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A High Throughput Microarray System of Polymer Surfaces for the Manipulation of Primary Pancreatic Islet Cells

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Abstract

We developed a high throughput micro-arrayed polymer system for the study of polymer surfaces for islet cell culture. A micro-arrayed library with 496 different polymers was synthesized and used to examine attachment and insulin expression of islet cells. While most polymers were not supportive, several related polymers were identified as suitable (“hit’s”). The “hit” arrays composed of “hit” polymers with 36 replicates were fabricated to confirm their capacities to support the attachment of islet cells, and these capacities were further validated in large surfaces. Notably, the attachment of islet cells on these synthetic polymeric films has been found to be as supportive as 804G supernatant coated tissue culture polystyrene dishes, one of the most extensively used substrates for the islet cell attachment. Interestingly, the polymeric surfaces optimal for a different cell type, hES derived cells, were distinct, highlighting the utility of these approaches for identifying cell-type specific surfaces.

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1. Introduction

Diabetes is a worldwide health problem, which is caused most commonly by a combination of the insulin resistance and inadequate insulin secretion from pancreatic beta cells. In the less common autoimmune diabetes (Type 1 diabetes), beta cells are almost entirely destroyed. Replacement of beta cells with either pancreas or islet cell transplantation has the potential to reverse diabetes, but the treatments are encumbered by the toxicity of immunosuppression and poor durability of the transplants [1]. One problem with islet cell transplantation is that it is difficult to maintain islet cells in tissue culture [2]. Presently, islet beta cells are usually cultured on 804G supernatant coated tissue culture plastic (TCP) dishes, however, the production of 804G supernatant is inconsistent, highly laborious and therefore limits the large-scale production [3]. Improvement of tissue culture conditions for islet cells may allow for better transplantation results as well as facilitating the study of beta cell biology. While some progress has been made in developing synthetic substrates to support the attachment of islet cells [4], further improvements are necessary. Herein, we developed a micro-arrayed polymer system for the study of polymer surfaces for islet cell manipulation in a high throughput manner. A micro-arrayed library with 496 different polymers was synthesized and used to examine attachment and insulin expression of islet beta cells. While most polymers were not supportive, several related polymers that support islet cell attachment were identified as suitable ("hit's). Arrays composed of "hit" polymers with 36 replicates were fabricated to confirm their capacities to support the attachment of islet cells, and these capacities were further validated in large surfaces. Notably, the attachment of islet cells on these synthetic polymeric films has been found to be as supportive as 804G supernatant coated tissue culture polystyrene dishes. Interestingly, the polymeric surfaces optimal for a different cell type, human embryonic stem cells derived cells, were distinct, highlighting the utility of these approaches for identifying cell-type specific surfaces.

2. Materials and Methods

2.1 Combinatorial array preparation

Polymers were printed in a humid Ar-atmosphere on epoxy monolayer-coated glass slides (Xenopore XENOSLIDE E, Hawthorne, NJ) which were first dip-coated in 4% (w/v) pHEMA (pHEMA = poly(2-hydroxyethyl methacrylate)), using modifications of robotic fluid-handling technology as described previously [5]. Spots were polymerized via 10 s exposure to longwave UV, and dried at < 50 mtorr (1 torr= 133.32 Pa) for at least 7 days. The chips are sterilized for 30 min for each side, and then washed with PBS twice for 15 min to remove the residue monomer or solvent. After that, the chips were coated with 25 µg/mL Fn (Sigma) for 1hr, and then washed with PBS, and medium before cell seeding.

2.2 Islet cell harvest and culture

Sprague Dawley rat islets were isolated by using a collagenase digestion followed by separation using a density gradient [6]. Briefly, under anaesthesia, a laparotomy was performed and the pancreas exposed. After ligation at the ampulla of Vater, 9 mL of a collagenase solution (Liberase RI, Roche, Indianapolis, IN) was injected into the pancreas via the common bile duct. The pancreas was removed and incubated in a stationary water bath for approximately 24 min at 37 °C. Islets were separated by a density gradient (Histopaque-1077, Sigma) and centrifuged at 1750g for 20 min. After washing, islets were hand picked and cultured overnight in RPMI 1640 with 10% fetal calf serum. For digestion, 1 mg/mL trypsin (bovine pancreas trypsin Sigma) and 30 µg/mL DNase (DNase I, Roche) was then added to the islets which were then incubated for 15 min in a 37 °C incubator. During the digestion, the islets were vortexed every 5 min for 10 seconds. Cold media with

serum was then added to stop the digestion. The cells were washed two times, then counted and plated.

