg/mL) blended in the full IPN is sufficient to exhibit microbicidal activity. Genipin has demonstrated antimicrobial properties and prevents the growth of selected pathogens as indicated herein associated with postsurgical brain infections. Initial screening of the IPNs against *Staphylococcus aureus* indicated that the semi IPN and Irgacure 2959 displayed no antibacterial activity (MBC > 40.00 mg/mL, SD: ±0.00) as expected, however, the Genipin individually and full IPN both displayed antimicrobial activities (MBC of 5.00 mg/mL, SD: ±0.00). These results proved that Genipin was the compound responsible for the antimicrobial properties. The Genipin individually was then carried forward to be tested by the eight other pathogens. Of the implicated meningitis causing pathogens namely, *Streptococcus agalactiae*, *Hemophilus influenzae*, and *Cryptococcus neoformans*, two pathogens (*H. influenzae* and *C. neoformans*) displayed the lowest MBC and MFC values in comparison to the other opportunistic pathogens. Because the outcomes for meningitis infections caused by these pathogens are generally considered to be poor, this result is important as the Genipin component of the full IPN scaffolds may assist in improving patient outcomes post TBI. The culture control displayed optimal growth for all the test pathogens. The negative control displayed clear wells indicating the diluent did not have any antimicrobial properties.

**2.9. Final Formulation.** Table 3 provides a summary of the optimized 3D printing parameters, scaffold design parameters, polymer and crosslinker concentrations, and postprocessing parameters presented herein for successful fabrication of the aECM biomaterial IPN scaffolds.

### 3. CONCLUSIONS

This study assessed the potential of two novel peptide-based 3D-printable biomaterials composed of GelMA, collagen, and elastin as potential novel biomaterials for neural tissue engineering applications. This study developed two IPN systems, which exhibited 3D printability at the optimized parameters. Through DSC, TGA, and XRD analyses, the formation of both a semi and full IPN system was confirmed as the full IPN was shown to have an increased thermal stability and a decreased amorphous nature in comparison to the semi IPN. Molecular vibrations indicated that the addition of the peptides to the GelMA polymer, during the formation of both IPN systems, retains the GelMA profile. Furthermore, small shifts in the wave number were suggestive of a molecular water interaction with the peptides in the biomaterials. Additionally, the crosslinkers were shown to have no effect on the native structure of the peptides nor does the 2 min of UV exposure affect the integrity of the peptide backbone. The real-time mechanical stiffness analysis indicated that the IPN systems, currently, could be utilized in developing scaffolds for several...
Table 2. Mean Microbicidal Activity (MBC Values in mg/mL) of Genipin against Six Bacterial Test Pathogens (n = 5)

| Gram positive strains | S. aureus (ATCC 25923) | L. monocytogenes (ATCC 19111) | S. agalactiae (ATCC 27956) | Ave. Genipin MBC value (mg/mL) and SD | Ave. positive control MBC (μg/mL) and SD | Ave. negative control MBC (μg/mL) and SD | Ave. culture control MBC (mg/mL) and SD |
|-----------------------|-------------------------|--------------------------------|-----------------------------|--------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| Ave. MBC value (μg/mL) and SD | 5.00 ± 0.00 | 5.00 ± 0.00 | 5.00 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.94 ± 0.31 | 4.00 ± 0.00 | 4.00 ± 0.00 |
| Ave. positive control MBC (μg/mL) and SD | 0.10 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 |
| Ave. negative control MBC (μg/mL) and SD | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 |
| Ave. culture control MBC (mg/mL) and SD | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.08 ± 0.00 |

Table 3. Processing Parameters for the Optimized IPN Biomaterials

| parameter | value |
|-----------|-------|
| GelMA concentration (% w/v) | 10.00 |
| collagen concentration (% w/v) | 0.50 |
| elastin concentration (% w/v) | 1.00 |
| cartridge temperature (°C) | 18.00 |
| platform temperature (°C) | 13.00 |
| plotting speed (mm/s) | 10.00–20.00 |
| internal bore of needle (mm) | 0.20 |
| dimensions of scaffold (mm) | 10.00 × 10.00 × 10.00 |
| scaffold design | rectilinear |
| material temperature equilibration (min) | 30.00 |
| Irgacure concentration (% w/v) | 0.50 |
| Genipin concentration—full IPN only (% w/v) | 1.00 |
| UV crossing time (min) | 2.00 |
| lyophilization time (h) | 24.00 |

