Screen printing tissue models using chemically crosslinked hydrogel systems: a simple approach to efficiently make highly tunable matrices

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ABSTRACT:

In vitro models provide a good starting point for drug screening and understanding various cellular mechanisms corresponding to different conditions. 3D cultures have drawn significant interest to mimic the in vivo microenvironment better and overcome the limitations of the 2D monolayered cultures. We previously reported a technique based on the screen printing process to pattern live mammalian cells using gelatin as the bioink. Even though gelatin is an inexpensive scaffolding material with various tissue engineering applications, it might not be the ideal hydrogel material to provide various mechanical and chemical cues to the cells. In this paper, we discuss the synthesis and characterization of two synthetic chemically crosslinked hydrogel systems based on poly (ethylene glycol) (PEG) and poly-L-lysine (PLL) to be used as the bioink in the screen printing process. These hydrogels are suitable as the bioinks for the screen printing process and serve as the barebone materials that can be tuned mechanically and augmented chemically to create a suitable in vitro microenvironment for the cells. This paper presents the synthesis, mechanical testing, and characterization of the hydrogel systems and their applications in the screen printing process.

KEYWORDS. Tissue models, PEG, Poly-L-lysine, in vitro models, 3D printing, bioprinting

Hydrogel biomaterials serve as an invaluable resource in tissue engineering because of their ability to mimic the in vivo physiological microenvironment in vitro. Hydrogels are hydrophilic polymeric networks that act as scaffolds providing mechanical, structural, and biochemical cues to the cells either alone or in conjunction to create a better mimic of the in vivo microenvironment[1-3]. Many natural and synthetic hydrogels have been used in the last few years to generate a plethora of in vitro models, in vivo scaffolds, and drug delivery systems[4-6].

In addition to seeding cells in the bulk hydrogel or on the hydrogel surface, hydrogels have been used as bioinks in 3D bio printing applications [7], to support spheroids and organoid cultures, in organ on a chip models, and in microfluidic devices.[8] Screen printing has been used traditionally for lithography and calligraphy, which in modern times was adopted to suit a range of applications like printing circuit boards[9-10], creating microfluidic devices[11-12], as an additive manufacturing technique to create 3D structures[13], to control the growth of bacterial cultures spatially[14]. We have adopted this traditional lithographic screen printing technique to pattern mammalian cells along with a suitable scaffold to generate in vitro models[15]. In that study, we have used gelatin as the bioink to develop and optimize the screen printing setup. However, gelatin-based hydrogels are challenging constructs due to their variable compositions and temperature-dependent mechanical properties, both of which can contribute to inconsistent gelation time and patterning. For the screen printing process, a hydrogel with a predictable gelation time between 3-10 min would be ideal since it allows for an expected timeframe to mix the precursor solutions, extracellular matrix (ECM) proteins, and the cells before printing, all while preserving the minute details of the print. A synthetic, chemically crosslinked gel with defined kinetics is optimal for this purpose since a synthetic gel would allow for tighter control over the gel properties and tunability, which can be

tailored to suit the needs of the cells and help to achieve a workable gelation time and ideal mechanics for screen printing.

In this paper, we present two PEG-PLL based, chemically crosslinked gel systems, one utilizing vinyl sulfone (VS) and thiol (SH) click chemistry and the other using succinimidyl and primary amine addition reaction to be used as the bioink in the screen printing process. This paper examines the synthesis, characterization of the precursors and gels, and optimization of the gel composition for maximum cell survival and its use in the screen printing process.

The first hydrogel is synthesized using a VS-PEG-PLL macromer and an SH-PEG-SH crosslinker. The schematic of the hydrogel formation and the reaction of the network formation is shown in Figure 1a, c. The VS-PEG-PLL is produced in two steps (Figure S1); the first step is synthesizing an imidazole carbamate active intermediate compound[16]. The formation of this intermediate compound is characterized using ^{1}H NMR, confirming the imidazole carbamate peaks with chemical shifts of 7.2, 7.5, and 8.2 ppm[17] (Figure S2). The second step, the synthesis of VS-PEG-PLL, by addition of PLL and end functionalizing the hydroxyl ends of PEG by divinyl sulfone, is confirmed using ^{1}H NMR (Figure S3).

The VS-PEG-PLL macromer has a PLL backbone with PEG branches which are end-functionalized by vinyl sulfone groups to be crosslinked with the thiol groups in the PEG dithiol crosslinker. To estimate the amount of PEGylation and the number of inyl sulfones on the VS-PEG-PLL macromer, we used the OPA (o-phthaldialdehyde) assay[18] and the ^{1}H NMR data. The OPA assay was used to quantify the number of free amines, and the ^{1}H NMR data was used to find the relative amounts of PLL and VS to PEG. The OPA assay and the 1H NMR data showed that PEG occupies 7.5% of free amines and 90% of those PEGs are end-functionalized by vinyl sulfone. Based on this data, we synthesized a hydrogel with a 1:1 ratio of vinyl sulfones to thiol groups

(Figure 1a). Different amounts of PEG and vinyl sulfone were used to alter the PEGylation and the functionalization of the VS-PEG-PLL macromer, but no significant alteration in the macromer was observed, slightly limiting the tunability of the system.

