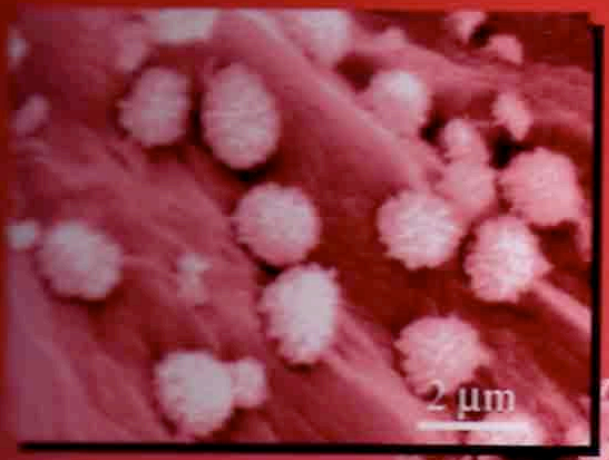
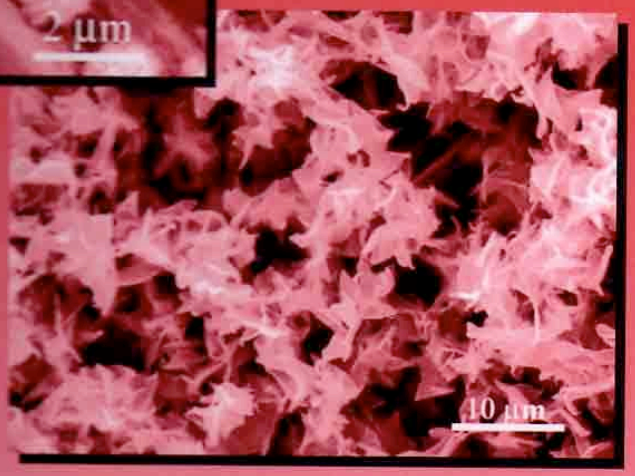
Scaffolding in Tissue Engineering





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15 PuraMatrix: Self-Assembling Peptide Nanofiber Scaffolds

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I. INTRODUCTION

The fields of tissue engineering and regenerative medicine require two key complementary components: (1) a suitable biological scaffold that creates a microenvironment niche for a given cell type, and (2) that the given cell type can rapidly integrate and coalesce into the needed tissue.

The three-dimensional assembly of cells in a biological microenvironment is designed to recapitulate normal tissue function.[1] Often, this involves the use of biomaterials. The task of the biomedical engineering is to design and to select optimal biomaterials with the properties most closely matched to those needed for a particular application.[2–6]

Both stem and progenitor cells hold promise for tissue engineering and for reparative and regenerative medicine. In most cases, these cells must be paired with microenvironments, which ensure proper expansion or differentiation through mimicry *in vivo* microenvironments, basement membranes with a critical constituent being the extracellular matrix (ECM).[7–9] This fibrous protein scaffold not only provides the appropriate three-dimensional architecture, but also promotes signaling pathways influencing critical cell functions such as proliferation, differentiation, and migration.[10,11] Moreover, researchers in the fields of tissue engineering, stem cell biology, and cancer biology are realizing that the ECM and a fine-tuned three-dimensional microenvironment are not only critical for fully understanding of cell biology, but also for developing successful clinically relevant therapies.

However, almost all cell biology research uses two-dimensional constructs to study single cell populations treated selectively with soluble factors in a homogeneous environment.[12] This greatly simplifies the research models, but also mitigates the powerful effects of ECM and three-dimensional culture conditions on the cells of interest and, in some cases, might be misleading. Recreating three-dimensional conditions and structures *in vitro*, rather than growing cells in two-dimensional Petri dishes, will provide tools for more accurate biological analyses of cells and tissues.[13,14]

A. Animal Derived ECM and Synthetic Scaffolds

The past two decades have witnessed the quest for synthetic biocompatible polymers, hydrogels, and or animal derived materials.[2–6] Until recently, three-dimensional cell culture has required either synthetic scaffolds, which fail to approximate the physical nanoscale size and chemical attributes of native ECM, or animal derived materials, which may confound cell culture with undefined or inconsistent variables.

