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Epidermal and dermal integration into sphere-templated porous poly(2-hydroxyethyl methacrylate) implants in mice

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Abstract

Percutaneous medical devices remain susceptible to infection and failure. We hypothesize that healing of the skin into the percutaneous device will provide a seal preventing bacterial attachment, biofilm formation, and subsequent device failure. Porous poly(2-hydroxyethyl methacrylate) [poly(HEMA)] with sphere-templated pores (40 μ m) and interconnecting throats (16 μ m) were implanted in normal C57BL/6 mice for 7, 14 and 28 days. Poly(HEMA) was either untreated, keeping the surface non-adhesive for cells and proteins, or modified with carbonyldiimidazole (CDI) or CDI reacted with laminin 332 to enhance adhesion. No clinical signs of infection were observed. Epidermal and dermal response within the poly(HEMA) pores was evaluated using light and transmission electron microscopy. Cells (keratinocytes, fibroblasts, endothelial cells, inflammatory cells) and basement membrane proteins (laminin 332, β 4 integrin, type VII collagen) could be demonstrated within the poly(HEMA) pores of all implants. Blood vessels and dermal collagen bundles were evident in all of the 14 and 28 day implants. Fibrous capsule formation and permigration were not observed. Sphere-templated polymers with 40 μ m pores demonstrate an ability to recapitulate key elements of both the dermal and the epidermal layers of skin. Our morphological findings indicate that the implant model can be used to study the effects of biomaterial pore size, pore interconnect (throat) size, and surface treatments on cutaneous biointegration. Further, this model may be used for bacterial challenge studies.

Keywords

wound healing; biointegration; biomaterial; skin; percutaneous

INTRODUCTION

Percutaneous medical devices play an important role in modern health care. However, despite advances in function and design, a combination of bacterial infection at the device/skin interface and mechanical instability leads to high device failure rate.¹ Use of percutaneous vascular catheters alone results in 80,000 to 250,000 blood stream infections per year.²⁻⁶ The most effective method for reducing device-related infections remains stringent care of the device/skin interface.⁷ The Centers for Medicare and Medicaid Services have proposed to implement more stringent restrictions for payment of hospital acquired infections,⁸ increasing the financial incentive for a simple, cost effective technological advancement to reduce infections at device/skin interfaces. Our goal is full biointegration of the skin into the percutaneous device, not only to prevent sinus track formation and bacterial infection, but to create a mechanically stable device/skin interface with an adequate blood supply for long-term use.

Implantation of solid (non-porous) materials in vivo result in marsupialization or downgrowth of the epidermis along the implant, which creates a sinus track between the skin and biomaterial and results in failure of the device.^{1·9·10} Others have shown that porous materials promote in vivo cutaneous biointegration into various porous (not merely surface roughened) geometries.¹¹ (review) and ^{10·12·26}

For our studies to promote biointegration into percutaneous devices, we first identified biomaterials that allow morphological study of implant/skin interfaces.²⁷ Crosslinked poly(2-hydroxyethyl methacrylate) [poly(HEMA)], a hydrogel used in the manufacture of soft contact lenses²⁸ proved to be an excellent biomaterial for our studies. Poly(HEMA) was precision-engineered by a sphere-templating method in which pore and interconnecting throat size could be controlled.^{29·31} These porous constructs were implanted in *in vitro* studies¹⁵ and in short-term in vivo mouse implants.¹⁴ The poly(HEMA)/skin interface remained intact for morphological studies for both *in vitro* and in vivo studies. Poly(HEMA) is essentially non-adherent for proteins and cells, however, 1,1'-carbonyldiimidazole (CDI) can be used to modify the poly(HEMA) surface to enhance adhesion of both proteins and cells.¹⁵ Thus, poly(HEMA) is an excellent biomaterial in which cutaneous integration can be evaluated in both adherent and non-adherent biomaterials and in response to changes in geometry such as pore and throat size. For example, poly(HEMA) implants with 20µm pores and 5µm pore interconnects (throats) elicited epithelial marsupialization while poly(HEMA) with 40µm pores and 16µm throats demonstrated cutaneous integration.¹⁵ The marsupialization response using the smaller pore and throat size could be rescued by modifying the biomaterial surface using CDI.¹⁵

The porous poly(HEMA) implants used for our studies were precision-engineered by sphere-templating with a uniform pore size of 40µm and throat (interconnect) size of approximately 16µm, and prepared with and without surface modification to enhance cell adhesion and protein absorption.

In this study, using the mouse implant model previously described,¹⁴ we further characterized cutaneous incorporation by histology and immunohistochemistry of not only 7 day implants, but also longer-term implants of 14 and 28 days. Epidermal biointegration, dermal-epidermal junction protein deposition, endothelial cell, monocyte/macrophage and fibroblast ingrowth, as well as collagen formation within the poly(HEMA) pores were evaluated. Transmission electron microscopy (TEM) was used to evaluate cellular organization within the microniches of the integrated porous biomaterial.