2.3 human embryonic stem cell (hESC) culture and embryoid body (EB) formation

Undifferentiated hESCs (H13, WiCell, Wisconsin) were grown on an inactivated mouse embryonic fibroblast (MEF) feeder layer, as previously described [5]. To induce the formation of EBs, undifferentiated hESCs were treated with 1 mg/mL type IV collagenase for 40 min, and then transferred (1:1) to low attachment plates (10 cm, Ref:3262, Corning) containing 10mL of differentiation medium [80% knockout-DMEM, supplemented with 20% fetal bovine serum (FBS, Hyclone), 0.5% L-glutamine, 0.2% b-mercaptoethanol and 1% nonessential amino acids (all from Invitrogen)]. EBs were cultured for 8 days at 37 °C and 5% CO₂, in a humidified atmosphere, with changes of media every 2 days. After that, EBs were trypsinized (0.25%) for 7 min and plated on the polymer array.

2.4 Immunohistochemistry

To stain the islet cells, chips were washed with PBS, fixed with 4% paraformaldehyde solution for 20 min, permeabilized with 0.3% Triton X-100 in PBS for 20 min, and then blocked with serum for 30 min. Primary antibody, guinea pig anti- insulin diluted in PBS was incubated on the chip for 1 hr, followed by biotin anti-guinea pig for 1 hr, and then streptavidin conjugated alexafluoro 488 diluted in PBS incubated with chip for 1 hr. The chips were then washed with PBS and water to remove the salts, and air dried. The chips were imaged and quantified with iCys Cytometry (Compucyte, Cambridge, MA) based on the intensities from three channels including one scattering channel and two fluorescence channels (405 and 488 nm). The intensity of scattering channel were used to determine the position of each polymer spot on the glass slide, and the fluorescence intensity at two different wavelengths (405 and 488 nm) was used to quantify the cell number and the amount of insulin on each polymer spot, respectively. In a similar manner, EB cells were stained with anti nuclei antibody.

2.5 Polymer film preparation

The polymerization solution was prepared by mixing 3 mL monomer with 40 mg 2,2-dimethoxy-2-phenyl-acetophenone dissolved in 1 mL DMF; 0.2 µL of this polymerization solution was spread into 1×1 cm² liquid thin film on the poly(HEMA) coated glass slide, and polymerized via 6 min exposure to longwave UV in a humid Ar-atmosphere, and dried at < 50 mtorr (1 torr= 133.32 Pa) for at least 7 days.

3. Results and Discussion

Photo-polymerized polymer microarrays have been recently developed for high throughput investigations of cell/materials interactions [5,7]. In this study, 22 acrylate monomers were used to create a library of 496 different surfaces in a combinatorial manner. The monomers used in this study are shown in Figure 1. Monomers were divided into 16 major monomers and 6 minor monomers groups, with the major monomers named numerically, and the minor monomers named alphabetically. In this study, each major monomer was mixed with each of 6 minor monomers at 6 different ratios (100:0, 90:10, 85:15, 80:20, 75:25, 70:30 (v/v)), and the mixtures were then printed and polymerized on a poly(HEMA) coated glass slide in triplicates, using a modified contact pin array printer [5,7]. In this way, a library with 16×5×6+16=496 different polymers was created on a conventional 25×75 cm² glass slide.

To generate polymers with chemically diverse properties, monomers were selected with a range of chemical groups and hydrophobicity/hydrophilicity. For example, the logarithm of partition coefficient (log P) can be used to quantify the hydrophobicity/hydrophilicity of

a molecule. In this study, the log P values differ from -0.03 (monomer 6) to 3.74 (monomer 14) [8]. Monomers were also selected to vary crosslinking density in the final material, as this can affect the mechanical properties of polymers [9]. Monomers were also selected to vary chemical and surface properties – for example, the fluorine containing monomer D was selected to reduce surface energy, and monomer E to provide tertiary amine groups.