Soft tissues such as the brain. However, because the brain stiffness can change with age, possible biomaterial modifications may need to occur to accurately mimic the stiffness of the patient’s brain at the time of injury. The full IPN presented an anomaly in terms of the degradation profile because an increased degradation rate was observed. It is recommended that further studies be performed to ascertain the reason for the anomaly. The SEM imaging indicated that the fabricated IPN scaffolds have a significant morphological and physiological similarity to the native rat cortex. Both IPN scaffolds promoted PC12 survival and cellular migration. Furthermore, through nuclei eccentricity and area studies of the PC12 cells, it was postulated that the cells were in a proliferative state. Despite this, it is advisable that further studies are conducted to ensure the proliferation of cells is in order with healthy tissue ranges. Finally, the full IPN was observed to have potential microbicidal effects against nine bacterial pathogens associated with post-surgical brain infections. It is recommended that further studies are conducted in other pathogens associated with brain infections to confirm these findings. It is concluded that a biomaterial composed of GelMA, collagen, and elastin in different crosslinked states is a suitable candidate for potential tissue engineering applications.

4. EXPERIMENTAL SECTION

4.1. Materials. Gelatin (Bloom 160, type B) derived from bovine skin was purchased from Fluka (Steinheim, Germany). Methacrylic anhydride (94%) containing 2000 ppm topanol A, collagen derived from bovine Achilles tendon, phosphate buffered saline tablets (pH 7.4), high retention cellulose dialysis tubing (MW cut off 12,400 Da), glacial acetic acid, acetone, ciprofloxacin, DAPI, and the radical photoinitiator 2-hydroxy-4’- (2-hydroxyethoxy)-2-methylpropophenone (Irgacure 2959, MW 224.25 g/mol, purity 98%) were purchased from Sigma-Aldrich (St Louis, MI, USA). Genipin was purchased from Challenge Bioproducts Co. Ltd. (Yun-Lin Hsien, Taiwan). Elastin derived from bovine neck ligament was purchased from Elastin Products Co., Inc (Owensville, MO, USA). Glycine (MW: 75.07 g/mol) was purchased from Merck (Merck KGaA, Darmstadt, Germany). DAPI and MitoTracker Orange were purchased from Thermo Fisher Scientific (Johannesburg, South Africa). aCSF (pH 7.40) was chosen as the hydration microenvironment and prepared using the method reported by Baumann et al., 2010: 1.40 mM CaCl2, 148.00 mM NaCl, 1.50 mM Na2HPO4, 0.20 mM NaH2PO4, 0.80 mM MgCl2, and 3.00 mM KCl.

All cultures that were used are laboratory reference strains of the American Type Culture Collection (ATCC) that were originally obtained from Davis Diagnostic (Johannesburg, South Africa) and available in the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg. The micro-organisms were selected for evaluation in this study due to their implication in brain infections. The bacterial micro-organisms were S. aureus (ATCC 25923), Listeria monocytogenes (ATCC 19111), and S. agalactiae (ATCC 27956), which are Gram-positive strains, and Escherichia coli (ATCC 8379), Pseudomonas aeruginosa (ATCC 27853), Klebsiella pneumoniae (ATCC 13887), H. influenzae (ATCC 19418), and Serratia marcescens (ATCC 14756), which are Gram-negative strains. The fungal micro-organism that was selected for this study was C. neoformans (ATCC 14113). Tryptone Soya (broth and agar), Sabouraud Dextrose (broth and agar), Mueller Hinton Broth, yeast extract, Hemophilus Supplement, and Hemophilus Test Medium Base were prepared using specified media.
obtained from Oxoide (Thermo Fisher Scientific, Johannesburg, South Africa). All chemicals were used as received and were of analytical grade.

4.2. Synthesis of the Neuroplatform. 4.2.1. Synthesis of Gelatin Methacryloyl (GelMA). GelMA was synthesized as previously reported. In brief, methacrylic anhydride was added in a dropwise manner (0.50 mL/min) to a 10% (w/v) solution of gelatin in PBS (pH 7.4) under constant stirring at 50 °C. To attain a high degree of functionalization in the polymer, 0.60 g of methacrylic anhydride was added per gram of gelatin. The reaction was left to react for 3 h to increase the degree of functionalization. The resulting polymer was then dialyzed against distilled water at 40 °C for 5 days to remove methacrylic acid and unreacted anhydride. The GelMA was frozen at −80 °C for 24 h and then lyophilized for 24 h to obtain a porous, white sponge. The polymer was stored at −20 °C before use. The degree of substitution of GelMA was determined utilizing a previously described method, and was ascertained to be about 95%. Finally, functionalization of the lysozyme residues was confirmed by 1H NMR analysis.