MATERIALS & METHODS

Porous poly(HEMA) synthesis

Porous poly(HEMA) implants used for this study have been described in previous studies.^{14·15·27·30·31} Briefly, 40µm diameter polymethyl methacrylate (PMMA) beads are compacted (Figure 1A) to maximize contact points. The PMMA is then heat-sintered to connect the PMMA beads and subsequently surrounded by poly(HEMA) (Figure 1B). The poly(HEMA) is then polymerized and the PMMA is extracted by solubilization, leaving behind the poly(HEMA) with uniform 40µm pores and 16µm pore interconnects (throats) at each sintered contact point of the PMMA beads (Figure 1C). Through the random packing of the PMMA beads, each pore has approximately 7-8 interconnecting throats, thus leaving the entire porous construct interconnected. A scanning electron micrograph shows open pores with interconnecting throats (Figure 1D). An example of a 6µm cryo-section of porous poly(HEMA) is shown in Figure 1E, with Figure 1F showing a higher magnification of 2 interconnected pores. Figure 1G shows 2 poly(HEMA) pores with a keratinocyte bridging the throat. The sphere-templated poly(HEMA) constructs were cut into 15mm × 2mm ×

2mm strips and were either 1) left untreated and stored in PBS, 2) surfaced modified with CDI, rinsed in dioxane and stored in PBS (CDI), or 3) surface modified with CDI and reacted overnight with 65µg/ml partially purified human laminin 332 (CDI/lam 332), as previously described.¹⁴

Mouse implantation

Animal studies were conducted with University of Washington Institutional Animal Care and Use Committee approval in compliance with the NIH Guide for the Care and Use of Laboratory Animals, 1985. Eight week-old male C57BL/6 mice (Charles River Labs, Wilmington, MA) were individually housed in a temperature-controlled animal facility. A total of 79 mice were used for the study [12 mice for 7 day implants, 32 mice for 14 day implants, and 35 mice for 28 day implants]. Each mouse was implanted with two poly(HEMA) strips as described.¹⁴ Briefly, mice were anesthetized, dorsal skin was shaved, treated with depilatory cream (1.5 min.), wiped with warm water gauze, and cleansed with 10% povidone-iodine followed by an alcohol wipe. Two full-thickness incisions, 0.5 cm apart, were created in the midline between the scapulae, through which the strips were implanted. The second implant was placed 1 cm inferior to the original implant. Exit sites were sealed with Dermabond® (Ethicon, Cincinnati, OH) to keep implants securely in place. Animals were placed on a warming pad following surgery and allowed to recover fully from anesthesia before recaging.

Documentation of implants

Mice with poly(HEMA) implants were checked daily for the first week, then twice a week to follow implant integrity. Macro photographs of mice were captured on day 0 and at time of harvest.

Tissue harvest

Mice were euthanized with a lethal dose of intraperitoneal pentobarbital. Implants and surrounding skin down to the level of the deep fascia were harvested 7, 14, and 28 days after implantation. Specimens were fixed in either 10% neutral buffered formalin (NBF) for paraffin embedding (histology), in half strength Karnovsky's³² (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer) for Poly/Bed® 812 (Polysciences, Inc., Warrington, PA) embedding³³ and electron microscopy, or frozen in Tissue Tek O.C.T. Compound (O.C.T.)(Sakura Finetek Inc., Torrance, CA) for cryosectioning and immunohistochemistry (see Table).

Histology and tissue staining

Six micron paraffin sections were deparaffinized and stained with hematoxylin and eosin for histology and with trichrome (Masson, Sigma-Aldrich, St. Louis, MO) or picrosirius red [Direct red 80 (Sigma-Aldrich, St. Louis, MO)] to evaluate presence of collagen within the pores.

Immunohistochemistry

Six-micron sections of O.C.T.-embedded implant specimens were immunolabeled using routine immunoperoxidase methods as previously described.¹⁵ Primary antibodies were selected to identify both epidermal and dermal cells and proteins. Primary antibodies used were: pankeratin (polyclonal rabbit, 1:100, Dako, Carpinteria, CA) for epidermal keratinocytes, β4 integrin subunit (monoclonal rat, 1:2000, BD Biosciences Pharmingen, San Jose, CA) to localize hemidesmosomes of the dermal-epidermal junction, P3C8 – laminin 332 (monoclonal rat, 1:2, gift from Dr. William Carter) to localize an epidermal-dermal basement membrane protein, type VII collagen (polyclonal rabbit, 1:14,000 gift from

Dr. David Woodley) to identify anchoring fibrils along the dermal-epidermal junction, platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD-31) (monoclonal rat, 1:400, Research Diagnostics, Concord, MA) to identify endothelial cells, and pan-macrophage/monocyte marker F4/80 (monoclonal rat, 1:50, MCA519G, SeroTec, Raleigh, NC) to localize macrophages and monocytes. Secondary antibodies used were biotinylated donkey anti-rat (1:1600, Jackson ImmunoResearch, West Grove, PA) or biotinylated goat anti-rabbit (1:300, Vector Laboratories, Burlingame, CA). Following secondary antibody labeling, sections were incubated with streptavidin-biotin complex [Vectastain Elite ABC kit (peroxidase), Vector Laboratories, Burlingame, CA], with 0.12% 3,3'-diaminobenzidine used as chromogen. Glycergel (Dako, Carpinteria, CA) was used as mounting media. Control slides were immunolabeled without incubation with primary antibody.

Photomicrography

Tissue sections were viewed using a Nikon Microphot-SA microscope and imaged with a Photometrics Sensys digital camera for brightfield and differential interference contrast (DIC) images. To capture the entire implant/skin section, a few sections were scanned with an Aperio ScanScope® CS digital scanner (Aperio Technologies, Vista, CA) equipped with a 20x objective. Photoshop® (Adobe Systems Inc., San Jose, CA) was used for image color adjustment.

Electron microscopy

The tissue region of interest was excised and processed for transmission electron microscopy.¹⁵ Excised tissues containing implanted biomaterial were fixed for a minimum of 24 hours in half strength Karnovsky's, then further dissected to remove excess tissue and allowed to fix for a minimum of an additional 48 hours at 4°C. Tissue blocks were post-fixed in 1% osmium tetroxide, stained en bloc with 1% uranyl acetate, dehydrated through a series of graded ethanols and embedded in Poly/Bed 812®.³³ Semithin sections, 1µm in thickness, were stained with Richardson's stain³⁴ for examination using light microscopy. Thin sections 60-80nm in thickness were picked up on formvar coated copper grids and stained with 1% phosphotungstic acid, 7% uranyl acetate, and lead citrate³⁵ and examined using a JEOL 1200 transmission electron microscope (JEOL, Tokyo, Japan). Film negatives were digitized at 2400 dpi using an Epson V700 scanner (Epson, Long Beach, CA). Photoshop® was used to view, montage and pseudocolor images for analysis and figure preparation.

