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Injectable supramolecular polymer-nanoparticle hydrogels for cell and drug delivery applications
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1   **TITLE:**
2   Injectable Supramolecular Polymer-Nanoparticle Hydrogels for Cell and Drug Delivery
3   Applications
4
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24   **KEYWORDS:**
25   drug delivery, cell encapsulation, biomaterials, hydrogels, biopharmaceuticals
26

27   **SUMMARY:**
28   This protocol describes the synthesis and formulation of injectable, supramolecular polymer-
29   nanoparticle (PNP) hydrogel biomaterials. Applications of these materials for drug delivery,
30   biopharmaceutical stabilization, and cell encapsulation and delivery are demonstrated.
31

32   **ABSTRACT:**
33   These methods describe how to formulate injectable, supramolecular polymer-nanoparticle
34   (PNP) hydrogels for the use as biomaterials. PNP hydrogels are composed of two components:
35   hydrophobically modified cellulose as the network polymer and self-assembled core-shell
36   nanoparticles that act as non-covalent cross linkers through dynamic, multivalent interactions.
37   These methods describe both the formation of these self-assembled nanoparticles through
38   nanoprecipitation as well as the formulation and mixing of the two components to form
39   hydrogels with tunable mechanical properties. The use of dynamic light scattering (DLS) and
40   rheology to characterize the quality of the synthesized materials is also detailed. Finally, the
41   utility of these hydrogels for drug delivery, biopharmaceutical stabilization, and cell
42   encapsulation and delivery is demonstrated through in vitro experiments to characterize drug
43   release, thermal biostability, and cell settling and viability. Due to its biocompatibility,
44   injectability, and mild gel formation conditions, this hydrogel system is a readily tunable platform

45   suitable for a range of biomedical applications.
46

47 **INTRODUCTION:**
48 Injectable hydrogels are an emerging tool to deliver therapeutic cells and drugs to the body in a
49 controlled fashion[1]. These materials can be loaded with drugs or cells and can be administered
50 in a minimally invasive manner through direct injection to superficial tissues or by catheter
51 delivery to deep tissues. In general, injectable hydrogels are composed of water-swollen polymer
52 networks that are crosslinked together by transient, physical interactions. At rest, these
53 crosslinks provide a solid-like structure to the gels, but upon application of sufficient mechanical
54 force these crosslinks are temporarily disrupted, transforming the material into a liquid-like state
55 that can easily flow[2]. It is these rheological properties that allow physical hydrogels to shear-thin
56 and flow through small needle diameters during injection[3]. After injection, the polymer network
57 of the material reforms, allowing it to self-heal and rapidly form a solid-like gel in situ[4,5]. These
58 structures can act as slow-release depots for drugs or scaffolds for tissue regeneration[6,7]. These
59 materials have been used in diverse applications encompassing drug delivery technology,
60 regenerative medicine, and immunoengineering[1,8-12].
61

62 Both natural materials (e.g., alginate and collagen) and synthetic materials (e.g., poly (ethylene
63 glycol) (PEG) or similar hydrophilic polymers) have been developed as biocompatible injectable
64 hydrogel materials[13-15]. Many natural materials exhibit batch-to-batch variation affecting
65 reproducibility[4,16]. These materials are often temperature-sensitive, curing upon reaching
66 physiological temperatures; thus, handling these materials poses additional technical and
67 logistical challenges[17]. Synthetic materials allow for more precise chemical control and excellent
68 reproducibility, but these materials can sometimes be subject to adverse immune responses that
69 limit their biocompatibility, a critical feature for in vivo therapeutic applications[6,18,19]. Recent
70 efforts have shown there are many complex design criteria involved in engineering an injectable
71 hydrogel material, including optimizing mechanical properties, polymer network mesh size,
72 bioactive molecular cues, biodegradability, and immunogenicity of the material[20-26]. All of these
73 factors must be considered depending on the application of interest, which means that a
74 modular, chemically tunable platform is ideal for satisfying a wide breadth of applications.
75

76 The present methods describe the formulation and the use of an injectable polymer-nanoparticle
77 (PNP) hydrogel platform that exhibits tunable mechanical properties, a high degree of
78 biocompatibility and low immunogenicity, and presents sites for conjugating bioactive molecular
79 cues[27-33]. These PNP hydrogels are composed of hydrophobically-modified cellulose polymers
80 and self-assembled core-shell nanoparticles comprising poly(ethylene glycol)-*block*-poly(lactic
81 acid) (PEG-PLA)[27,34] that interact to produce a supramolecular network. More specifically, the
82 dodecyl-modified hydroxypropylmethyl cellulose polymers (HPMC-C_{12}) dynamically interact with
83 the surface of PEG-PLA nanoparticles and bridge between these nanoparticles to form this
84 polymer network[27,34]. These dynamic, multivalent interactions allow the materials to shear-thin
85 during injection and rapidly self-heal after administration. The PNP hydrogel components are
86 easily fabricated through simple one-pot reactions and the PNP hydrogel is formed under mild
87 conditions by simple mixing of the two components[35]. Due to the ease of fabrication, this
88 hydrogel platform is highly translatable at scale. The mechanical properties and mesh size of PNP

89   hydrogels are controlled by altering the weight percent of the polymer and nanoparticle
90   components in the formulation. Prior studies with this platform indicate that PNP hydrogels are
91   highly biocompatible, biodegradable, and non-immunogenic[28,30,31]. Overall, these hydrogels
92   present wide utility in biomedical applications encompassing post-operative adhesion
93   prevention, tissue engineering and regeneration, and sustained drug delivery and
94   immunoengineering.
95
96   **PROTOCOL:**
97   Prior to beginning this protocol, it is necessary to synthesize HPMC-C_{12} and PEG-PLA using
98   previously published methods[27-31,36-38].
99
100 **1.   Nanoparticle (NP) synthesis by nanoprecipitation**
101
102 NOTE: This section describes synthesis of a single batch of NPs, producing 250 µL of 20 wt% NPs
103 in buffer solution (50 mg of dry PEG-PLA polymer per batch). Notes for scaling up the number of
104 batches are provided at relevant steps.
105
106 1.1.   Measure 50 mg of PEG-PLA polymer, put into an 8 mL glass scintillation vial and add 1 mL
107 of acetonitrile. Vortex to fully dissolve.
108
109 NOTE: To scale up the number of batches, linearly scale this step and add the total amount of
110 polymer and solvent needed in a single vial.
111
112 1.2.   Add 10 mL of ultrapure water into a 20 mL glass scintillation vial with a small stir bar. Put
113 on a stir plate set to 600 rpm.
114
115 NOTE: If step 1.1 has been scaled, it is still necessary to have an individual scintillation vial to
116 precipitate into for each equivalent batch. For example, for 200 mg of polymer dissolved in 4 mL
117 of acetonitrile, prepare 4 x 20 mL scintillation vials.
118
119 1.3.   To form NPs by nanoprecipitation, add 1 mL of polymer solvent solution dropwise into
120 the water using a 200 µL pipette. Stir for 2 min. The PLA block of the PEG-PLA is not soluble in
121 water, and as a result, core-shell NPs will self-assemble with the hydrophobic PLA blocks as the
122 core and the hydrophilic PEG blocks as the shell.
123
124 1.4.   Verify the particle size by dynamic light scattering (DLS).
125
126 NOTE: This procedure is specifically written for a commercially available plate reader with the
127 associated software package (see **Table of Materials**). For using alternative instruments, refer to
128 the sample preparation methods described by the instrument's manufacturer.
129
130 1.4.1.   Dilute 20 µL of NP solution with 80 µL of ultrapure water (analysis concentration: 1 mg/mL
131 PEG-PLA NPs). Add 30 µL per well to a clear bottom black 384-well plate (analyze in triplicate).
132

133   1.4.2.   Measure the hydrodynamic radius and polydispersity of each sample with a DLS plate
134   reader using preset protocol options in the software package. As an example of a typical protocol,
135   set the data collection parameters to acquire 5-10 DLS measurements of 2-5 s duration per
136   acquisition and then report a mean particle size and distribution per well, calculated from the
137   globular proteins model. To form hydrogels with consistent rheological properties, the resulting
138   particles should be 30-50 nm in hydrodynamic diameter with a polydispersity (PD) < 0.2.