4.2.2. Preparation of the Semi IPN 3D-Printed Biomaterial Hydrogel. To prepare the semi IPN hydrogel, 0.5% collagen solution was heated to 37 °C under constant stirring. Under dark conditions, 10% GelMA, 1% elastin, and 0.5% Irgacure 2959 were added to the peptide mixture and rapidly mixed until homogenous. The stirring speed was reduced for 1 h to mitigate excess foam formation. Following this, the biomaterial was placed into a UV chamber set to 365 nm and exposed to UV light for 2 min for GelMA crosslinking. The biomaterial was placed at −80 °C for 24 h and then lyophilized for 24 h. Samples were stored at −20 °C until used.

4.2.3. Preparation of the Full IPN 3D-Printed Biomaterial Hydrogel. Under constant stirring at 37 °C, the full IPN hydrogel was prepared by allowing 0.5% collagen solution, 1% elastin, 10% GelMA, and 1% Genipin to chemically crosslink for 2 h at 37 °C. This ensured that the collagen and elastin fibers would form a network in the presence of the GelMA polymer to meet the requirement for the formation of a full IPN. Under dark conditions, 0.5% Irgacure 2959 was then added to the mixture and rapidly mixed until it becomes homogenous. The stirring speed was reduced for 1 h to mitigate excess foam formation. Following this, the biomaterial was placed into an ultraviolet (UV) chamber set to 365 nm and exposed to UV light for 2 min for GelMA crosslinking. The biomaterial was placed at −80 °C for 24 h and then lyophilized for 24 h. Samples were stored at −20 °C until used.

4.3. 3D Printing of the IPN Biomaterial Hydrogel Scaffolds. A standard rectilinear scaffold structure (0–90° cross hatch), 10 × 10 mm with varying heights, was designed using the Magics V18 design software. The two different IPN biomaterial formulations were prepared as previously stated and was then loaded into an amber barrel (30 cm³), low-temperature dispensing head (30 mL) extrusion printing cartridge and a standard rectilinear 10 × 10 × 10 mm scaffold structure (0–90° cross hatch), designed using the Magics V18 design software, was 3D-printed using a standard Envisiontec 3D Bioplotter (Envisiontec GmbH, Gladbeck, Germany) at 18 °C. The cartridge was kept at 18 °C for 30 min to allow for material–temperature equilibration (biomaterial gelation). The IPN biomaterial scaffolds were then printed onto a cold platform through a 0.20 mm bore needle at a predetermined pressure and speed. After completion of the printing, the scaffold was immediately placed into a UV chamber set to 365 nm and exposed to UV light for 2 min. The scaffolds were then placed at −80 °C for 24 h and then lyophilized for 24 h. Scaffolds were stored at −20 °C until used.

4.4. Physicochemical Characterization of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. 4.4.1. Analysis of the Molecular Vibration Transitions of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. The FTIR spectra of the lyophilized biomaterials and pristine polymers were analyzed using a PerkinElmer Spectrum 2000 ATR-FTIR (PerkinElmer100, Wales, UK) to identify molecular transitions and interactions. The FTIR conditions were set as follows: wave range 4000–650 cm⁻¹, 20 scans per spectrum with a resolution of 4 cm⁻¹. The spectrometer was fitted with a diamond, single-reflection MIRTGs detector.

4.4.2. Thermophysical Characterization of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. The thermal properties of the lyophilized biomaterials, GelMA, and pristine peptides were analyzed using a differential scanning calorimeter (Mettler Toledo DSC, STARes system, Schwerzenbach, Switzerland). The samples (between 3.00 and 10.00 mg) were sealed in aluminum crucibles and a small hole was pierced into the crucible lids to allow for pressure dissipation. Samples were subjected to two cycles of heating at a rate of 10 °C/min under constant nitrogen gas flow: (1) 0–125 °C to allow for the molecular water to be released and (2) 0–300 °C for analysis. The thermograms were then plotted as heat flow against temperature. Determining the percentage weight change as a function of temperature was performed using a thermovinmetric analyzer (TGA) (PerkinElmer, TGA 4000, Llantrisant, Wales, UK). Samples were heated at a rate of 10 °C/min from 30 to 900 °C under continuous nitrogen purging. Thermograms were generated as percentage weight versus temperature.