The second hydrogel is synthesized by using PLL (70-150kDa) and PEG-succinimidyl glutarate (4 arm 10kDa or 2 arm 4.6kDa) as the crosslinker (Figure 1b, d) (the main text uses the 4arm 10kDa PEG the data corresponding to the bifunctional PEG 4.6kDa is used in the supplementary information). The PEG-SG is synthesized starting from hydroxyl PEG using the succinimidyl reaction (Figure 1d)[16]. The formation of the PEG-SG is confirmed by using ^{1}H NMR (Figure S4). From the ^{1}H NMR data, the hydroxyls on the PEG are found to be completely converted to succinimides. The hydrogel is then synthesized using 10% w/vol solutions of the PEG-SG and the PLL in PBS at 1:1 ratio of free amines in PLL to succinimides in PEG. Successful gelation was achieved using different molecular weights of PLL and PEG, as well as by varying their relative amounts i.e. by altering the ratio of free amines in PLL to succinimides in PEG. These findings show that the succinimidyl-amine reaction based hydrogel system has a high level of tunability, which will be highlighted in more detail.

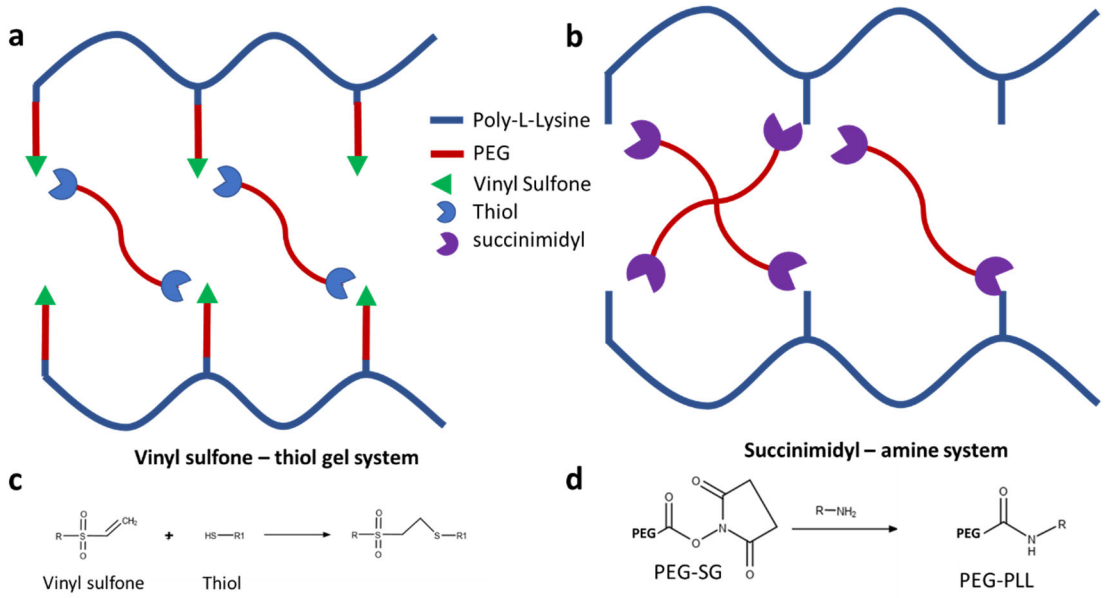

Figure 1. a) Schematic of VS-SH based gel system based on VS-PEG-PLL macromer with PLL backbone and the vinyl sulfone ends; b) Schematic of the succinimidyl-amine gel formation based on PLL molecule directly reacting to 4 arm PEG succinimidyl glutarate or a bifunctional PEG-SG; c) VS-SH reaction mechanism; d) SG-NH_2 reaction mechanism