140   NOTE: If the NPs are smaller than desired, use a solution of 75% acetonitrile / 25% dimethyl
141   sulfoxide (DMSO) in step 1.1. Increasing the percentage of DMSO in the solvent solution will
142   generally increase particle size.

144   1.5.   Transfer the NP solution from the 20 mL scintillation vial into a centrifugal filter unit.
145   Centrifuge at 4500 x g for 1 h to concentrate the NP solution to <250 µL.

147   1.6.   Resuspend in desired buffer, such as phosphate-buffered saline (PBS), to 20 wt% NPs.
148   Pipette the contents of the centrifugal filter unit into a tared microcentrifuge tube or glass
149   scintillation vial on a mass balance. Use a small amount (50-100 µL) of buffer to rinse the filter
150   and ensure collection of all NPs. Add buffer to reach a total mass of 250 mg.

152   NOTE: Batches can be pooled during resuspension. NP stock solutions can be stored at 4 ˚C for
153   approximately 1 month. Do not freeze. For longer storage, verify size and polydispersity by DLS
154   before use.

156   **2.   Hydrogel formulation and encapsulation of drugs or cells**

158   NOTE: This section describes preparation of 1 mL of 2:10 PNP hydrogel formulation, with 2:10
159   denoting 2 wt% HPMC-C_{12} and 10 wt% NPs (12 wt% total solid polymer) and 88 wt% buffer
160   solution, drug cargo solution, or cell suspension. The formulation percentages can be varied to
161   yield hydrogels with a range of mechanical properties. For example, 1:5 PNP hydrogels were used
162   for the cell settling and viability experimental results shown.

164   2.1.   Prepare stock solution of 6 wt% HPMC-C_{12} in PBS (or other buffer of choice). Dissolve for
165   48 h to ensure the polymer is fully dispersed.

167   NOTE: The HPMC-C_{12} stock solution is stable for months at room temperature. However, storage
168   at 4 ˚C is recommended to inhibit microbial growth.

170   2.2.   Add 333 mg of 6 wt% HPMC-C_{12} stock solution into a 1 mL Luer lock syringe.

172   2.3.   Add 500 µL of 20 wt% NP stock solution to a microcentrifuge tube. Add 167 µL of PBS and
173   pipette to mix. Using a needle, fill another 1 mL Luer lock syringe with the diluted NP solution.

175   NOTE: To load drug cargo, calculate the desired final concentration of the drug in the hydrogel
176   and load the appropriate amount into the 167 µL of PBS that is mixed with the NPs. If a molecular

177   probe is necessary for an in-situ assay, such as for monitoring drug biostability, load the probe in
178   a similar manner as described above for loading drug cargo. To load cells, calculate the desired
179   final cell concentration in the hydrogel and load the appropriate number of cells into the 167 µL
180   of PBS that is mixed with the NPs.
181
182   2.4.   Mix the two hydrogel components (HPMC-C_{12} and NPs) using an elbow mixing method[35].
183
184   2.4.1. Attach the Luer elbow connector to the syringe containing NP solution (optionally also
185   containing drug cargo or cells). Push the NP solution through the elbow until the meniscus is
186   visible at the open end. Pull back slightly and connect the syringe containing HPMC-C_{12} solution.
187
188   NOTE: It is important to minimize air in the elbow connection to prevent the formation and
189   dispersal of bubbles throughout the hydrogel during the mixing process. When mixing cells with
190   the elbow mixer, take care to mix more gently as mixing too rapidly may subject the cells to high
191   shear forces, leading to cell death.
192
193   2.4.2. Pump the two solutions back and forth through the elbow mixer for approximately 60
194   cycles until a homogeneous, opaque white hydrogel material has formed.
195
196   2.4.3. Push the entire volume of hydrogel into one syringe. Remove the empty syringe and draw
197   the plunger back on the gel-loaded syringe to recover material from the elbow connector. Cap
198   with a needle or plug.
199
200   NOTE: It is necessary to account for ~300 µL of lost hydrogel volume due to the dead space in the
201   mixing process. For example, if 700 µL of final hydrogel volume is desired, 1 mL of hydrogel should
202   be prepared. The hydrogel formulation process can be scaled up by using larger syringes.
203   However, for stiff hydrogel formulations, such as 2:10, it can become difficult to mix and inject
204   from syringes larger than 3 mL in volume due to the increasing ratio of syringe barrel to elbow or
205   needle diameter.
206
207   2.4.4. Store the hydrogel in the syringe at room temperature. However, if drugs are encapsulated,
208   storage at 4 ˚C is recommended unless the drug manufacturer specifies otherwise. Do not freeze
209   the material.
210
211   **3.   Measuring rheological properties of hydrogel formulations**
212
213   NOTE: This protocol is specifically used with the commercial rheometer mentioned in the **Table**
214   **of Materials** with a 20 mm serrated plate geometry. For using other instruments, refer to the
215   manufacturer's instructions for sample preparation.
216
217   3.1.   Formulate at least 700 µL of PNP hydrogel for rheological characterization.
218
219   3.2.   Inject material in the center of the serrated rheometer plate. The amount will vary
220   depending on the chosen geometry gap. For reference, a 700 µm gap requires ~400-500 µL of

221   <mark>material.</mark>

222

223   3.3.   Lower the rheometer to the trim gap (500-1000 µm) and slowly turn the top rheometer
224   plate as it makes contact with the PNP hydrogel to ensure the gap is filled evenly and completely.

225

226   3.4.   Inspect the loading of the PNP hydrogel such that it covers the entire rheometer plate
227   surface. Use a spatula or plastic trimmer to gently trim and remove any excess material, such
228   that it has a very slight bulge out of the plate.

229

230   3.5.   Lower the rheometer to the final geometry gap and verify the sample is cleanly loaded.

231

232   3.6.   <mark>Measure the mechanical properties of the sample using oscillatory tests, such as</mark>
233   <mark>amplitude or frequency sweeps, or flow tests, such as flow sweeps or step tests.</mark>

234

235   NOTE: In the representative data shown, oscillatory amplitude tests are run at a constant
236   frequency of 10 rad/s. Oscillatory frequency sweeps are run at a constant 1% strain, within the
237   linear viscoelastic regime of the amplitude sweep. Flow sweeps were run from high shear rates
238   to low shear rates[39]. All tests are completed with 10 points per decade of data collected and at
239   room temperature. The test parameters may need to be adjusted depending the properties of
240   the formulation. Subjecting stiffer PNP materials such as 2:10 formulations to high shear rates
241   can cause the material to be ejected from the rheometer plates, resulting in inaccurate
242   mechanical characterization, and requiring reloading of the sample between subsequent tests.
243   Representative data shown below can be used for comparison during quality control testing.

244

245   **4.   <mark>Characterizing in vitro drug release</mark>**

246

247   4.1.   <mark>Prepare capillary tubes by cutting glass capillary tubes to desired length. Seal one end of</mark>
248   <mark>each tube by using a disposable spatula or pipette tip to push a small amount of epoxy into the</mark>
249   <mark>end of the tube to form a plug</mark>. Allow epoxy to set per manufacturer's recommended time.

250

251   NOTE: The tube must be shorter than the length of the injection needle. Tubing with 2-3 µm inner
252   diameter is recommended such that a length of 2.5 in will contain at least 300 µL of total volume.