After contact with serum containing medium, serum proteins rapidly adsorb to materials surfaces to varying degrees [10]. The layer of the adsorbed serum protein typically plays an important role in determining cellular response to a material [11,12]. In this work, we precoat the polymer array with fibronectin (Fn) because of its role in the extracellular matrix responsible for cell adhesion, neurite growth, and tissue repair [13]. It has been reported that the chemical composition of substrates can affect the amount and conformation of adsorbed Fn, and thereby effect cell behavior [14-17].

In this study, rat islet cells were harvested and seeded on the polymer array. After 24 hr of culture, they were fixed and the nucleus stained with Hoechst 34580, a blue fluorescence dye binding to DNA. They were co-stained with a green fluorescent insulin antibody to confirm they were insulin producing islet cells [5]. The merged images from fluorescence and scattering channels of islet cells attachment on the entire array has been shown in the Figure 2. Several representative images of polymer spots have been enlarged and shown in the Figure 3a-3d. Notably, the islet cells in the Figure 3c and 3d were double stained by Hoechst 34580 and insulin antibody, while the fibroblasts-like cells in the Figure 3b were only stained with Hoechst 34580. The difference in green fluorescence staining between islet cells and fibroblast like cells indicates the specificity of the insulin antibody, and the capacities of the islet cells to produce insulin. One advantage of the microarray format is its small scale – 496 different polymer materials were studied in triplicate with only 3 million islet cells.

These results were quantified using laser scanning cytometry and automated image analysis. Islet cell attachment was measured first by quantifying the average green fluorescence (insulin) intensity per polymer spot [18,19]. As shown in the Figure 4a, most polymers do not support the attachment of islet cells. However, a small percentage does support attachment, and those that do share the amine-containing minor monomer E. The material with the best islet cell attachment are 7E (15%) and 12E (25%). To confirm the results from primary screening, “hit” arrays composed of 36 replicates of 7E and 12E with varying E compositions (2%, 5%, 10%, 15%, 20%, 25%) were fabricated. As shown in the Figure 4b, the composition of minor monomer E in the copolymers is critical to their support to islet cell attachment. There are optimal ranges for minor monomer E compositions for each major monomer. For example, the optimal minor monomer E composition for major monomer 7 is 15% while the optimal composition for major monomer 12 is 25%. These results show that both the chemical structures and the compositions of major and minor monomers are critical for polymeric materials’ biological activities. Importantly, the islet cell attachment data collected from the “hit” array was found similar to the primary array indicating a high consistence among polymer array fabrication process, and the results collected from the primary array based on three replicates can be reliably reproduced.

Previous studies with partially differentiated human embryonic stem cells, human embryoid body (hEB) cells, showed that Fn-coated polymers containing monomer E only marginally promote hEB cell attachment, while monomers B, D, and F significantly increase hEB cell attachment [17]. For instance, 3E (10%) and 3E (15%) are low adhesion polymers for hEB cells while they can considerably promote the attachment of islet cells. (Figure 4c) On the other hand, 15B (25%) can significantly promote the attachment of hEB cells although its capacity to support islet cells are minimal. These results demonstrate the ability of polymers

to direct cell attachment in a cell type specific manner by altering their compositions. Although the biological basis for these differences is unclear, it could be attributed to the difference in conformations of Fn adsorbed on polymer surfaces [15,17]. Our recent data indicated a range of Fn conformation can be induced by polymers within the library [17]. The differential conformation of Fn on polymer surfaces may expose protein regions with differing affinity for cell surface proteins, and result in cell type specific adhesion. For instance, Yamada and coworkers identified two different regions within Fn which are able to induce cell type specific adhesion for melanoma and fibroblastic cells, respectively [20,21]. Although cell type-specific adhesion has been shown in previous research, most of the research has been limited to using peptides or protein segments to interact with cell type specific surface receptors. The procedures to identify peptide/protein segments that can interact with cell type specific surface receptors usually are laborious [20-22]. These results indicate that the polymer array technology developed here can facilitate the rapid discovery of polymer surfaces that can direct the cell type specific adhesion. The ability to direct cell type specific adhesion by altering polymer composition may facilitate the creation of complex medical devices with multiple cell types.