To compared the gels, we added macromers from the two systems, either vinyl sulfones to thiols in one gel system and free amines to succinimides in the other gel system, in a 1:1 ratio. A frequency sweep was performed on the gels, and from that it was found that the VS-SH system has a lower storage modulus(G') of 108.68 Pa compared to the succinimidyl amine system, which has a strength of 859.69 Pa (Figure 2a). It can also be noted that the crosslinker length in the VS-SH system is around ~12.6kDa, while it is ~ 5kDa in the succinimidyl amine system, which tells that VS-SH system inherently has a lower crosslinking density compared to the other system, which results in the lower storage modulus of the gel. Gelation time is an important aspect for consideration in the design of materials for the screen printing setup. To determine the gelation time for both the systems, a constant force time sweep was performed. Gelation for the VS-SH system occurred in 12 mins, while the succinimidyl amine system gelled within 10 secs (Figure 2b, Figure S5). While both of these times are not optimal for the screen printing process which ideally involves gelation tines on the order of 3-5 minutes, the high tunability of the succinimidyl amine system allows one to develop a range of hydrogels with a gelation time in the workable time range (Figure S6). SEM micrographs (Figure 2c, d) of both the gels reveal a porous structure suggesting a uniform pore distribution, which is helpful in synthesizing a homogenous scaffold that provides consistent chemical and mechanical cues to the cells.

The time to synthesize the components for is lower for the SG system (5-7 days for the VS-PEG-PLL macromer, 2 days for the PEG-SG crosslinker). Furthermore, the VS-PEG-PLL macromer

takes 10-15 mins to dissolve into the solution whereas as the others are readily dissolved. Also the SG-based system exhibits greater tunability of the mehcanics and gelation time and clear optical properties while the VS-SH gels are white. Based on this, we chose the succinimidyl amine system going forward, to test its biocompatibility and its application in the screen printing process (Supplementary Information).

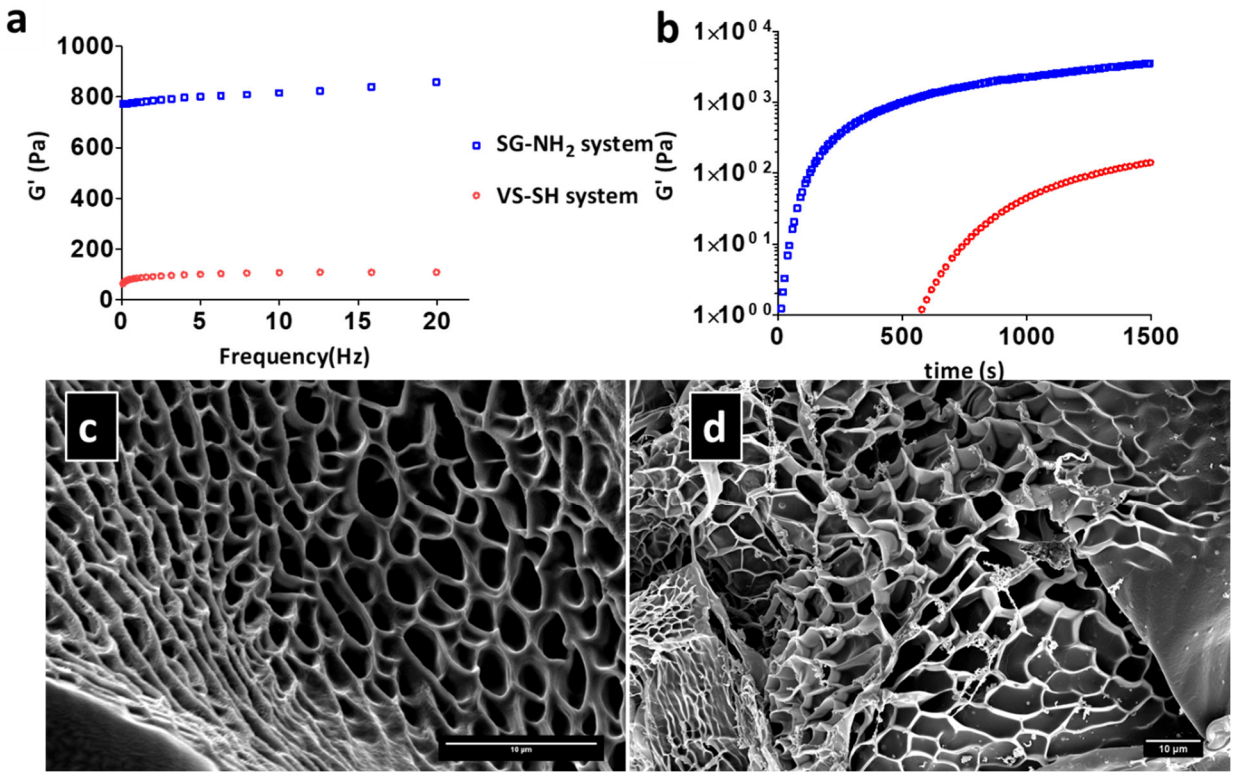

Figure 2. a) Frequency sweep comparing both the gel systems synthesized by using 1:1 reactive groups; b) Time sweep to determine the gelation time of the gel systems (the G' axis is on the log scale for easy juxtaposition of the gelation times.); SEM micrograph of the porous structure of the gels formed by using 1:1 ratio of the reactive groups in the c)VS-SH system and d) SG-NH2 system (Scale bar is 10µm)

Since the screen printing process utilizes the hydrogel as both a bioink and a substrate to print the cells, we created a library of hydrogels by altering the ratio of free amines to succinimides as

the substrate for the printing process. Next, we seeded Caco-2 cells on the hydrogels and stained them with a live/dead stain. It should be noted that no significant cytotoxicity was observed after 12 hours in the gels compared to the control (Figure 3a), indicating the usability of the hydrogel as substrate.