253

254   4.2.   Formulate at least 500 µL of a PNP hydrogel material in a syringe containing the drug of
255   interest. Prepare each sample group in a separate syringe.

256

257   4.3.   <mark>Inject 100-200 µL of the PNP hydrogel at the bottom of each capillary tube using a long</mark>
258   <mark>hypodermic needle (22G, 4 inch).</mark> Prepare at least three tubes (triplicate) per sample group.

259

260   4.4.   (Optional) Place filled capillary tubes in a conical centrifuge tube and centrifuge for 1 min
261   at 1000 x g to ensure the surface of the hydrogel is uniform. This step may need to be repeated,
262   altering time and speed as necessary to smooth the surface of the material.

263

264   CAUTION: Ensure centrifuge is well balanced.

265

266   4.5.   Carefully fill 200-300 μL of PBS on top of the hydrogel in the capillary tube using a syringe
267 and needle or pipette. Do not contact or disturb the surface of the hydrogel. Seal the tube with
268 a cap or plug or cover with at least two layers of paraffin film.

269

270   4.6.   (Optional) Incubate samples at 37 ˚C to simulate in vivo conditions.

271

272   4.7.   Carefully completely remove the PBS from each capillary, without disturbing the hydrogel
273 surface, using a syringe and needle at chosen time points depending on the anticipated time scale
274 of drug release. Replace the volume removed with fresh PBS. Store aliquots under appropriate
275 conditions.

276

277 NOTE: The recommended volumes and time points in steps 4.3, 4.5 and 4.6 can be optimized to
278 capture in vitro drug release over a range of timescales, depending on how much drug is loaded
279 in the material and how quickly it releases into the supernatant. A sample of selected time points
280 could be 6 h, 1 day, 3 days, 1 week and 2 weeks for a slow-releasing drug. Aliquots can also be
281 analyzed as they are acquired rather than stored.

282

283   4.8.   At the completion of the study, analyze aliquots with an appropriate method such as
284 ELISA, HPLC or fluorescence assay to quantify the amount of drug released at each time point[40-
285 42]. The appropriate detection method will vary depending on the drug of interest.

286

287 NOTE: In vitro release studies are useful for comparing release between different hydrogel
288 formulations or drug cargo. The in vitro release timescale does not often directly indicate an
289 expected time scale of release in vivo.

290

291 **5.   Characterizing thermal biostability of gel-encapsulated insulin**

292

293   5.1.   Formulate at least 1.2 mL of PNP hydrogel per sample group. Following the procedure
294 described in section 2.3, load both insulin (drug cargo) and thioflavin T (ThT) (molecular probe)
295 into the PNP hydrogel.

296

297 NOTE: The primary mechanism of aggregation and, therefore, inactivation of insulin is through
298 the formation of amyloid fibrils. ThT is a suitable molecular probe because it produces a strong
299 fluorescence signal in the presence of amyloid fibrils, allowing for in situ monitoring of insulin
300 aggregation. Depending on the drug cargo of interest, aggregation may be monitored through
301 different methods. For the representative data shown, insulin was loaded to a final concentration
302 of 6.7 or 10 mg/mL and ThT to a final concentration of 25 μM.

303

304   5.2.   Using a 21 G needle, inject 200 μL of PNP hydrogel per well into a black 96-well plate.
305 Each sample group should be measured in at least triplicate. Seal plate with an optically clear
306 adhesive   plate   seal   to   prevent   evaporation.

307

308   5.3.   Insert plate into a plate reader equipped with temperature control, shaking, and kinetic

309 read programming and begin read protocol. Representative data was acquired with a Plate
310 Reader using the following conditions.

312 5.3.1. Stressed aging conditions: continuous linear shaking (410 cpm, 5 mm) at 37 ˚C.

314 5.3.2. Data acquisition: excitation/emission 450 nm/482 nm at 20 min intervals

316 NOTE: If a plate reader with temperature control, shaker, and kinetic read capabilities is not
317 available, the plate can be placed on a shaker plate in an incubator and read manually at above
318 wavelengths at selected time points.

320 5.4.   Plot data as mean fluorescence signal over time for each group. Time to aggregation can
321 be quantified by defining an arbitrary signal threshold[43].

323 NOTE: For the representative data shown below, the threshold was defined as 750,000 arbitrary
324 fluorescence units (AFU). This value was chosen to be above the measured baseline while still
325 sufficiently capturing the onset of aggregation indicated by a sharp fluorescent signal increase.

327 5.5.   Terminate assay when samples aggregate or visually begin to dehydrate.

329 **6.   Assessing cell viability**

331 6.1.   Formulate at least 2 mL of PNP hydrogel containing the desired cell concentration
332 following above protocols (normally 1 - 5 x 10^6 cells/mL). Prepare each sample group in a separate
333 syringe.

335 6.2.   Using a 21G needle, inject 150 µL PNP hydrogel into each well in a clear bottom 96-well
336 plate; each well is one replicate. Each sample group should have 3-5 replicates per time point.
337 Centrifuge the plate at 50 x g for 2 min to spread the hydrogel evenly in the well.

339 6.3.   Add 100 µL of the appropriate cell media on top of the hydrogel. Remove this media each
340 day and add 100 µL new media.

342 6.4.   On Day 1, remove the media on top of the hydrogel for the designated replicates for that
343 time point for each sample group. Add 50 µL of 2 mM calcein AM solution on top of the hydrogels.
344 Incubate for 30 min.

346 NOTE: Calcein AM can be used to identify and label live cells. In live cells, the non-fluorescent
347 calcein AM is converted to a green-fluorescent, calcein, by intracellular esterases after
348 acetoxymethyl ester hydrolysis.

350 6.5.   Image the center of each well in a 96-well plate using a confocal microscope. Survey an
351 area of least 300 µm² with a z-stack spanning at least 150 µm. Use confocal wavelength settings
352 to capture the fluorescence of calcein (excitation/emission: 495 nm/515 nm).

6.6.   Repeat step 6.6 and 6.7 for each subsequent time point as desired.

6.7.   To analyze each image, collapse all z-stack images into a single plane maximum intensity image using FIJI or similar software. Quantify the number of fluorescent cells in each image. The ratio of the number of fluorescent cells at each time point compared to the number of fluorescent cells at Day 1 is the relative cell viability in the PNP hydrogels.

7.   Assessing cell settling

7.1.   Calculate the number of cells required to formulate 500-700 µL of PNP hydrogel at a final concentration of 5 x 10^6 cells/mL. Suspend cells in 1 mL of PBS at a concentration of 1 x 10^6 cells/mL. Stain cells by adding 50 µL of 2 mM calcein. Incubate the cells with the dye for 10 min.

7.2.   Centrifuge cells at appropriate conditions, remove the PBS and resuspend the cells in the volume of PBS needed to form 500-700 µL of the desired PNP hydrogel.

NOTE: The recommended speed and duration to centrifuge each specific cell type is typically provided in the product documentation.

7.3.   Formulate 500-700 µL of PNP hydrogel with the stained cells (5 x 10^6 cells/mL) following protocol section 2.

7.4.   Using a 21G needle, inject 100-200 µL of PNP hydrogel containing the stained cells in the bottom of a cuvette. Three replicates should be performed for each sample. Move the needle back and forth within the cuvette while injecting to prevent bubble formation.

7.5.   Immediately (time t=0), image the cuvettes lying on their side over the entire flat cuvette rectangle area at the base of the cuvette. Use the confocal tile scanning capabilities to image the entire well area and image a z-stack in 3D across a 100 µm depth. For later visualization, either use the confocal microscope software to stitch together all of the individual tiles and perform a maximum intensity projection to form a single image of the large area or use FIJI software on a personal computer[44,45].

7.6.   Following imaging, stand the cuvettes upright.

7.7.   Image at 1 h and 4 h to observe if cells have settled in the hydrogel or whether they remain suspended.

NOTE: These time points are suggestions and can be modified as desired.