To test the performance of hit islet growth materials in large scale, a $1 \times 1 \text{ cm}^2$ polymer film was prepared from the top candidates (7E (15%) and 12E (25%)), and islet cells attachment was studied. Similar to its micro-scale counterpart, the attachment of islet cells was reproduced on the polymer 7E (15%) rapidly (<2hr). Insulin antibody staining shown in the Figure 5a confirmed the attached cells are insulin producing cells. For comparison, several commonly used ECM proteins were used to coat tissue culture polystyrene dishes. As shown in the Table 1 and Figure 5, the results presented here are consistent with the previous finding that most commonly used ECM proteins such as gelatin and collagen do not support islet cell attachment on tissue culture polystyrene dishes. However, laminin from human placenta (Sigma), matrigel basement membrane matrix (BD bioscience) and 804G supernatant do support the attachment of islet cells. 804G supernatant has been most extensively used for the islet cell attachment, and as shown in Figure 5, the attachment of islet cells on the polymeric film 7E (15%) is similar to the 804G supernatant coated tissue culture polystyrene dishes. Of note the major component of matrigel basement membrane matrix and 804G supernatant is laminin, and laminin is a major component of the basement membranes in pancreas [3, 23-26]. Importantly, only a subset of laminin can support the attachment of islet cells [24, 25]. For instance, laminin (from BD Bioscience) coated dishes did not support the attachment of islet cells. Moderate islet cell attachment was found on the Fn coated poly(D-lysine) surfaces, which is consistent with the hypothesis that amine containing group maybe important to modulate the conformation of adsorbed Fn to support the attachment of islet cells.

Conclusion

In summary, a library with 496 different polymers was designed and prepared, and it was used to examine the attachment of islet cells. The amine containing minor monomer E was shown critical for the attachment of the islet cells, and the copolymers 7E (15%) and 12E (25%) was found to be the best performing polymers for the attachment of islet cells, and these results were further confirmed with the “hit” array with 36 replicates. It is thought that the amine containing minor monomer E could induce the exposure of certain epitopes of the adsorbed Fn to support the attachment of islet cells, and this capacity can be used to direct cell adhesion in a cell type specific manner. To generate industry applicable products to support the islet cell attachment, two polymeric films have been prepared from 7E (15%) and 12E (25%). The islet cell attachment was reproduced on the 7E (15%), and the attachment of islet cells on the polymer 7E (15%) was found to be similar to that seen in 804G supernatant-coated tissue culture polystyrene dishes. The polymer micro-array

technology presented herein can provide an efficient manner to screen polymeric substrates for a variety of cell biology applications.

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Figure 1.

Biomaterials array design a) monomers used for array synthesis b) 36 different combinations for the major monomer 1 with all 6 different minor monomers

Figure 2.
The attachment of islet cells on the polymer array. The chemical compositions of each polymer spots were labeled in white. The white boxes are polymer spots which are detached from the slides during the culture.

Figure 3. The representative merged images collected by iCys cytometry from scattering and fluorescence channel. a) no cell attachment, b) fibroblast like cells attachment, c,d) the islet cell attachment

Figure 4a.

The map of the relationship between islet cell attachment and polymer composition has been established. The number in the map represents the green fluorescence intensity on the polymer spots.

Figure 4b.

The attachment of islet cells on polymer 7E and 12E with varied minor monomer (E) composition.

Figure 4c.

The adhesion of two different cell types (islet cells and EB cells) on polymers 15B (25%), 3E (10%) and 3E (15%), respectively.

Figure 5.

The attachment of islet cells on the a) Fn coated polymeric film 7E (15%); b) matrigel coated TCP; c) G408 supernatant coated TCP; d) Sigma laminin coated TCP; e) poly(D-lysine) coated TCP; f) Fn coated poly(D-lysine) surfaces. TCP stands for tissue culture polystyrene.

Table 1

The attachment of rat islet cells on the ECM proteins coated tissue culture polystyrene dishes ^[a]

Collagen	Gelatin	Laminin (BD)	Poly(D-lysine)	Matrigel	Laminin (sigma)	G-408 supernatant
-	-	-	+	+	++	++

^[a] - represents little cell attachment; + represents moderate cell attachment and ++ represents high cell attachment