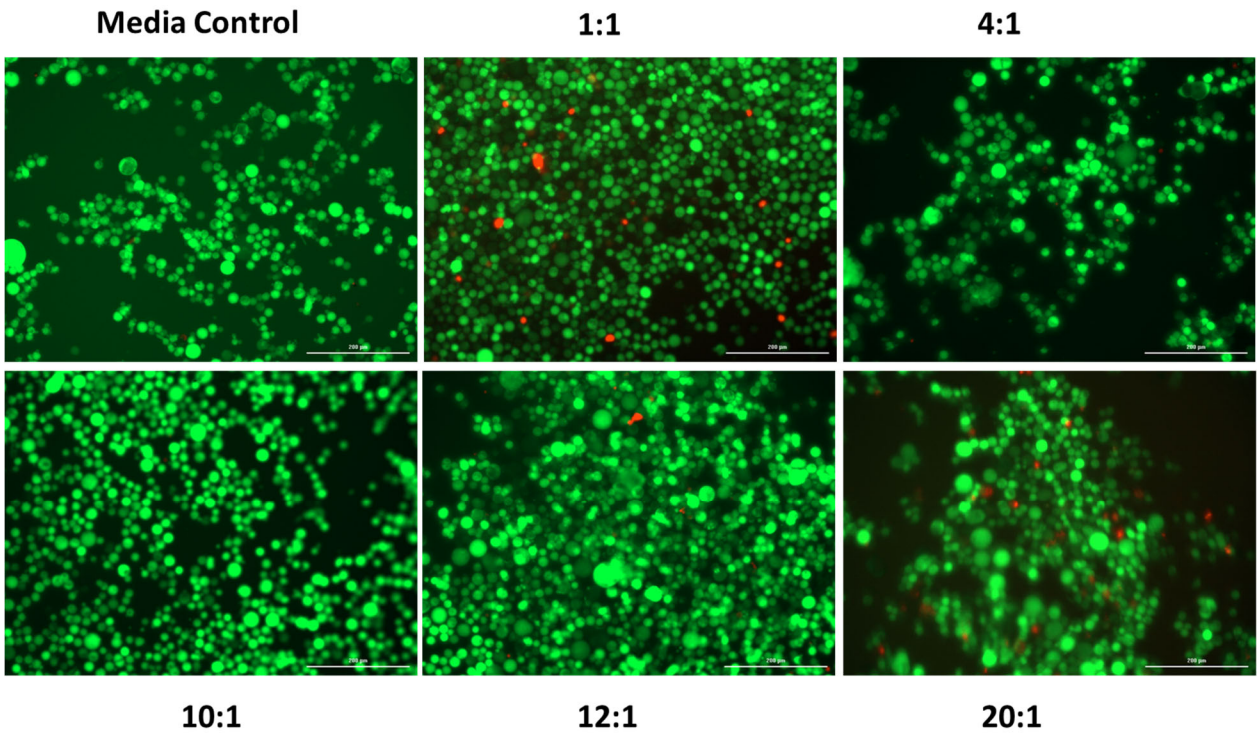

Figure 3. Live/Dead scans of the Caco-2 cells seeded on PEG-PLL hydrogel base synthesized using different ratios of free amines to succinimide groups; calcein-AM stain is used to stain the live cells and an ethidium homodimer-1 stain for the dead cells (Scale bar is 200µm)