RESULTS

Gross appearance

All (24/24) implants remained intact 7 days post-implantation, 63/64 (98.4%) remained intact at 14 days, and 57/70 (81.4%) remained intact at 28 days. There was no clinical evidence of infection at the implant/skin interface for all implants. Figure 2 shows mice with implants on day 0 and 7, 14, and 28 days post-implantation. Dried Dermabond® can be seen in the 7, 14 and 28 day implants.

Morphology

All tissue samples with implants for the 7, 14, and 28 day time points remained intact throughout processing and sectioning for NBF, O.C.T. and half strength Karnovsky's fixation and embedding. An illustration depicting the implant orientation and general morphology is shown in Figure 3. All implants showed crust-like structures at exit sites with serum apparent in some pores towards the outer regions of the implants of the 7 and 14 day implants. Neutrophils, as identified by morphology, were seen between the crust and

epidermal region. There was evidence of slight downgrowth of the epidermis along the implant. The space between exit sites was approximately 4-5mm and was occupied by dermis. The panniculus carnosus was retained in the “upper cap of skin” with fat located above the panniculus carnosus.

Immunohistochemistry and histochemistry

Epidermal integration—*Pankeratin* staining to identify keratinocytes in the O.C.T. embedded cryosections of implants revealed that staining was seen within the pores for all time-points and surface treatments. A total of 8, 10, and 11 samples from all three time-points were evaluated for poly(HEMA), CDI and CDI/lam 332, respectively. Figures 4A, B, C show examples of keratinocyte incorporation within the pores of 7, 14, and 28 day post-implantation sections, respectively. The epidermal tongue appeared initially to migrate down along the poly(HEMA) with keratinocytes adjacent to the poly(HEMA) migrating into the pores.

Dermal-epidermal junction proteins—*Laminin 332* (formerly laminin 5) is a basement membrane protein found along the dermal-epidermal junction of skin. Laminin 332 immunostaining was seen within the pores for all time-points and surface treatments. A total of 8, 12, and 13 samples from all three time-points were evaluated for poly(HEMA), CDI and CDI/lam 332, respectively. The laminin 332 appeared to be both cellular and extracellular within the poly(HEMA) pores as shown in Figures 4D, E, F, of 7, 14, and 28 day post-implantation sections, respectively. There appeared to be a trend of greater evidence of laminin 332 deposition in the CDI and CDI/lam 332 implants.

$\beta 4$ integrin subunit: Integrin $\alpha 6 \beta 4$ is the receptor for laminin 332 and is found in hemidesmosomes along the basement membrane of skin. A total of 5, 4, and 8 samples from all three time-points were evaluated for poly(HEMA), CDI and CDI/lam 332, respectively. A linear pattern of $\beta 4$ integrin subunit immunostaining was apparent along the dermal-epidermal junction of the adjacent mouse skin. Beta4 integrin immunostaining was seen within the pores of all time-points and surface treatments. (Figures 4G, H, I).

Type VII collagen is the major structural component of anchoring fibrils, found along the dermal-epidermal junction of skin. Type VII collagen immunostaining was evident along the dermal-epidermal junction in the skin of all 7, 14, and 28 day implants (**Figure 5A** arrowheads). A total of 9, 12, and 12 samples from all three time-points were evaluated for poly(HEMA), CDI and CDI/lam 332, respectively. For the 7 and 14 day implants, type VII collagen immunostaining within the pores appeared to emanate from the exit sites and from the upper cap of skin (Figure 5B, pink overlay of 14 day implant). Note the strong linear pattern within the pores associated with the region of the presumptive epidermal stratum (**Figure 5C** arrows) and in the dermis directly adjacent to the implant (**Figure 5C** asterisks). By 28 days, staining was concentrated in a strong linear pattern associated with the region of the presumptive epidermal stratum as seen in 14 day specimens (Figure 5D). There appeared to be a reduction of Type VII collagen in pores adjacent to the dermis by 28 days.

Dermal integration—*PECAM-1* is expressed constitutively on the surface of endothelial cells and weakly expressed on some peripheral lymphoid cells and platelets. A total of 10, 14, and 12 samples from all three time-points were evaluated for *PECAM-1* immunostaining for poly(HEMA), CDI and CDI/lam 332, respectively. PECAM-1 immunostaining of endothelial cells within the pores of the 7 day implants appeared to emanate from the exit sites and from the upper cap of skin (**Figure 5D** pink overlay). Vessels within the pores adjacent to the upper cap of skin are shown in Figures 5E, F, G, for 7, 14, and 28 days

respectively. By 14 and 28 days, PECAM-1 staining was seen throughout all implants. Larger vessels were present in the 28 day specimens (Figure 5G).

Immunohistochemical analyses of *fibroblasts* were inconclusive (data not shown). Cells, morphologically identified as fibroblasts, are described in pores using transmission electron microscopy (see Ultrastructural evaluation).