7.8.   To analyze each image, collapse all z-stack images into a single plane maximum intensity image. Using FIJI or similar software, quantify the cell distribution by measuring the fluorescence intensity down the center vertical profile of the cuvette to determine the degree of settling.

REPRESENTATIVE RESULTS:

PNP hydrogel fabrication and characterization

PNP hydrogels are formed through the mixing of the two primary components – hydrophobically-modified HPMC polymers and PEG-PLA nanoparticles (**Figure 1a**). Therapeutic cargo is most easily incorporated into the additional buffer used to dilute the nanoparticle component prior to hydrogel preparation. For downstream biomedical characterization, it is convenient to use an elbow mixing method that enables simple and reproducible mixing of the two components (**Figure 1b**). After adequate mixing, the hydrogel should feel firm in the syringe, but yield under pressure and extrude from a standard needle (21 G shown) (**Figure 1c**). After injection, the hydrogel should rapidly set into a solid-like material that resists flow from gravity. To fully characterize the hydrogel and ensure consistent batch-to-batch products, samples should be analyzed using several different experiments on a rheometer. The shear-thinning and self-healing capabilities of the gel will be easily observed using a flow sweep protocol and step-shear protocol, respectively (**Figure 2a,b**). For stiffer gels, such as the 2:10 formulation, the user should look for viscosity to decrease at least two orders of magnitude during the flow sweep as the shear rate is increased from 0.1 to 100 s^{-1}, which simulates the mechanical conditions during injection. The step-shear protocol should reveal an orders-of-magnitude decrease in viscosity under the high-shear steps, and a rapid return (<5 s recovery time) to baseline viscosity during the low shear steps. Characterization of the storage and loss moduli using an oscillatory shear frequency sweep experiment in the linear viscoelastic regime should reveal solid-like properties at frequency ranges from 0.1-100 rad s^{-1} (**Figure 2c**). In particular, there should typically not be a crossover of the shear storage and loss moduli that is observable at low frequencies for stiffer formulations like the 2:10 hydrogels. Such a crossover event may indicate issues in the quality of the starting materials, either the modified HPMC or PEG-PLA polymer, or the size and dispersity of the PEG-PLA nanoparticles. It should be noted that a crossover event can be expected for weaker hydrogel formulations, such as the 1:5 hydrogel. Oscillatory shear amplitude sweeps on PNP hydrogels reveal that the materials do not yield until high stress values are applied, indicating these materials possess a yield stress, a threshold amount of stress required for the material to flow.

Characterizing release kinetics from PNP hydrogels

An essential step in designing PNP gels for drug delivery is the characterization of drug release kinetics from a chosen formulation. There are several techniques for this, but a simple in vitro methodology provides useful data during early formulation development (**Figure 3a**). Varying the polymer content of the PNP hydrogels through modulating the amount of HPMC-C_{12} or NPs is the most straightforward way to tune the mechanical properties and mesh size of these hydrogels, which can have a direct impact on the diffusion of cargo through the polymer network and rate of release from the materials (**Figure 3b**). For cargo that is larger than the dynamic mesh size (i.e., high molecular weight or large hydrodynamic radius), researchers should expect a slow, dissolution-mediated release of cargo from the hydrogel depot. Formulations with dynamic mesh sizes greater than or equal to the size of the cargo will allow for diffusion-mediated release that can be described using traditional models of cargo diffusion and release[46-49]. Based on the shape of the release curve, researchers can reformulate the hydrogel to tune it towards slower (e.g.,

441   increase the polymer content) or faster (e.g., decrease the polymer content) release.

442

443 **Assessing stability of therapeutic cargo**

444   Determining the stability of the therapeutic cargo in a hydrogel formulation is critical before
445   commencing preclinical or cellular studies. Compared to other synthetic methods for
446   encapsulating drugs, PNP hydrogels incorporate cargo in a gentle manner by mixing into the bulk
447   material, and it is unlikely that encapsulation will damage the cargo. These studies indicate that
448   PNP hydrogels can also stabilize cargo that is susceptible to thermal instability, such as insulin,
449   considerably extending shelf life and reducing reliance on cold storage and distribution (**Figure**
450   **4**). It is important to evaluate the condition of the cargo immediately after encapsulation into
451   the hydrogel as well as after extended periods of storage. These data show that insulin remains
452   stable in hydrogels after 28 days of storage under continuous thermal and mechanical stress,
453   using a simple fluorescence assay for measuring insulin aggregation. An alternative technique for
454   cases where an appropriate plate assay is unavailable would be to perform circular dichroism
455   measurements of the cargo, which is particularly useful for determining the secondary structure
456   of protein drugs.

457

458 **Determining cell viability and dispersion in PNP hydrogels**

459   Many therapeutic cells require adhesion motifs to remain viable, and thus inclusion of integrin
460   motifs like arginine-glycine-aspartic acid (RGD) peptides is an important step in adapting PNP
461   hydrogels for cellular therapies[50]. The modular PEG-PLA polymer comprising the NPs enables
462   chemical functionalization of the PEG corona through simple "click" chemistries[28,51]. In this
463   example, cell-adhesive RGD peptides were attached to the PEG-PLA polymer to promote cell
464   engagement with the PNP hydrogel structure. Formulations lacking adhesion sites will have low
465   cell viability as encapsulated cells fail to proliferate compared to cells encapsulated in
466   formulations with these adhesion motifs (**Figure 5a,b**). Encapsulated cells can be labeled with
467   calcein AM or another appropriate fluorescent dye (e.g., CFSE) to facilitate cell counting with a
468   fluorescence microscope. During optimization, viability should be compared to unmodified PNP
469   hydrogels to assure integrin-functionalized formulations are providing enhanced viability and
470   proliferation. If integrin-functionalized formulations are providing similar efficacy as unmodified
471   hydrogels, this may indicate a failure in the conjugation chemistry used to incorporate the
472   adhesion motifs.

473

474   Researchers should expect encapsulated cells to be evenly dispersed through the hydrogel
475   medium when using an appropriate hydrogel formulation. This will allow for consistent and
476   predictable dosing of cells during hydrogel administration and should translate to local retention
477   of cells in the hydrogel after administration. The distribution of cells can be easily determined
478   using fluorescence microscopy techniques. Cells can be labeled with an appropriate dye and then
479   imaged using confocal microscopy. The images can be assessed visually (**Figure 5c**) and also
480   quantitatively (**Figure 5d**) using ImageJ software to measure the average fluorescence intensity
481   along the vertical axis of the image (or along whichever axis cell-settling due to gravity is expected
482   to occur). If the hydrogel formulation is too weak to support the cells in suspension over
483   prolonged timeframes, cell settling will occur, as observed in the 1:1 formulation in **Figure 5**.
484   Increasing the polymer content can resolve issues with inhomogeneous cell dispersion due to

485 settling.

486

487 **FIGURE AND TABLE LEGENDS:**

488

489 **Figure 1: Polymer-nanoparticle (PNP) hydrogels are easily formed by mixing two components.**
490 (**a**) The first component is a solution of dodecyl-modified hydroxypropylmethyl cellulose (HPMC-
491 C_{12}), and the second component is a solution of poly(ethylene glycol)-*block*-poly(lactic acid) (PEG-
492 PLA) nanoparticles along with any therapeutic cargo. Gentle mixing of these two components
493 yields an injectable hydrogel, where the HPMC-C_{12} polymers are physically crosslinked by
494 dynamic, multivalent interactions with the PEG-PLA nanoparticles. (**b**) Photograph demonstrating
495 gel formulation by mixing with two syringes, each one containing one component of the PNP
496 hydrogel. By connecting the two syringes with a Luer-lock elbow connector, the two components
497 can be easily mixed under sterile conditions to yield a bubble-free hydrogel pre-loaded into a
498 syringe for immediate use. The NP solution is dyed blue for the purpose of demonstration. (**c**)
499 Demonstration of the injection of PNP hydrogels and their re-solidification. (i) PNP hydrogel in a
500 syringe with an attached 21 G needle. (ii) Injection places the hydrogel under shear which
501 temporarily breaks the interactions between polymer and nanoparticles, creating a fluid-like
502 consistency. (iii) Post-injection, the dynamic polymer-nanoparticle interactions rapidly reform,
503 allowing the hydrogel to self-heal into a solid. (iv) The solid hydrogel does not flow under forces
504 weaker than its yield stress, such as gravity. The PNP hydrogel is dyed blue for the purpose of
505 demonstration.