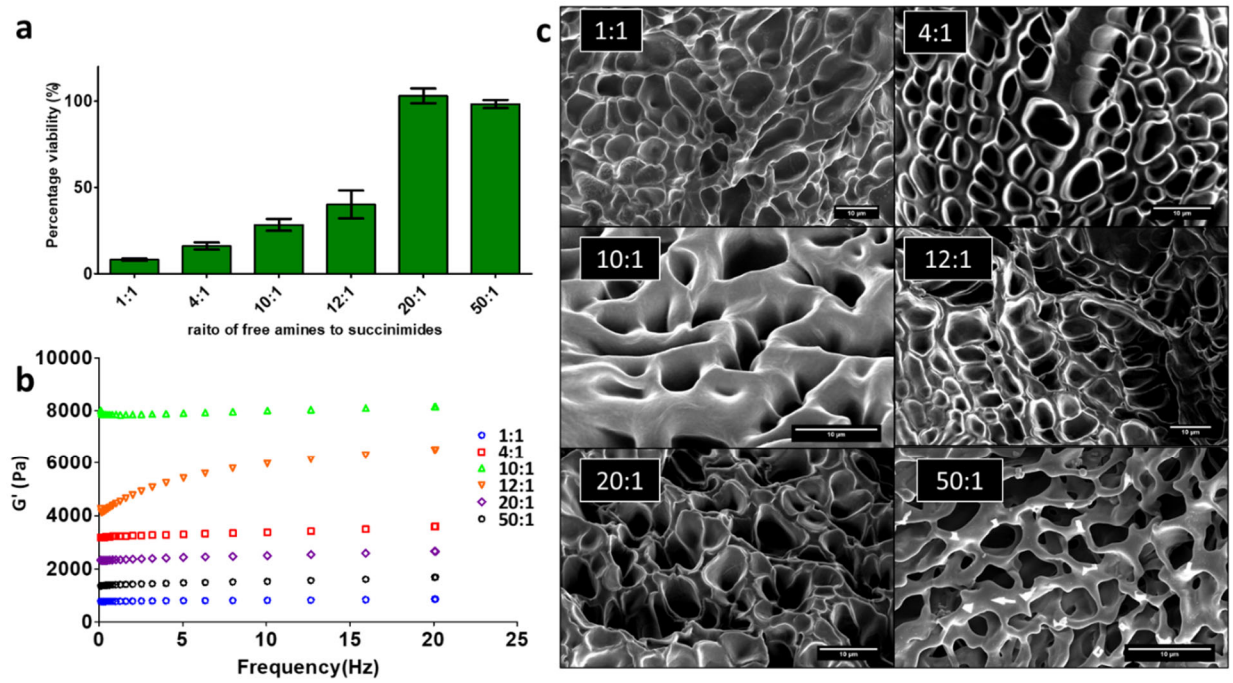

Figure 4. a) Viabilities of Caco-2 cells seeded in different PEG-PLL hydrogels synthesized; b) Storage modulus (G') of the different hydrogels obtained by tuning the PEG-PLL hydrogel system; c) Scanning electron microscopy images of PEG-PLL hydrogels (Scale bar is 10 µm) synthesized using different ratios of free amines in PLL to succinimides in PEG;

The cells were encapsulated in the hydrogels to screen the gels for cytotoxicity. Different hydrogels were synthesized by altering the crosslinker to PLL ratio, and the precursor solutions were mixed along with the cell suspension in the requisite amount to encapsulate the cells in the hydrogels. The MTT assay is used to measure the survival of the cells seeded inside the hydrogel scaffold (Figure 4a, Figure S7), and it is observed that reducing the amount of crosslinker increases cell survival. There is no correlation between the hydrogel system's storage modulus, G' (Figure 4b), and survival. From the SEM micrographs(Figure 4c, Figure S8) and the pore size analysis (Figure S9), there is no correlation between the pore size and the survival of the cells. Also, it can be noted that the gel formed with the lower amount of crosslinker (50:1 ratio) has a slightly larger

pore size compared to the other gels, which might be helping in improving the cell survival, but more detailed testing is needed to establish the correlation between the gels and the survival of the cells.