Collagen was detected within the pores using trichrome staining (**Figures 6A-D** dark blue). Six samples from all time-points were evaluated for poly(HEMA), CDI and CDI/lam 332 implants. Collagen can be seen both in surrounding tissue (Figure 6A) and within the poly(HEMA) pores of 7, 14, and 28 day implants (Figures 6B, C, D, respectively). Thicker collagen bundles can be seen in the 28 day implant (Figure 6D). Using picrosirius red staining and visualizing by polarized light, we distinguished collagen types by color. Bifringent greenish fibers (typical of collagen III) could be distinguished from the thicker red collagen bundles (typical of collagen I). Thinner fibers are seen in the 7 day implants (Figure 6E), with presence of thicker collagen bundles in the 14 and 28 day implants (**Figures 6F and G**, respectively).

Monocytes and macrophages identified by F4/80 staining, were present within the pores of 7, 14, and 28 day implants (Figures 7A-C, respectively). A total of 12, 14, and 13 samples from all time-points were evaluated for poly(HEMA), CDI and CDI/lam 332, respectively. These monocytes or macrophages appeared to line the inner surface of the pores with very thin cytoplasmic extensions and were still present in the 28 day samples (**Figures 7C and D**).

Ultrastructural evaluation—Fourteen and 28 day poly(HEMA), CDI, and CDI/lam332 samples were evaluated by TEM to assess epidermal and dermal biointegration. Our ultrastructural evaluation revealed the first detailed glimpse of cellular organization within the microniches of the integrated porous biomaterials. Cell types and locations were influenced on two levels: first, on a macro level in which the normal stratification of the skin adjacent to the biomaterial dictates which cell types are available for incorporation (Figure 8A), and second, on a microniche level in which the cell types present within each pore organize into zones of preferential location (**Figures 9A and 10A**).

At the macro level, tissue incorporation was organized into four zones similar to a normal wound: first, the crust or exit site where the percutaneous implant is at the interface between the skin and the outer environment, second, the inflammatory stratum, showing a predominance of leukocyte infiltrate, third, the epidermal stratum, in which keratinocytes actively migrate into the pores, and fourth, the dermal stratum in which fibroblasts, collagen deposition and vessel formation can be found (Figure 8A).

The pores located within the crust stratum of the implant contained serum exudate, fibrin matrix and cellular debris (data not shown). We also observed the occasional presence of bacterial clusters (Figure 8B).

Beneath the crust we observed the inflammatory stratum where a predominance of neutrophils (identified by polymorphic lobular nuclei and small cytoplasmic granules) could be observed filling pores in a band across the implant and transitioning to a mixture of neutrophils, monocytes (large leukocyte with kidney-shaped nucleus, loose chromatin and pronounced nucleolus), and macrophages (larger than monocytes with cytoplasmic vacuoles, lysosomes, phagocytic inclusions and nuclei which shows irregular outline of dense chromatin). When found, the bacteria were never observed to extend past this region of inflammatory infiltrate (Figure 8C).

Within the epidermal stratum, stratum corneum-like keratinocytes could be observed filling spaces between the skin and implant as well as adjacent pores (Pink colorized region above normal keratinocytes, Figure 8A). Leukocytes often occupied the space between the epidermis and biomaterial when a gap or sinus was present (**Figure 8D**, neutrophils). Even in regions of tight juxtaposition between the keratinocytes of the migrating epithelial tongue and the biomaterial, leukocytes occupied an intermediary position between the keratinocytes and outer surface of the biomaterial (**Figure 8E** macrophage). Keratinocytes, as characterized by keratin filaments and desmosomes, within the pores were typically separated from directly contacting the inner surface of the poly(HEMA) by leukocytes, most commonly macrophages (Figure 8F) as evidenced by morphology. Within the epithelial stratum we observed a dramatic increase in cell types and a preferential positioning of the cells within each pore (Figure 9). Three spheres of stratification were observed. First, leukocytes [neutrophils, macrophages, mononuclear cells, plasma cells (lymphocyte B-cell characterized by extensive endoplasmic reticulum and a large round nucleus exhibiting a cartwheel-like dense chromatin around the perimeter), T-cells (small lymphocyte with a large nucleus/cytoplasmic ratio), and eosinophils (granulocyte with large cytoplasmic granules containing distinct crystalline structures)] were observed closest to the inner surface of biomaterial, while keratinocytes occupied a secondary or middle sphere interior to leukocytes. The third or central core of the pore was invariably occupied by fibroblasts (spindle-shaped cell with distinct ribosomal endoplasmic reticulum) with a robust deposition of banded collagen (Figure 9A). There were, however, occasions in which keratinocytes were observed directly adjacent to the biomaterial inner surface with hemidesmosome-like structures present at the cell/biomaterial interface (**Figures 9B and C**). Keratinocytes found within the pores, as in normal skin, were connected to other keratinocytes via desmosomes (Figure 9D), but we were particularly interested in observing the interface between the groups of keratinocytes and dermal components to determine if a dermal-epidermal junction formed within the pores similar to normal skin. Thus far, we have observed no formation of a basement membrane or hemidesmosomes between keratinocytes and extracellular matrix within this region. In fact, keratinocytes were observed in tight juxtaposition to other cells types, both leukocytes and fibroblasts, rather than extracellular matrix (Figure 9D).

The dermal stratum was the most predictably organized region of ingrowth. Prior to the observation of blood vessels, fibroblasts were seen occupying the central core of each pore with an organized deposition of collagen fibers aligned with the location of pore throats (Figure 9A). When blood vessels were observed within these fibroblast/collagen cores, they occupied the most central location, with alignment and branching directed towards the inner pore throats (Figure 10A). We observed endothelial cells with tight junctions and surrounding basement membrane (**Figures 10B and C**), forming functional vessels as indicated by circulating red blood cells and leukocytes (Figure 10A). Vessels were surrounded by fibroblast/collagen sheaths, which in turn, were insulated from the inner surface of the biomaterial by macrophages (**Figures 10A and B**). This tri-layered organization was highly conserved among all specimens.