506

507 **Figure 2: Rheological characterization of two PNP hydrogel formulations.** Formulations are
508 denoted as polymer wt.%: NP wt.%. (**a**) Steady shear flow sweeps from low to high shear rate of
509 PNP hydrogels. Viscosity as a function of shear rate characterizes shear-thinning properties. (**b**)
510 Viscosity as a function of oscillating shear rates between low shear rates (white background; 0.1
511 s^{-1}) to high shear rates (red background; 10 s^{-1}) demonstrating self-healing properties of PNP
512 hydrogels. Shear rates are imposed for 30 s each. (**c**) Elastic storage modulus G' and viscous loss
513 modulus G'' as a function of frequency at a constant 1% strain for various PNP hydrogel
514 formulations. (**d**) Amplitude sweeps at a constant frequency of 10 rad/s to characterize elastic
515 storage modulus G' and viscous loss modulus G'' of PNP hydrogels as a function of stress. This
516 rheological characterization can be used as comparison for quality control. This figure has been
517 adapted from Grosskopf et al.[28]

518

519 **Figure 3: In vitro release of bovine serum albumin (BSA) from PNP hydrogels.** Formulations are
520 denoted as polymer wt.%: NP wt.%. (**a**) Schematic describing the experimental in vitro release
521 protocol. Aliquots are removed from PNP hydrogel-loaded capillary tubes over time. (**b**) The in
522 vitro release of BSA from 1:10 PNP, 2:5 PNP and 2:10 PNP reported as the mass collected by the
523 specified time point divided by the total mass collected during the assay (data shown as mean ±
524 SD; *n* = 3). BSA was detected through absorbance measurements.

525

526 **Figure 4: Thermal stability of insulin encapsulated in PNP hydrogels by ThT assay.** Formulations
527 are denoted as polymer wt.%: NP wt.%. Insulin encapsulated in both 1:5 and 2:10 PNP hydrogel
528 remained unaggregated for over 28 days at stressed aging conditions of 37 ˚C and constant

529 agitation. Time to aggregation for insulin formulated in PBS was 20 ± 4 h (mean ± SD, aggregation
530 threshold 750,000 AFU). Data presented as an average of n = 4 experimental replicates (AFU,
531 arbitrary fluorescence units). This figure has been adapted from Meis et al.[38]

533 **Figure 5: Cell viability and cell settling in PNP hydrogels. (a,b)** Cell viability studies in PNP
534 hydrogels with human mesenchymal stem cells (hMSCs). (**a**) Representative images of viable
535 hMSCs in 1:5 PNP hydrogels with and without the cell-adhesive arginine-glycine-aspartic acid
536 (RGD) motif conjugated to the PEG-PLA NPs. hMSCs were calcein-stained for 30 min prior to
537 confocal imaging. Scale bar represents 100 µm. (**b**) Cell viability on Day 6 defined as number of
538 fluorescent cells in the image relative to number of fluorescent cells on Day 1 (data shown as
539 mean ± *SD*; n = 3). (**c,d**) Cell encapsulation and settling experiments with hMSCs. (c) Maximum
540 intensity images of calcein AM-stained hMSCs encapsulated in 1:1 PNP hydrogel (top row) and
541 1:5 PNP hydrogel (bottom row) across 4 hr to quantify cell settling. Scale bar represents 1 mm.
542 (**d**) Average horizontal pixel intensity of hMSCs along the vertical profile of the hydrogel. This
543 figure has been adapted from Grosskopf et al.[28]

545 **DISCUSSION:**
546 Polymer-Nanoparticle (PNP) hydrogels are easily fabricated and enable the long-term local
547 delivery of therapeutic cells and drugs through minimally invasive administration via direct
548 injection or catheter delivery. These protocols describe the formulation of PNP hydrogels and the
549 characterization methods for assuring quality of the resulting materials. Supramolecular PNP
550 hydrogels are scalable to manufacture and are formed through the simple mixing of modified
551 cellulose polymers and polymeric core-shell nanoparticles. The present methods describe facile
552 procedures to form gels pre-loaded in syringes through simple elbow mixing protocols. Through
553 quality control metrics of each of the component parts, such as DLS to monitor the NP size and
554 distribution, one can reproducibly formulate PNP hydrogel materials with consistent rheological
555 properties. Through varying the amount of HPMC-C$_{12}$ or NPs, one can modulate the mesh size
556 and stiffness of the resulting PNP hydrogel. These properties can be tuned to best suit a particular
557 biomedical application, and with the rheological methods detailed here researchers can
558 characterize the shear-thinning and self-healing properties of PNP hydrogels as they optimize the
559 platform for their specific applications. Methods for in vitro release studies are also described;
560 researchers can use these studies to characterize the relative timescale of release of drugs of
561 interest, informing future *in vivo* studies. Using stability studies, researchers can also assess the
562 ability of these materials to help preserve the biological structure and stability of sensitive
563 biotherapeutics over time and extreme temperatures, with compelling potential applications to
564 reduce the cold chain dependence of biotherapeutics. Finally, with simple cell viability assays,
565 cell growth and migration within PNP materials can be evaluated, with potential applications in
566 cell therapies and scaffolds.

568 Our group has found many compelling applications for the PNP hydrogel platform[27]. PNP
569 hydrogels have been used for slow delivery of subunit vaccines, enabling matched kinetic release
570 profiles of antigens and adjuvants to boost the magnitude, duration, and quality of the humoral
571 immune response[31]. PNP hydrogels have been found to have a smaller mesh size than most
572 commonly used hydrogels, so they are effective at slowing diffusion and slowly releasing

molecular cargo. The unique tissue adherence properties and mechanical properties of PNP hydrogels have also been utilized to form physical barriers to prevent adhesions arising from surgery by spraying the hydrogels over large surface areas of organs following surgery[30]. PNP hydrogels have also been shown to be effective cell delivery vehicles, and the mechanical properties actually shield cells from the mechanical forces occurring in the syringe needle during injection, improving cell viability[29]. When the NPs are conjugated with a cell-adhesive peptide, cells can attach and engage with the PNP matrix to remain viable. Using this approach, PNP hydrogels have been shown to improve the local retention of injected stem cells compared to methods using liquid vehicles[28]. In addition, PNP hydrogels have been shown to prevent thermally-induced aggregation of encapsulated insulin, even under harsh stressed aging conditions, suggesting that these materials may be able to reduce the need to refrigerate temperature-sensitive drugs[38].

Overall, the methodologies described here will allow research groups to fabricate and explore PNP hydrogels as a biomaterial. These protocols provide the lab-scale synthesis techniques to fabricate enough hydrogel material to pursue both in vitro and in vivo studies. The studies described above indicate that the dynamic crosslinks of these materials enable it to be suitable for a range of biomedical applications by allowing active motility of entrapped cells while restricting passive diffusion of molecular cargo. It is anticipated that researchers will find the PNP platform an accessible and powerful tool to improve clinical outcomes through controlled drug delivery and to study basic biological mechanisms such as cell recruitment and mechanobiology.

DISCLOSURES:
These authors have nothing to disclose.