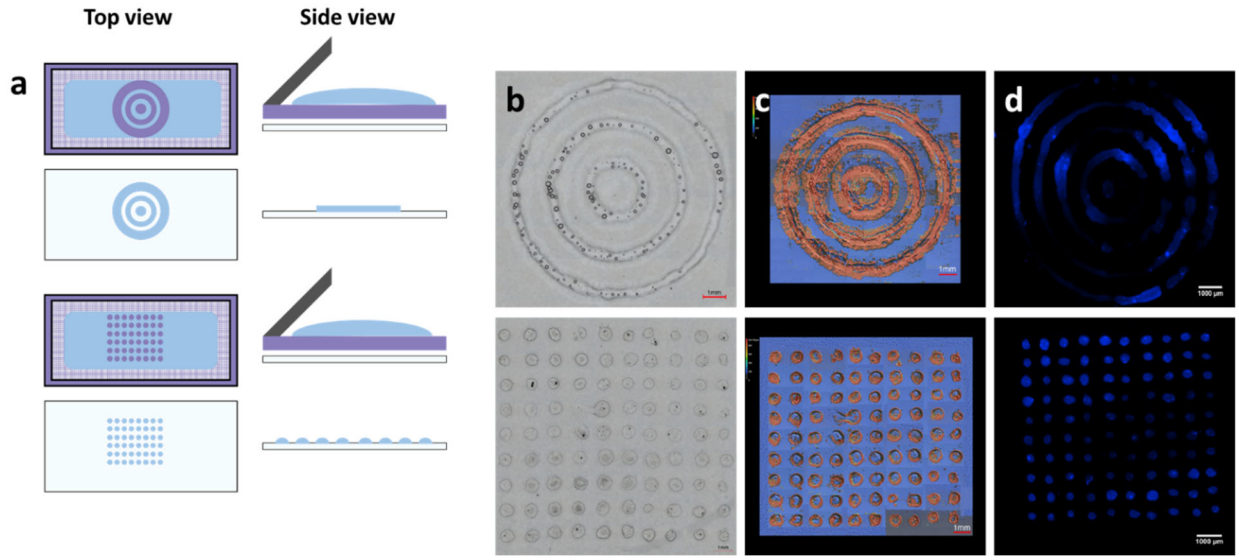

Figure 5. a) Schematic of the screen-printing process showing a monolayered concentric circle and a grid of dots as the pattern; b) Bright field images, c) heat maps and d) florescent images of prints obtained using carboxylate microspheres embedded succinimidyl amine hydrogel as the bioink. The images are a composite of a grid of images a 400 µm dot pattern, a concentric circles pattern (Scale bar is 1mm)

To investigate the application of the gel system to screen printing, we mixed florescent carboxylate microspheres (0.10 µm) in the precursor solution and analyzed the quality of the features printed. As reported previously with the gelatin bioink[15], we used a similar set of patterns consisting of a grid of dots, concentric circles, and a set of parallel lines to print the PEG-PLL based bioink. Figure 5a shows the screen printing setup with the grid of dots and the concentric circle stencil on the screen. From the qualitative analysis of the patterns (Figure 5b-d, Figure S10),

the PEG-PLL gel system is on par with the previously tested gelatin bioink system[15] in terms of the resolution and ease of printing.

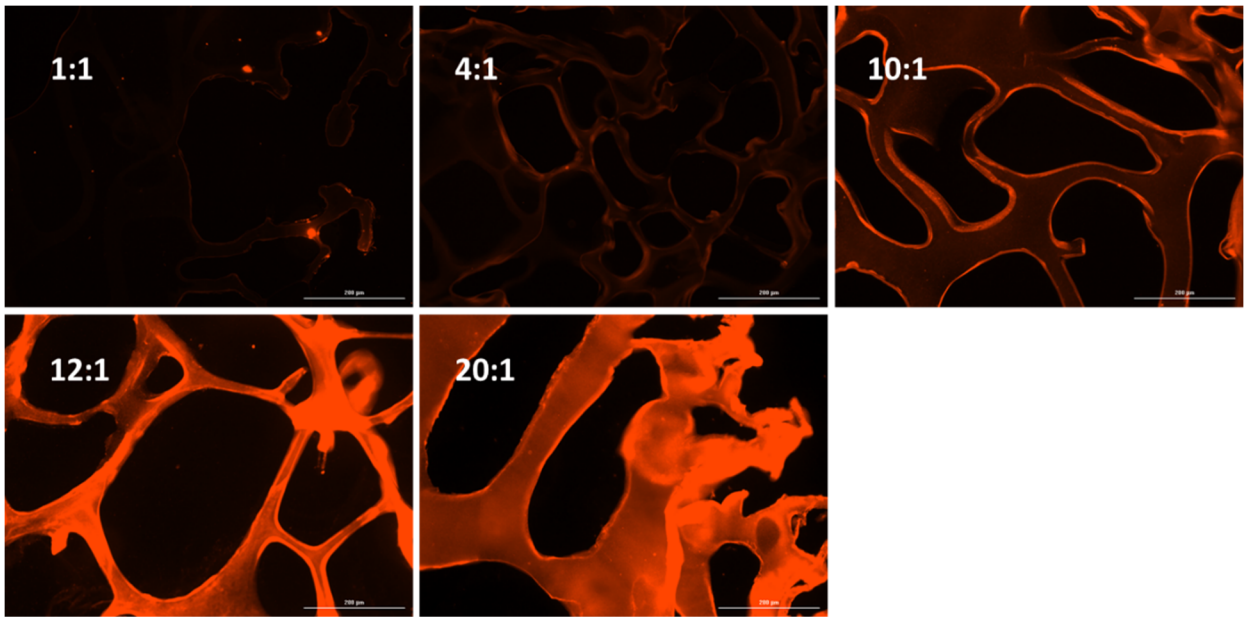

Figure 6. Florescent images showing the variation of laminin in different succinimidyl amine gels obtained by altering the relative amounts of PEG and PLL. Laminin is used as a part of the PLL solution, and the amount of laminin can be seen increasing as the relative amount of PLL in the gel increases. All the images are taken at the same exposure and focal length to observe the variation in florescence (Scale is 200 µm)