Macrophages occupied all strata except the crust. The macrophages showed great structural diversity. Some macrophages exhibited extraordinary membrane folds oriented toward the interior of the pore, creating a high surface area with low numbers of phagocytic inclusions (Figure 11A). Clathrin coats (organized protein coatings easily seen at the ultrastructural level, involved in protein and membrane transport) and adjacent budded off clathrin-coated endosomes were often observed at the basal terminations of the membrane folds. (**Figures 11B and C**). Other macrophages exhibited a more activated phenotype, showing phagocytic activity with multiple inclusions and lysosomes and a paucity of thin membranous projections (Figure 11B). Macrophages were also found to occasionally fuse together into

multinucleated giant cells ultrastructurally lacking separating plasma membranes (Figure 11D).

DISCUSSION

In this study we characterized cutaneous incorporation of long-term implants using immunohistochemistry, tissue stains, and TEM. We demonstrated that 40 μ m sphere-templated porous poly(HEMA), 2mm $\times$ 2mm $\times$ 15mm in size, with and without surface modification, can be implanted and maintained percutaneously in C57BL/6 mice for up to 28 days. Since a 2mm excisional wound in mice would be expected to fully heal by 28 days³⁶, we anticipated that by 28 days, epidermal and dermal ingrowth into the implant would be robust.

None of the implants showed any clinical evidence of infection. Our results are in contrast to studies by Shin and Akao²⁰ in which they demonstrated acute infection within a month post-implantation in dogs using 2 types of porous (50-150 μ m) hydroxyapatite (spongy-type or close pore implants). The difference in pore size, biomaterial, or immune response between mice and dogs may account for the difference in infection result.

The implant/skin interface withstood routine processing for paraffin-, ultrastructural- and frozen-sectioning. The tissue response to the implanted poly(HEMA) progressed through the normal course of wound healing stages identified: injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue formation, and foreign body reaction.³⁷ Because of the extensive vascularization seen in our implants, we were not surprised that avascular fibrous capsule formation, the last stage identified by Anderson, et al.,³⁷ was not detected in our porous implants. Likewise, permigration was not observed in the 144 implants available for study.

The laminin 332 used in these studies was partially purified from cultured human keratinocytes. The laminin 332 antibody used to detect laminin 332 protein expression in mouse keratinocytes was specific for mouse laminin 332 and did not cross-react with the human 332 used for surface treatment. In addition, we evaluated laminin 332 protein expression in biointegrated keratinocytes to determine if the migrating keratinocytes deposited their own laminin 332 within the poly(HEMA) pores.³⁸ There was an indication that CDI and CDI/lam 332 improved laminin 332 expression within the pores. Greater numbers of samples need to be evaluated to determine whether this trend is statistically significant. In vitro studies by Fitton et al., suggest that porous membranes need to be adhesive toward protein/cells for tissue outgrowth from explants to be supported.³⁹ Our results indicating that keratinocytes migrated into 40 μ m pores of untreated non-adhesive poly(HEMA) suggest that it may be unnecessary to add exogenous proteins; however, changing the property of non-adhesive poly(HEMA) to an adhesive surface with CDI, or CDI adsorbed with laminin 332 or fibronectin or use of a more adhesive material such as medical grade silicone, may augment biointegration.

In vitro corneal explant studies by Fitton, et al.³⁹ using pores sizes ranging from 0.22 μ m to 3 μ m of trabecular pore membranes (Millipore) and 0.1 μ m to 3 μ m track-etched membranes and in vivo studies by Squier et al.²⁴ using Millipore filters with pore sizes ranging from 0.025 μ m-8.0 μ m, indicate an inverse relationship between pore size and the extent of epithelial migration; the larger the pore, the shorter the epithelial migration. The homogeneous surface properties of our porous material, for which the pores are contiguous, are geometrically very different from membranes with porous holes or tracks. The spaces between pores in membrane filters vary greatly and the rough surface property of the filter is very different from the porous gap. Length of migration on the non-porous part of the filter

may greatly impact epithelial migration. The columnar track-etched membranes in Fitton's studies are a very different geometry compared to our porous poly(HEMA) constructs and may account for the difference in results, as we saw epithelial migration into 40µm pores (large in contrast to the 3µm pores in membranes).

The cells found within the implants with all treatments express normal keratinocyte wound healing proteins. Keratinocytes within the pores express basement membrane proteins such as laminin 332 and the β4 integrin subunit necessary for epidermal adhesion and migration. Although we found keratinocytes within the poly(HEMA) pores, migration into the pores was limited. The keratinocytes never created a contiguous epidermal layer across the entire epidermal stratum, nor did the keratinocytes show signs of permigration. Our results are in contrast to those observed by von Recum, who showed epidermal permigration by 10-20 days in 2mm thick Dacron velour implants.¹ This difference may be due to the very different geometry between interconnected round pores and the more fibrous Dacron mesh. Despite the fact that the epidermis, in our studies, did not appear to fully migrate across the entire implant to create "an epidermal seal", there was no evidence of infection.

Type VII collagen, the major component of anchoring fibrils within the basement membrane zone, exhibits a linear staining pattern along the dermal-epidermal junction in normal skin. Both human keratinocytes and fibroblasts synthesize type VII collagen.⁴¹⁻⁴³ The majority of type VII collagen was thought to be produced primarily by keratinocytes.⁴³ However, Goto et al. using a human skin graft/nude mouse model showed that fibroblasts supplied more type VII collagen to the new dermal-epidermal junction than keratinocytes.⁴⁴ The type VII collagen staining within our implants progressed from an early diffuse pattern within the pores to a contiguous concentrated zone of expression within the presumptive epidermal stratum, despite limited keratinocyte integration. We hypothesize that the concentrated type VII collagen along the presumptive epidermal stratum was produced primarily by fibroblasts occupying the poly(HEMA) pores.