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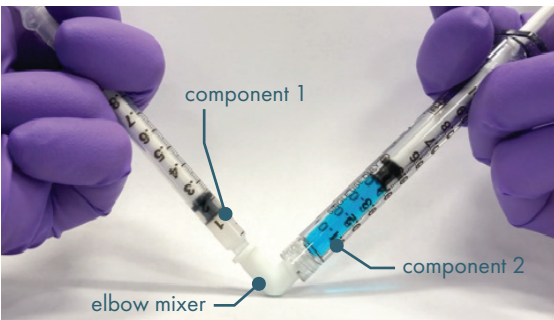
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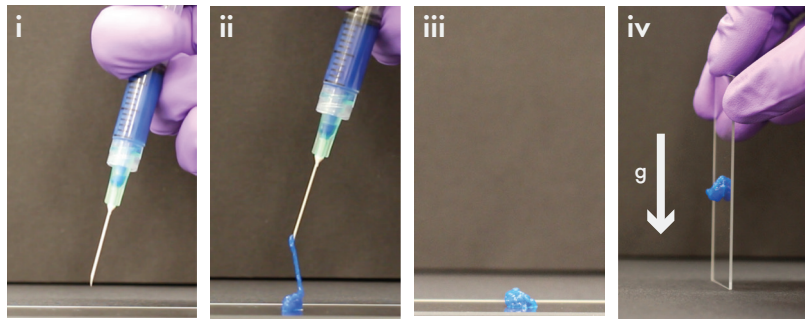
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(a) Figure

component 1

dodecyl-modified HPMC

+

component 2

poly(ethylene glycol) -b- poly(lactic acid) nanoparticle

drug

and/or

cells

cargo

injectable polymer-nanoparticle (PNP) hydrogels

gelation via dynamic, multivalent polymer-nanoparticle interactions
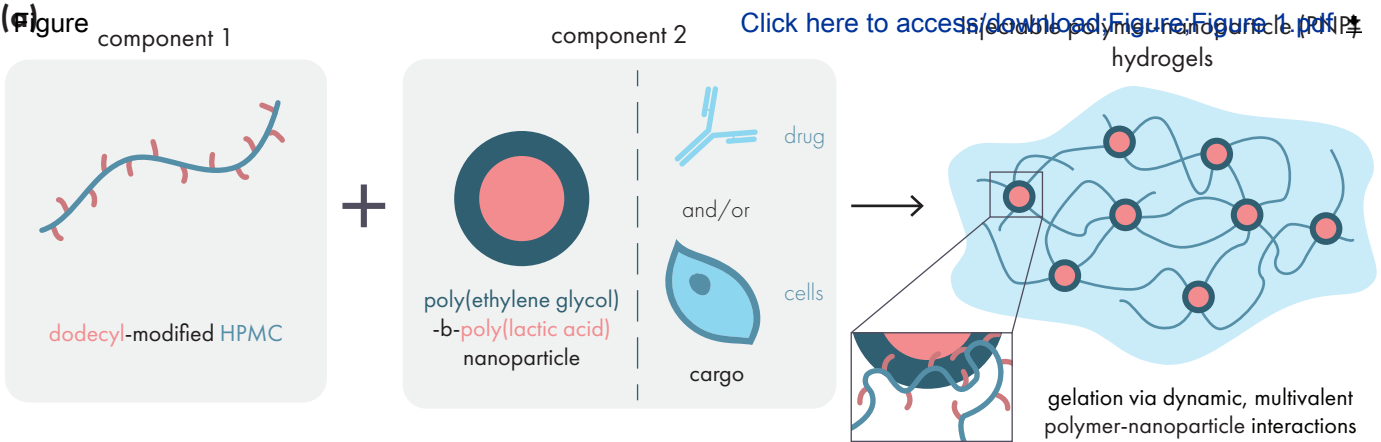
(b) mixing components yields hydrogel

component 1

component 2

elbow mixer

(c) hydrogel injection through needles

i

ii

iii

iv

g

Figure 3

(a) Remove Buffer

Buffer

PNP Hydrogel

Time Zero → Time Infinity

(b)

- 1:10 PNP
- 2:5 PNP
- 2:10 PNP

M_t/M_{inf}

Time (days)