Since the succinimides promote protein adhesion[19-20] and the PLL is used to coat culture ware[21-22] to promote cell adhesion, the succinimidyl amine system provides an ideal scaffold to conjugate the required proteins and biomolecules, in a facile manner, providing the necessary chemical cues for cell survival and proliferation. We utilized laminin, a common ECM protein, to probe adsorption and distribution in the gel. Laminin is added to the PLL precursor at a concentration of 100ug/ml, and the gel system is synthesized by altering the relative amount of PEG-PLL. From the immunofluorescent staining of laminin in the hydrogel sections, a uniform distribution of

laminin is observed, and the variation in concentration of the laminin in the gels is similar to the amount of PLL (Figure 6). Also, it can be noted that the laminin-absorbed gels showed no toxicity compared to the barebones gels (Figure S11).

As a proof of principle that the screen printing process can be used to fabricate in vitro models, we utilized the Caco-2 and Rat endothelial cells (REC) cells. Caco-2 cells have been used as an in vitro intestinal model for many applications[23-27], and it has been shown that endothelial- colonic cell interactions are critical to modeling aspects of the colon [28]. The coculture is the first step in creating a model of the intestinal barrier to mimic the inflammatory bowel disease[29]. Here we used the PEG-PLL hydrogel precursors (25:1 ratio) and the Caco-2 cells to print the line pattern and the REC cells to print a dot pattern adjacent to the Caco-2 cells (Figure 7). Caco-2 cells stained with calcein-AM (green) and RECs stained with DAPI. The pattern is an example of different geometries in which the cells can be patterned to understand the cell signaling pathways and create better models. Being able to pattern different cells efficiently is a critical first step in developing meaningful 3D tissue models. This proof of principle work lays the foundation for using these hydrogels in these 3D models and opens the door to be able to build tissue models and investigate the paracrine and autocrine signaling in coculture systems in vitro in an efficient and accessible manner.

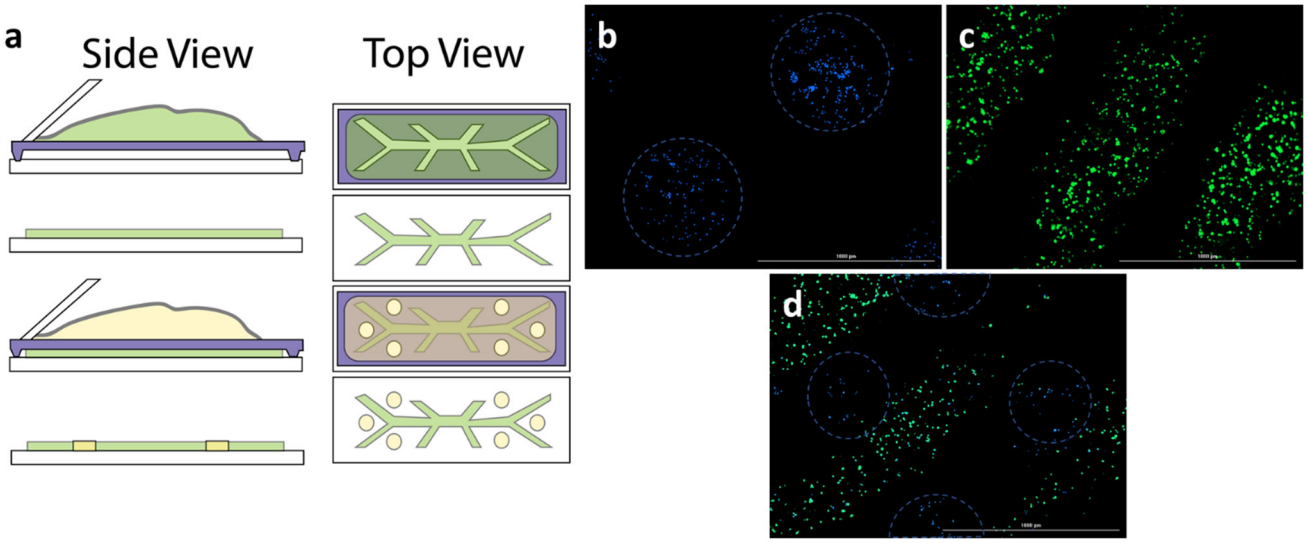

Figure 7. a) Schematic of the screen printing process using the hydrogel precursor solutions along with the cells as the bioink to print a multilayered pattern; b) Rat endothelial cells stained with DAPI printed using a 600µm diameter dot pattern, c) Caco-2 cells stained with calcein-AM printed using a 600µm parallel line pattern d) multilayered print obtained using both the cell lines using a dot pattern and a parallel line pattern. (Scale is 1000µm)

PEG-PLL based hydrogels have been used in several in vitro and in vivo applications [30-33]. Besides hydrogels, PLL has been used in many in vitro applications as culture ware coating to promote cell adhesion [21-22], and as a component of block polymers in drug delivery systems to deliver DNA and proteins[34-36]. PLL is also enzymatically degradable[36], making it a good candidate as a scaffold material for the cells so that the hydrogel can be remodeled accordingly with the cell growth.[37-38]

PEG is one of the most widely used materials in hydrogel synthesis and can be functionalized with various end groups to achieve different crosslinking reactions with various synthetic and natural polymers to form hydrogel networks[39]. These properties make a PEG based hydrogel

system an excellent candidate to tailor the gel strength and other properties that mimic native tissue microenvironment [2, 39].