The precise pore size, interconnectivity and alignment indicated that the geometry,⁴⁵ not surface properties [(poly(HEMA) implants with and without surface treatment)], supports vascularization as indicated by staining of endothelial cells with the PECAM-1 antibody. Our results are in contrast to studies by Kidd, et al., who showed that untreated porous expanded polytetrafluoroethylene (ePTFE) implanted in rats remained avascular and required rat squamous cell carcinoma cells to be pressure-sodded to the ePTFE to promote vascularization.⁴⁶ The pattern of vascularization emanating from the dermis in the upper cap may indicate that the thick fascial plane of the lower dermis may be a barrier for vascular sprouting. The presence of red blood cells and leukocytes indicate functional, viable vessels, important for providing nutrients and oxygen and removing waste from the cells found within the pores. Vascularization may be the essential component to preclude implant encapsulation in our experiments.

Since fibroblasts were only identified by morphology in the absence of a reliable fibroblast antibody for immunohistochemistry, we used trichrome and picrosirius red stains to identify collagen assumed to be produced by viable fibroblasts. Our results indicate that the tightly woven collagen bundles throughout the interconnecting pores will provide both strength and elasticity necessary for implant stability.

Morphological differences in the inflammatory response in hematoxylin and eosin-stained sections were not apparent among the different surface treatments. Other than immunostaining for macrophages and monocytes with the F4/80 antibody, further characterization of inflammatory cell-types (i.e. neutrophils, eosinophils, etc.) was based solely on ultrastructural morphology. Despite the presence of macrophages in our 28 day

implants, our implants showed a paucity of foreign body giant cells normally associated with a foreign body reaction to biomaterials.³⁷

The ultrastructural morphology has brought to light both provocative insights and new questions. The open interconnecting porosity of the biomaterial may be viewed as an easy route of entry for bacteria existing at the surface of the skin to invade the underlying murine tissues. However, we have never observed signs of systemic or clinical infection in the many mouse implants performed to date. Examining the cellular organization within the pores leads us to hypothesize a possible explanation. The porous configuration of the biomaterial may create small, manageable spaces in which bacteria can be cleared via leukocyte activity in a divide-and-conquer strategy. The ratio of bacteria to leukocytes within micro spaces, along with the limited number of bacteria that can physically occupy a pore, may prevent pathogenic invaders from overwhelming host defenses, especially as bacteria migrate through the narrow pore throat into the next pore. Our observations, thus far, suggest that leukocytes occupy pores early and may be primed defenders against bacterial invaders.

Our ultrastructural analysis supports the hypothesis that cutaneous incorporation into the biomaterial recapitulates normal skin and wound healing on both the macro and micro level of stratification. It suggests that tissue invasion into the biomaterial occurs in two phases. The primary invasion by leukocytes has altered the microniche environment prior to the secondary invasion by keratinocytes and fibroblasts.

One departure from normal healing is the sustained presence of leukocytes, in particular, macrophages. In normal acute wound healing the inflammatory phase resolves within one week of wounding.⁴⁷⁻⁴⁹ The extraordinary folds of cellular membranes exhibited by many of the macrophages may, in fact, be an indicator of a shift toward quiescence. Macrophages that shift from a phagocytic phenotype to a remodeling or quiescent phenotype may exhibit an increase in surface area via membrane folds.⁵⁰ The presence of clathrin coats at the basal terminus of the folds that we observed may indicate robust sampling of the intercellular space within the pore with subsequent cytokine production that encourages vascularization.⁵¹⁻⁵⁴ The sustained presence of macrophages is inexorably tied to the sustained presence of biomaterial³⁷ and the positioning of the macrophage gives the impression of isolating the inner core of cutaneous tissues from the biomaterial.

Another departure from normal wound healing is the apparent lack of a dermal-epidermal junction. Keratinocytes quickly reestablish a dermal-epidermal junction during normal wound healing, with the formation of hemidesmosomes anchoring the basal aspect of the cell to an underlying extracellular wound matrix.⁵⁵⁻⁵⁷ In this study, however, keratinocytes even after 28 days, form tight associations with other cell types (leukocytes and fibroblasts) as opposed to extracellular matrix. This suggests interactive communication similar to that seen in early normal wound healing.⁵⁷⁻⁵⁸ Even though both collagen and fibrin were found within pores, keratinocytes were not observed to be forming a dermal-epidermal junction. The rare observation of hemidesmosome-like structures forming at 14 days where a keratinocyte is juxtaposed to CDI/lam 332 treated poly(HEMA) is provocative and could suggest that adhesion is being promoted by the surface treatment.⁵⁹⁻⁶⁰ The use of a more adhesive biomaterial such as silicone may encourage keratinocyte adhesion. Further investigation using immunogold localization of hemidesmosome-associated proteins within treated vs. untreated implants is necessary in order to conclusively identify hemidesmosomal structures at both the light and electron microscopic levels.⁵⁵⁻⁶¹ The ability of the keratinocyte to adhere to the biomaterial surface may profoundly affect keratinocyte phenotype of activation vs. quiescence.