4.4.3. Crystallinity of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. Due to the static nature of the GelMA polymer, the lyophilized sponges of the biomaterials as well as the GelMA polymer were first flattened and then cut into a circular shape using steel equipment (to reduce interfering fibers) to mimic the shape of the sample holder. The peptides were also analyzed in the powder form. All materials were fixed to the sample holder using double-sided tape. The phases and crystallinity of the samples were then determined using a benchtop Rigaku MiniFlex 600 X-ray diffractometer (Rigaku, Japan) with Cu Kα radiation at 15 mA and 40 kV. The parameters were as follows: 2θ range between 5 and 60° at a scan rate of S°/min.

4.5. Physicomechanical Characterization of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. 4.5.1. Viscoelastic Properties and Real-Time Material Stiffness Assessments of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. The IPN hydrogels were prepared (without crosslinkers) as previously stated and subsequently cooled to 20 °C on an ice bath. The viscoelastic properties of each IPN hydrogel were assessed using the ElastoSens Bio (Rheolution, Montreal, Canada). In brief, following the instrument system calibration, 4.00 mL of the biomaterial (20 °C) was syringed into the sample holders and then placed into the thermal chamber for analysis. The analysis was performed for 4 h at a constant temperature of 10 °C with a soak time and temporal step of 30.00 s. All analyses were performed in triplicate (n = 3).

To assess the soft mechanical properties, a novel real-time experiment was conducted in two ways, whereby lyophilized
disks of the biomaterial \((n = 3)\) were (1) placed in the sample holders and 2.00 mL aCSF was placed over the disks and the material stiffness during hydration \((G')\) was monitored for 8 h at 25 °C and (2) lyophilized disks of the IPN systems were placed in the sample holders and 2.00 mL aCSF was placed over the disks and samples were allowed to swell to saturation for 8 h prior to analysis and the material stiffness \((G')\) was monitored for 8 h at 25 °C. The soak time and temporal step were the same as for the viscoelastic studies.

4.6. Morphological Analysis of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. 4.6.1. Scanning Electron Microscopy for the Mapping of Surface Morphology of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. SEM and energy dispersive spectroscopy (EDS) analysis were performed on both IPN systems using the Zeiss Sigma 03-39 field emission scanning electron microscope (Zeiss, Oberkochen, Germany) with an accelerating voltage of 0.02–30.00 kV. Prior to analysis, 3D-printed scaffolds were mounted, using double sided tape, onto aluminum stubs and coated with two coats of gold palladium (AuPd).

4.7. Architectural Performance of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. 4.7.1. Degradation, Fluid Uptake, and Swelling Studies. Material hydration due to fluid uptake and degradation was performed on both IPN biomaterials under simulated in vitro conditions. The IPN biomaterials were prepared as above. Briefly, 1.00 mL of the material was placed into each well of a 48-well plate, crosslinked, frozen at −80 °C, and lyophilized for 24 h. The disk was then removed and placed in a 24-well plate \((n = 5)\) with 2.00 mL of aCSF. The plate was then placed into an orbital shaking incubator (LM-530-2, MRC Laboratory Instruments Ltd., Holon, Israel) set at 37 °C and 25 rpm for 8 h. Swelling equilibrium was reached within the 8 h, where excess fluid was blotted before measurement. The mass swelling ratio, fluid uptake, and degradation were calculated using eqs 1−3, respectively.

\[
\text{Mass swelling Ratio} = \frac{W_s}{W_i} \tag{1}
\]

\[
\% \text{fluid uptake} = \left(\frac{W_s - W_i}{W_i}\right) \times 100 \tag{2}
\]

\[
\% \text{degradation} = \left(\frac{W_i - W_d}{W_i}\right) \times 100 \tag{3}
\]

where \(W_s\), \(W_i\), and \(W_d\) are the swollen blotted mass, dry initial mass, and the postdry mass of the IPN biomaterials, respectively.

4.7.2. In Vitro Cell Analysis of the 3D-Printed IPN Biomaterial Hydrogel Scaffolds. 4.7.2.1. PC12 Cell Culture and MTT Proliferation Assay. Adherent rat adrenal gland pheochromocytoma PC12 cell line from Cellonex (Separations, South Africa) was cultured in tissue culture-treated (TPP) T-75 flasks using DMEM supplemented 5% (v/v) FBS and 1% (v/v) P/S/AB solution in a humid 5% CO₂ atmosphere at 37 °C. The culture medium was replaced every 2 days.