Decreasing Mesh Size

Figure 4

Figure 5

(a) Day 1 / Day 6 — No RGD, RGD; 100 µm

(b) % Cell Number (Relative to Day 1) vs No RGD, RGD

(c) 1:1, 1:5 at 15 min, 1 hour, 4 hour; 1 mm

(d) Profile (mm) vs Average Horizontal Pixel Intensity; 1:1, 1:5
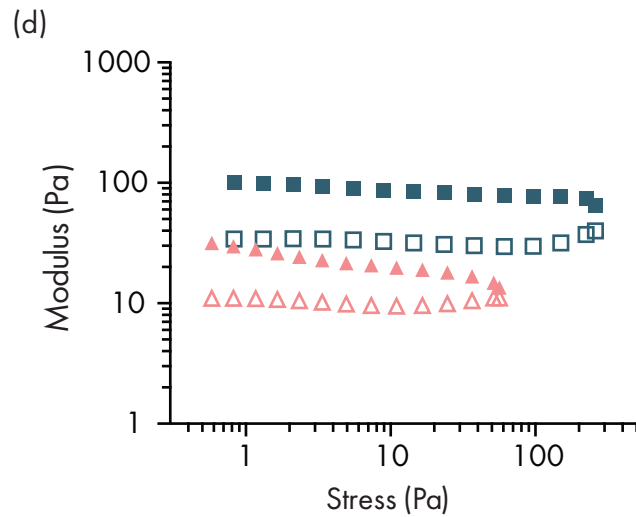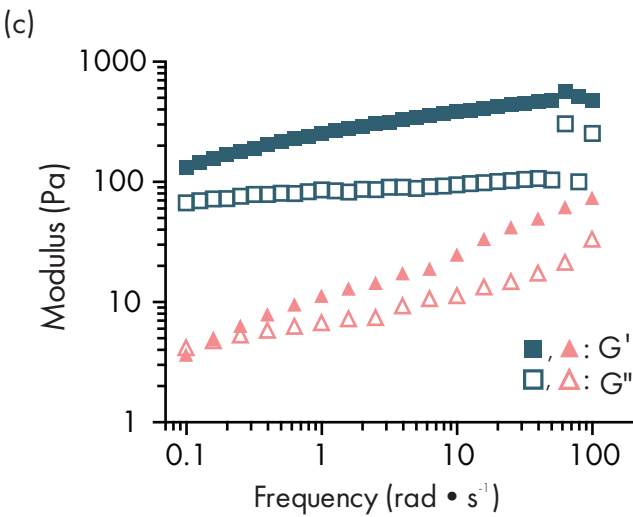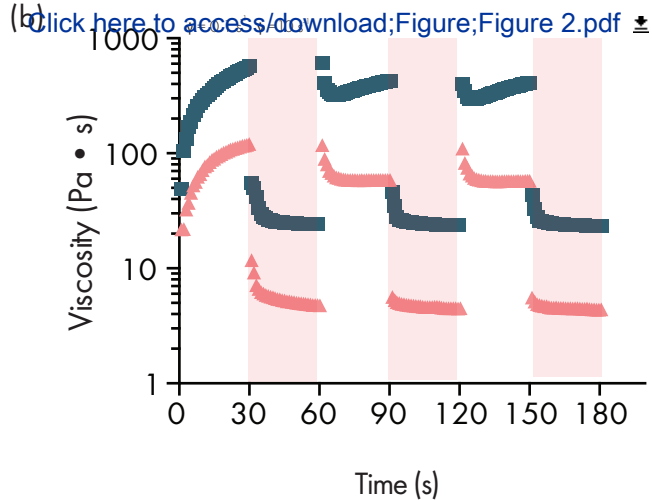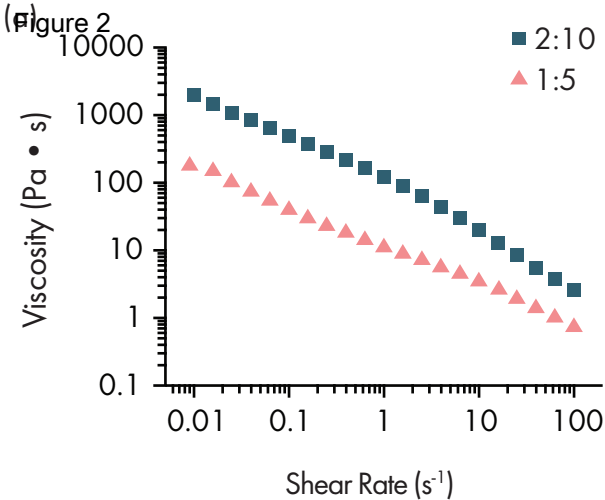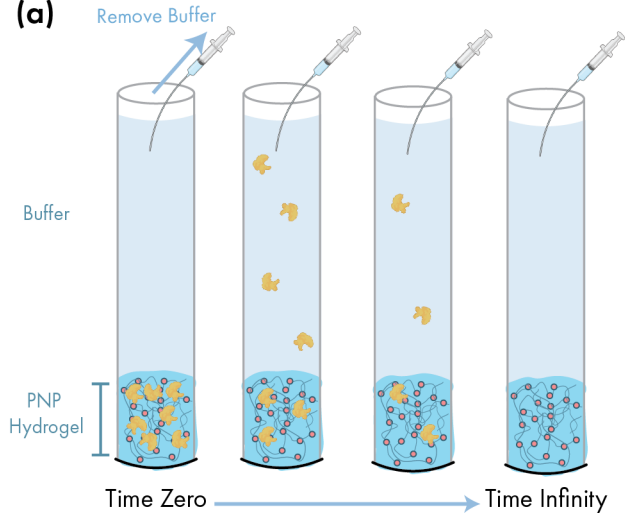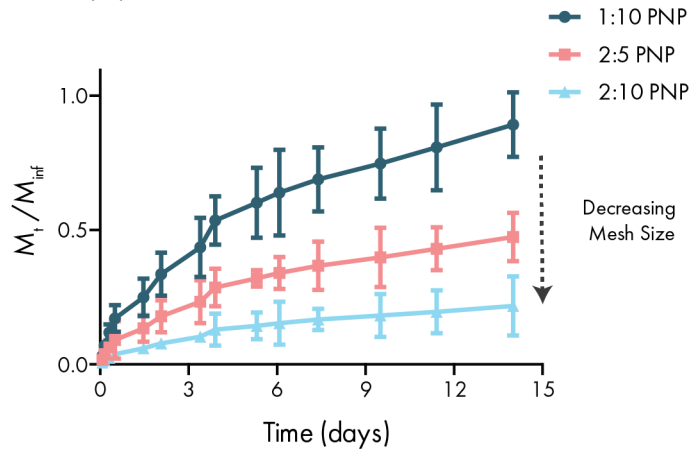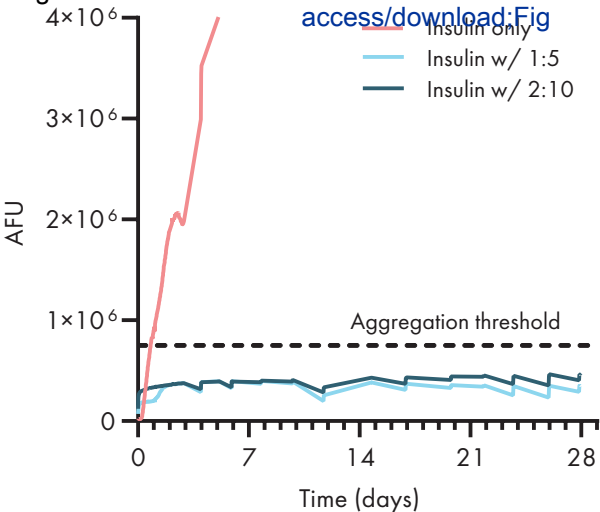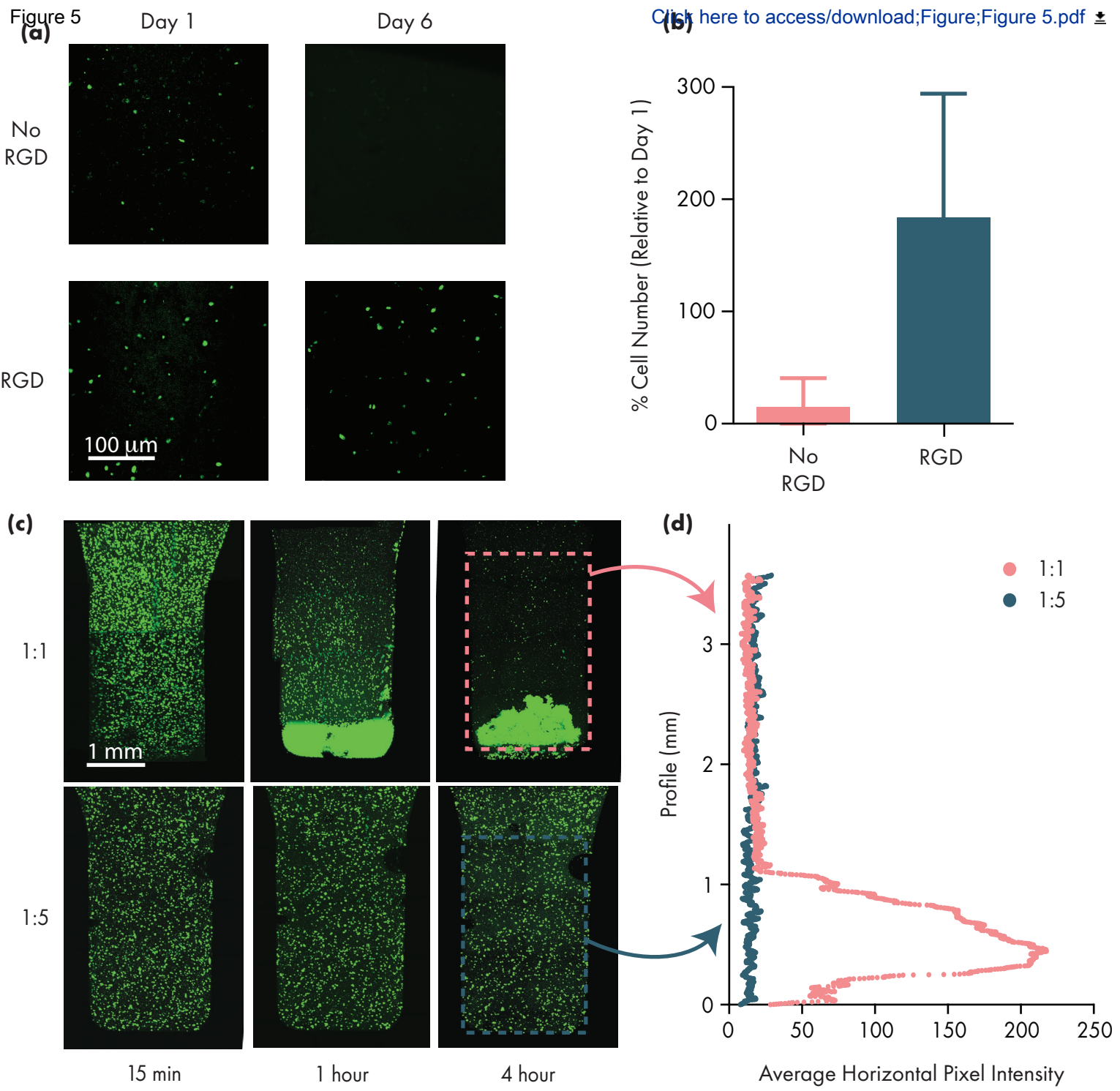
Name of Material/ Equipment	Company
21 G needles	BD
22 G, 4 in hypodermic needles	Air-Tite
384-well plates, black, clear bottom	Corning
96-well plates, black	Fisher Scientific
96-well plates, clear	Corning
Calcein AM	Thermo Fisher Scientific
Capillary tubes	McMaster-Carr
Centrifugal filter units	Fisher Scientific
Cuvettes	Millipore Sigma
DLS Plate Reader	Wyatt Technology
Epoxy	VWR International
Hypodermic needles	Air-Tite
Luer elbow connector	Cole-Parmer
Luer lock syringe	Fisher Scientific
Phosphate Buffered Saline (1x)	Fisher Scientific
Plastic Spatula	Thomas Scientific
Plate Reader	BioTek
Plate seals	Excel Scientific
Recombinant human insulin	Gibco
Rheometer	TA Instruments
Thioflavin T	Sigma-Aldrich