The cellular and subcellular organization within this unique microenvironment suggests that precision engineered sphere-templated porous materials have a profound influence on tissue incorporation from cellular migration, directionality of matrix deposition, vessel formation and branching, regulation of activation vs. quiescence and even fighting infection. While integration could be found in all TEM samples, a greater number of sites and quality of integration were observed in CDI and CDI/lam 332 implants. We cannot conclude, however, that these results indicate better adhesion or biointegration in the modified implants. Quantification studies are being conducted to determine the effects of pore size and implant surface treatment on biointegration. Future projects will pursue insights derived from molecular morphology utilizing ultrathin cryomicrotomy with immunogold labeling and transmission electron microscopy^{55,62-64} to localize proteins of interest involved in adhesion, migration and communication. These studies will help clarify influence of specific porous biomaterial configuration on keratinocyte and macrophage phenotype.⁶⁵ Likewise, studies involving bacterial challenges are currently being conducted. Understanding the *in vivo* cellular biological response to biomaterials is a necessary step in the development of biomaterials that optimize cutaneous biointegration. Successful biointegration of the skin with medical devices is expected to greatly expand clinical applications for percutaneous devices by reducing infection risk, improving device integrity, favorably impacting healthcare costs as well as quality of life.

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Figure 1.

Sphere templated poly(HEMA). (A) Polymethyl methacrylate (PMMA) beads of uniform size are compacted. Contact points between beads are indicated by red stars. (B) Compacted PMMA beads are heat-sintered, then surrounded by poly(HEMA). (C) The PMMA is extracted, leaving interconnected poly(HEMA) pores. (D) Scanning electron micrograph showing the pores of poly(HEMA). Yellow arrows show open pores with black, circular pore throats (red stars). (E) Cryo-section of poly(HEMA) showing interconnecting pores and pore throats (red stars). (F) Higher magnification of (E) showing 2 interconnected pores. Red stars indicate pore throats. (G) Cryo-section immunostained with pankeratin antibody. Body of keratinocyte is seen within one pore with its cytoplasmic extension traversing through a pore throat into an interconnected 2nd pore. Large yellow arrow indicates the

nucleus of the keratinocyte. Small yellow arrow indicates immunolabeled keratin filaments. Red stars indicate pore throats.

Figure 2. Poly(HEMA) strips implanted into mice. (**A-D**) Mice with two poly(HEMA) strips implanted and secured in place with Dermabond® at days 0, 7, 14, and 28, respectively.

Figure 3.

Illustration of implant orientation and general morphology. Area dorsal to the implant is the “upper cap of skin”. Implant rests on a “lower fascial plane” of skin (ventral to the implant). Serum (violet circles) and crust (blue circles) occupy the region of the implant at the air/implant interface, inflammatory cells (green circles) are found beneath the crust area, followed by epidermal cells (red circles.) The core of the poly(HEMA) becomes occupied by dermal components (beige circles). Fat (yellow circles) and the panniculus carnosus muscle layer (pc) (brown dashed line) are seen within the normal surrounding tissue. Regions 1 indicate interface at exit sites: region 2 is located at the dermal /implant interface of the upper cap; region 3 located in the central core of the poly(HEMA) implant; and region 4 indicates the dermal/implant interface of the lower fascial plane.

Figure 4.

Tissue sections immunolabeled for presence of epidermal proteins showing intact epidermis to the left of implants. (A-C) Tissue sections immunostained with a pankeratin antibody showing immunoreactive keratinocytes within the poly(HEMA) pores adjacent to mouse epidermis. (D-F) Tissue sections immunostained with laminin 332 show deposition of laminin 332 in the poly(HEMA) pores. (G-I) Tissue sections immunostained with $\beta 4$ integrin subunit show presence of $\beta 4$ within the poly(HEMA) pores. (A, D, G) are 7 day implants, (B, E, H) are 14 day implants, and (C, F, I) are 28 day implants. Immunostaining within pores are indicated by arrows.

Figure 5.

Tissue sections immunolabeled for type VII collagen and PECAM-1. (A) Tissue section of a 14 day poly(HEMA) implant immunolabeled with an antibody to type VII collagen showing type VII collagen along the dermal-epidermal junction of normal mouse skin (arrowheads) in the cap region. (B) Fourteen day implant with pink shaded area highlighting the pattern of type VII collagen deposition which appears to emanate from dermis near the poly(HEMA) exit sites and from the upper cap of skin. Area between dotted blue lines indicates porous implant. Upper box is the region seen in Figure 5A. (C) Higher magnification of boxed area in Figure 5B. Strong dermal type VII collagen is present along the implant/skin interface (*) and within the pores in the presumptive epidermal stratum, (small arrows). Arrowheads indicate tip of migrating epidermis. (D) Tissue section immunolabeled with type VII collagen in a 28 day implant showing similar immunostaining pattern to the 14 day implant in Figure 5C. Type VII collagen is concentrated within the pores in the presumptive epidermal stratum, (small arrows). Arrowheads indicate tip of migrating epidermis. (E) Tissue section of a 7 day poly(HEMA) implant immunostained with PECAM-1 antibody to localize endothelial cells. Endothelial cells appear to emanate from dermis near the poly(HEMA) exit sites and the region from the upper cap of skin. The pink shaded area highlights the pattern of endothelial cells present in the poly(HEMA) pores. Blue dashed line indicates porous implant. Arrowheads indicate epidermal/implant interface. (F-H) Immunoreactive endothelial cells are present within the pores of 7, 14, and 28 days implants, respectively. The box in (D) indicates region represented for images (F-H). By 28 days, vessel formation is apparent (H). Dashed lines in (F and G) indicate upper cap of skin/implant interface.

Figure 6.

Implant sections stained with trichrome and picrosirius red for presence of collagen. **(A)** A 28 day implant stained with trichrome shows presence of collagen within the poly(HEMA) pores and in the dermis of the surrounding skin (blue stain). **(B-D)** Trichrome-stained 7, 14, and 28 day implants respectively, show maturation of collagen as a function of time. **(E-G)** Picrosirius red-stained 7, 14, and 28 day implants respectively, show increasing collagen deposition by 28 days. Thinner collagen bundles, using polarized light, are green (7 day) with thicker collagens bundles appearing red/orange (28 day). Area between white dashed lines indicates porous implant.