For the detection of cell proliferation and cytocompatibility of the 3D-printed scaffolds, the MTT-based Roche cell proliferation kit I was utilized. The IPN scaffold was prepared as previously indicated; however before UV crosslinking, the hydrogels were washed several times with 0.5% acetic acid and then capped in 2% glycine for 15 min to prevent ketone cytotoxicity from the exposed ketone groups on the radical crosslinker. Samples of size 1 cm × 1 cm × 1 cm were sterilized under UV light (256 nm) for 12 h before overnight incubation with cells in 100.00 μL culture medium containing 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) P/S/AB (penicillin/streptomycin/amphotericin B) in a 96-well plate maintained at 37 °C at 5% CO₂. PC12 cells were seeded into a 96-well plate at a density of 7.50 × 10⁵ cells/well and incubated for 24, 48, and 72 h. Thereafter, the culture medium was removed, and each well was washed once with PBS before 100.00 μL culture medium was returned to each well. Following this, 12.00 μL MTT solution was added to each well, followed by a further 4 h incubation period after which the culture medium was removed and 100.00 μL solubilizing agent was added to dissolve the formazan crystals. The 96-well plate was then measured for absorbance at 570 nm, with a background subtraction at 690 nm, using a multiplate reader (BioTek, USA).

Relative cell viability was calculated as follows

\[
\text{cell viability} (%) = \frac{A_{\text{sample}}}{A_{\text{positive control}}} \times 100 \tag{4}
\]

where \(A_{\text{sample}}\) is the absorbance of the treated cells and \(A_{\text{positive control}}\) is the absorbance of the positive control (the absence of treatment).

4.7.2.2. Evaluation of Nuclear Morphology and Migration in Response to 3D-Printed Scaffolds. A leachable and extractable assay was performed to assess the nuclear morphological response of PC12 cells to the extracted components (chemical cues) of the IPN scaffolds over a period of 24 h. This would give an indication if the cells were able to flourish in the absence of physical cues (the physical scaffold for anchorage etc.). Briefly, both IPN scaffolds were weighed out to a concentration of 0.02 g/mL of media, sterilized overnight (265 nm) and then mixed with growth media, and incubated at 37 °C for 1 h. After the 1 h leach time point, any residual scaffolds were removed from the media and the resulting solution was filtered to remove any particulate matter. The PC12 cells were seeded at a density of 1.00 × 10⁵ cells/well into a 96-well plate and 100.00 μL culture medium containing 5% (v/v) FBS and 1% (v/v) P/S/AB and left to attach overnight at 37 °C at 5% CO₂ in a humid incubator. The culture medium was then aspirated off and the cells were treated with the filtered solution for 24 h at 37 °C at 5% CO₂ in a humid incubator. Following this, the filtered solution was removed, the cells were washed twice with PBS, and then 100.00 μL culture medium containing 300 nM MitoTracker Orange was placed in each well and left to incubate for 45 min at 37 °C at 5% CO₂ in a humid incubator. The media were then removed, and all wells were washed with PBS to remove any unbound MitoTracker Orange. The cells were then fixed with 5% formaldehyde in PBS for 20 min. The cells were then incubated with 1.00 μg/mL solution of DAPI for 15 min. The cells were washed several times with PBS to remove unbound DAPI and reduce the fluorescence of the background. Plates were kept in the dark until analysis. Plates were analyzed on the Logos Biosystem CELENA X High Content Imaging System and all further analyses were performed using their proprietary software. Using the Logos Biosystem software, a pipeline was set up to determine the nuclei count, eccentricity, and area. The following definitions were used:
The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) are defined as the lowest concentration of an antimicrobial agent required to kill a micro-organism. After the microdilution assay, the MBC and MFC were obtained by subculture whereby a loop-full of the sample from each well of Genipin and positive control columns at all concentrations were streaked onto subdivided agar of the appropriate culture medium. The plates were then incubated at 37 °C for an appropriate incubation period. Microbicidal activity was indicated by the complete absence of growth on the agar plate. MBC and MFC values of ≤10.00 mg/mL were recorded as important activity as these were the concentration (10 mg/mL) incorporated into the 3D-printed full IPN scaffold itself.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01903.

Summary of the previously utilized ECM biomaterials for neural tissue engineering; real-time material stiffness (hydrated and nonhydrated states) for the semi and full IPN; and percentage of fluid uptake (aCSF) of the semi and full IPN (PDF)

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**Notes**

The authors declare no competing financial interest.

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