Catalog Number	Comments/Description
305165	PNP hydrogel injection
N224	In vitro release studies
3540	Dynamic light scattering (DLS)
07-200-627	Biostability studies
3599	Cell viability and settling studies
C3100MP	Cell viability and settling studies
8729K66	In vitro release studies
UFC901024	NP concentration
BR759015-100EA	Cell viability and settling studies
DynaPro II Plate Reader	Dynamic light scattering (DLS)
300007-392 (EA)	In vitro release studies
8300015027	In vitro release studies
EW-30800-12	PNP hydrogel formulation
14-955-456	PNP hydrogel formulation
10010049	PNP hydrogel formulation
1229F13	Rheological characterization
Synergy H1 Hybrid Multi-Mode Plate Reader	Biostability studies
TS-RT2-100	Biostability studies
A11382II	Biostability studies
DHR-2 Rheometer	Rheological characterization
T3516-5G	Biostability studies

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have reviewed the manuscript and believe it is free of editing errors.

2. Please provide the complete address of all the affiliations.

We have updated the manuscript with the complete addresses of affiliations of each author.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed all personal pronouns.

4. Please ensure that when giving a reference in the text, the corresponding number from the reference list must appear superscripted without a space after the word/group of words it applies to but before any punctuation (in the case of et al., place the superscripted number after et al. but before other punctuation).

The punctuation has been corrected throughout.

5. Line 36-38: Please revise the following lines to avoid previously published work: Paul, Arghya, Anwarul Hasan, Hamood Al Kindi et al. "Injectable Graphene Oxide/Hydrogel-Based Angiogenic Gene Delivery System for Vasculogenesis and Cardiac Repair", ACS Nano; Kim, Kyung Hoon; Zheng, Nan; Gabrielson, Nathan P.; Cheng, Jianjun Advanced Materials (Weinheim, Germany) (2013), 25 (22), 3063-3070CODEN: ADVMEW; ISSN:0935-9648.

We believe these lines avoid these previously published works. In these lines, we are introducing the general scope of our manuscript.

6. Lines 78- 80: Please revise the following lines to avoid previously published work: Bioeng Transl Med. 2019 Oct 22;5(1):e10147. doi: 10.1002/btm2.10147. eCollection 2020 Jan.Injectable supramolecular polymer-nanoparticle hydrogels enhance human mesenchymal stem cell delivery. Abigail K Grosskopf 1, Gillie A Roth 2, Anton A A Smith 3, Emily C Gale 4, Hector Lopez Hernandez 3, Eric A Appel 3

The text has been edited to avoid previously published work. The noted work is also cited in the text.

7. Please use standard abbreviations for SI Units when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL

The abbreviations have been changed to the correct format.

8. Line 135: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

The following lines have been added or updated to explain how the DLS step is performed:

"Note: This procedure is specifically for using a Wyatt DynaPro Plate Reader DLS with the Wyatt DYNAMICS software package."

"Measure the hydrodynamic radius and polydispersity of each sample with a DLS plate reader using preset protocol options in the DYNAMICS software package. As an example of a typical protocol, set the data collection parameters to acquire 5-10 DLS measurements of 2-5 s duration per acquisition and then report a mean particle size and distribution per well, calculated using the globular proteins model."

9. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute and Italicize g in centrifugation speeds. Example: 1,000 x g

The centrifuge speeds have been converted and denoted as requested.

10. Line 253: Please elaborate on how the mentioned tests are performed.

Additional details were added to further describe the methods of the mechanical tests for testing viscoelasticity and flow properties. Additionally, a reference with explanations of these tests was added.

11. Line 275: Please specify how the sealing step is performed.

The following text has been added to clarify this step:

"Seal one end of each tube by using a disposable spatula or pipette tip to push a small amount of epoxy into the end of the tube to form a plug. Allow epoxy to set per manufacturer's recommended time."

12. Line 281: Please mention the specifications of the hypodermic needle.

The needle specifications have been added to both the text and the table of materials.

13. Line 310-312: Please provide the details for the analysis techniques mentioned. Appropriate citations would suffice.

The appropriate detection method will vary depending on the drug of interest. We have added relevant citations with protocols that could be adapted for this particular step.

14. Line 353: Please mention how the arbitrary signal threshold is defined. Insert references if required.

We have added references as well as the following text to address the definition of a signal threshold:

"Note: For the representative data shown below, threshold was defined as 750,000 arbitrary fluorescence units (AFU). This value was chosen to be above the measured baseline while still sufficiently capturing the onset of aggregation indicated by a sharp fluorescent signal increase."

15. Line 400: Please mention the centrifugal conditions (speed, time)

The centrifugal conditions will vary depending the chosen cell type for the experiment. We have added the following text for clarification of the procedure:

"Note: The recommended speed and duration to centrifuge each specific cell type is typically provided in the product documentation."

16. Line 412: Please elaborate on image stitching. Cite references wherever necessary.

Additional details were added to explain the image processing. Additionally, citations were added to explain the algorithm that performs image stitching and maximum intensity projections in FIJI image processing software.

17. Please Do not use the &-sign or the word "and" when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

We have corrected the reference formatting using the Jove style in our reference manager and also manual updates.

18. Please do not use any abbreviations for the journal titles and book titles.

We have corrected the reference formatting using the Jove style in our reference manager and also manual updates.

19. Figure 2: Please use the multiplication dot for compound units. Examples: N·m, m/s, eV·s/rad, m2·kg·s-3A-2, m2·kg/s3

The multiplication dot has been used in the updated figure.

20. Figure 5: Please include a scale bar for all images taken with a microscope to provide context to the

magnification used. Please define the term RGD in the figure or the text (5a). Please make sure that the X-axis is defined correct (5d).

The requested edits have been made in the updated figure. RGD has been defined in the text.

21. Please revise the table of materials in alphabetical order.

The table has been alphabetized.

Reviewer #1:

Well written and full of details

Thank you for the positive comment. We hope this method will be descriptive and useful to those seeking to formulate these types of hydrogels.

Reviewer #2:

This manuscript describes the formulation and characterization of PNP hydrogels for use as biomaterials. These materials have potential utility for drug delivery, stabilization of biomolecules, and cell encapsulation and delivery. The authors do a great job of thoroughly describing representative protocols associated with these applications.

Thank you for the kind remarks. We hope and anticipate that these methods will help other researchers formulate and test the materials described as well as other soft biomaterials.

Major Concerns:
I have no major concerns.

Minor Concerns:
There are a few small typos throughout the manuscript. In addition, some lines are highlighted in yellow, and it is not clear why.

The highlighted lines are to indicate which sections should be adapted for video scripting. We believe we have corrected any typos after careful editing; thank you for bringing this to our attention.