Figure 7. Tissue sections immunolabeled with F4/80 antibody to localize monocytes/macrophages. (A-C) Monocytes/macrophages are present in all pores of 7, 14, and 28 day implants respectively. Monocytes/macrophages appear to be in direct contact with the pore walls (arrows). (D) Higher magnification of the boxed region in (C). Black dashed lines (A-C) indicate upper cap of dermal interface with porous implant.

Figure 8.

Poly/Bed® embedded tissue sections of poly(HEMA) implants. **(A)** Composite brightfield light micrographs of a Richardson's stained one micron section of a 28 day CDI implant showing the four strata of cutaneous incorporation [crust (scab), inflammatory, epidermal, and dermal]. Figure colorized to facilitate separation of epidermis with stratum corneum (pink) from dermis (green). **(B)** Transmission electron micrograph (TEM) within the crust region of a 28 day poly(HEMA) implant shows a bacterial cluster. Active proliferation is indicated by binary fission (arrows). The crust matrix surrounds the clusters of bacteria. **(C)** TEM within the upper inflammatory stratum of a 14 day CDI/lam 332 implant shows neutrophils within pores. **(D)** TEM of a 14 day CDI/lam 332 implant showing keratinocytes of the epithelial migrating tongue adjacent to a sinus or gap that can exist between the skin and biomaterial. A neutrophil, in this example, lines the outer face of the epithelial tongue. Desmosomes are seen between keratinocytes (arrows). **(E)** TEM of a 14 day CDI/lam 332 implant showing that when no sinus is present, keratinocytes are separated from the outer surface of the biomaterial by macrophages which are also present within adjacent pores (arrow). **(F)** TEM of a 14 day CDI implant showing that keratinocytes have migrated within the pores. Arrow indicates a desmosome between keratinocytes. The keratinocytes are separated from the poly(HEMA) by an intervening macrophage. [keratinocyte (ker), macrophage (mac), neutrophil (neu)]

Figure 9.

TEM micrographs within the epithelial stratum. **(A).** Composite of TEM micrographs showing a single 40µm pore within the epithelial stratum of a 14 day CDI implant, colorized to facilitate identification of cell types. Fibroblasts with deposited collagen matrix occupy the inner core, while leukocytes (macrophages, neutrophil, and T-cell), occupy the outer zone. A keratinocyte can be seen migrating into the pore through the pore throat on the left. **(B)** TEM of a 14 day CDI/lam 332 implant shows a region in which a keratinocyte and a B-cell (plasma cell) are observed adjacent to the inner surface of biomaterial. **(C)** Higher magnification of the designated region-of-interest boxed in **(B)**. The keratinocyte in juxtaposition to biomaterial exhibits a hemidesmosome-like adhesion structure (arrow). **(D)** TEM of a 28 day CDI implant showing keratinocytes with a desmosome (arrow) in tight juxtaposition with an eosinophil and a fibroblast. [fibroblast (fibro), keratinocyte (ker), macrophage (mac), neutrophil (neu)]

Figure 10.

TEM within the dermal stratum. (A) Composite TEM micrographs showing a branched blood vessel within the dermal region of a 28 day CDI/lam 332 implant. Macrophages are adjacent to the biomaterial while the functioning vessel, noted by circulating red blood cells and monocytes, occupies the inner core. (B) TEM of a 28 day CDI implant within the dermal stratum showing macrophages enveloping a blood vessel that is surrounded by a collagen sheath. (C) Higher magnification TEM of boxed region in (B) showing the formation of tight junctions (arrow) between the endothelial cells of the blood vessel as well as the surrounding basement membrane indicative of vessel functionality. [macrophage (mac), fibroblast (fibro), red blood cell (RBC), basement membrane (bm)]

Figure 11.

Macrophage phenotypes within the poly(HEMA) pores. **(A)** TEM of a 28 day CDI implant shows a phenotype of macrophages in which the cell surface opposite the biomaterial exhibits extraordinary membrane folds. A neutrophil is also present in the lower right of the micrograph. **(B)** TEM of a 14 day CDI implant shows two distinct phenotypes of macrophages. The macrophage toward the bottom of the micrograph shows the redundant membrane folds opposite the poly(HEMA) while the macrophage above it shows a highly active phagocytic phenotype with phagocytic inclusions and a paucity of membrane folds. An eosinophil can also be seen in the upper right. **(C)** Higher magnification micrograph of the boxed region of interest shown in **(B)** shows clathrin coats forming at the terminus of the membrane folds (arrows). **(D)** Composite TEM micrograph showing that giant cells or multinucleated macrophages can be found within the pores of a 28 day CDI implant.

Table

Tissue fixation for 7, 14, and 28 day implant samples. Table indicates tissue fixation of harvested implants. Tissues fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin were used for tissue staining, fixed in half strength Karnovsky's and embedded in Polybed® were used for ultrastructural studies and embedded in O.C.T. were used for immunohistochemistry.

Implant age	# of mice	# of implants at harvest	Implant treatment	10% NBF (tissue stain)	1/2 Karnovsky's (electron microscopy)	O.C.T. (cryo-sectioning/IHC)
7 day	12	8	poly(HEMA)	4		4
		8	CDI	4		4
		8	CDI/lam 332	4		4
14 day	32	21	poly(HEMA)	8	7	6
		22	CDI	8	7	7
		20	CDI/lam 332	7	6	7
28 day	35	17	poly(HEMA)	6	6	5
		20	CDI	5	8	7
		20	CDI/lam 332	5	8	7