Click chemistry reactions are highly selective and efficient reactions with faster and defined kinetics used in various hydrogel syntheses [2, 40-41]. The free amine and succinimidyl reaction is a fast, selective reaction used in coupling many PEG compounds to proteins and other amine-containing compounds [16]. PEG can be easily functionalized with vinyl sulfone and thiol groups to be used in the click chemistry and also can be functionalized using carbonyl and succinimidyl, which can be used to form stable carbamate linkages with the free primary amines in PLL [19-20]. Exploiting these reactions, we have synthesized precursor molecules based on PEG and PLL backbone.

VS-SH click chemistry has been used for various applications[2, 42-43]; we tried to leverage that reaction using the VS-PEG-PLL macromer. We hypothesized that having three components in the macromer would lead to high control over the final hydrogel via altering the ratio and molecular weight of the macromers. However, the range of properties of the VS-PEG-PLL hydrogels was limited. This limited range might be due to the high molecular weight of the final macromer formed or due to the steric hindrance between the PEG chains attached to the PLL backbone. However, we examined a range of macromers (PLL of 30-70kDa, 70-150kDa, and 150-300kDa and PEG of 4.6kDa and 8kDa), without much change in properties. Further research may be needed to determine the potential of this hydrogel.

Succinimidyl groups are used in protein adsorption[19-20], and the PLL is used to coat tissue culture plates to promotes cell adhesion, so using a hydrogel with both succinimides and the PLL in the hydrogel system would provide an opportunity for ECM protein adsorption and also cell adhesion, which was evident with the laminin distribution (Figure 6). Also, the synthetic nature of the

hydrogel gives a greater level of control on the hydrogel, unlike using natural materials like Matrigel, collagen, or gelatin, which have batch-to-batch variability [44-45]. Furthermore, it is known that chemical crosslinking can be bad for cell survival [1], so we tuned the hydrogel to optimize the short-term survival without any external chemical cues. It can be noted that previously, we discussed the long term survival of the cells in a similar hydrogel system albeit with different crosslinking chemistry[33].Also, we showed the adsorption of laminin and a uniform gel structure to create a homogenous microenvironment for the cells. So, this system shows the potential of a tunable system both in terms of providing the chemical cues and the optimal mechanical properties for the cells. Also, it can be noted from the preliminary results, the PEG-PLL system completely degrades in trypsin over 24 hours, which might be helpful for the cells to degrade the hydrogel over time to produce their own ECM.

In this paper, we tested the utility of the succinimidyl amine gel in the screen printing process. We utilized the fluorescent microspheres embedded in the gel as an analog to the cells to compare the current setup with our previous framework based on the gelatin bioink. From the initial testing and qualitative analysis, the resolution of the prints coupled with the facile bioink formulation was on par with the older bioink system. Finally, we have demonstrated the use of the screen printing process to pattern multiple cell types with the bioink to generate small in vitro models in a simplistic fashion.

The screen printing process can be used to create 3D in vitro models to mimic the in vivo microenvironment without specialized equipment in an economical way. This paper presents two potentially useful hydrogels that can be used as bioinks in the screen printing process and traditional tissue culture applications. Even though from our testing over a small range of molecular weights of PEG and PLL, the VS-PEG-PLL macromer showed limited tunability,

further research may be needed before we can rule out the potential of this hydrogel. However, the succinimidyl amine hydrogel system shows much promise even as an unmodified hydrogel system, so when supplemented with suitable adhesive motifs, ECM proteins, and other chemical cues, this hydrogel system can be tuned to achieve an ideal scaffolding material for tissue engineering applications. Also, given the ease of synthesizing the succinimidyl precursors, the succinimidyl system can be exploited with other free amine-containing molecules to create other gels for different applications.

ASSOCIATED CONTENT

Supporting Information. Experimental Section, NMR data corresponding to the synthesis of the macromers and the supporting information for the linear PEG based PEG PLL hydrogel.

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Notes

The authors declare no competing financial interest

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Screen printing tissue models using chemically crosslinked hydrogel systems: a simple approach to efficiently make highly tunable matrices

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