Biomaterials such as PLLA and PGA biopolymers, calcium phosphate mesh, PEG gels, methylcellulose, alginates, and agarose have shown only limited success, partly due to either their large microfiber size relative to cells (about the same size as most tissue cells), acidic breakdown products, charge density, lower nutrient diffusion rates, or their inability to allow the creation of functional microenvironments by the cells.

Animal derived biomaterials including bovine collagen and gelatin, fibronectin, intestinal submucosa, cadaver tissue, fibrin, and Matrigel may help to create the right microenvironments, but complicated research and therapies, with their potential risk of other unknown material contaminations, such as viruses, prions, unknown proteins, and factors, raises issues about cell signaling, protein content, and reproducibility.

We have described a family of synthetic self-assembling peptide scaffolds (SAPS), now called *PuraMatrix* because of its synthetic nature and extreme purity of a single peptide component. Its basic constituents are the natural L-amino acids, thus the breakdown products can be reused by cells. PuraMatrix not only can be used as a two-dimensional coating or a three-dimensional encapsulation of cells similar to the ECM, but can also be tailor-made for particular cells, tissues, and cell-based therapies.

B. PuraMatrix Peptide Nanofiber Scaffolds

Over the past 10 years, a new class of biologically inspired peptide biomaterials has been discovered and developed in the context of cell culture, stem cell biology, and tissue engineering. These self-assembling peptide scaffolds have been used successfully as a synthetic *in vitro* and *in vivo* ECM, proving themselves as a critical component to successful three-dimensional cell growth.[15–22,35]

1. Nanofiber Scale Synthetic ECM

PuraMatrix is a 16 amino acid synthetic peptide that is resuspended in water to generate a range of solution concentrations. Upon the introduction of millimolar amounts of monovalent cations, either through the addition of salt solution, cell culture media (e.g., DMEM), or injection of the material *in vivo*, PuraMatrix undergoes self-assembly into nanofibers, ~10 nm in diameter, on a scale similar to the *in vivo* ECM. The physical size relative to cells and proteins, the amphiphilic peptides' charge density, and water structuring abilities mimic the *in vivo* ECM. These well-ordered nanofibers create three-dimensional porous scaffolds that are very difficult or impossible to synthetically produce by other fabrication techniques. The nanofiber density and average pore size (between ~5 and 200 nm) correlates with the concentration of peptide solution that is used to produce the material, which can be varied from 0.1 to 5% in water (1 to 50 mg/ml w/v) depending on the application.

2. Defined Three-Dimensional Microenvironments for Cell Biology

PuraMatrix mimics several important aspects of the *in vivo* environment — a synthetic ECM — enabling defined cell culture conditions while allowing cells to proliferate and differentiate within a three-dimensional context, easily migrate within that microenvironment, and create their own microenvironments quickly including production of their own ECM.

C. Three-Dimensional Cell Culture vs. Two-Dimensional

The advancement of biological study often requires the development of new materials, methods and tools. The introduction of the Petri dish over 100 years ago provided an indispensable tool for culturing cells *in vitro*, thus permitting the detailed dissection of seemingly intractable biology and physiology systems into manageable units and well-defined studies. This simple dish has had profound impact on our understanding of complex biology, especially cell biology and neurobiology.

However, the Petri dish culture system, including multiwell plates, glass cover slips, and so on, is less than ideal for several reasons:

1. It is a two-dimensional system that sharply contrasts to the three-dimensional environment of natural tissues both animal and plant.
2. The Petri dish surface without coating is rigid and impermeable, again, in sharp contrast to the *in vivo* environment where cells intimately interact with the ECM and with each other.
3. The tissue cell monolayers on a coated two-dimensional surface, such as poly-L-lysine, collagen gels, fibronectin, and laminin as well as other synthetic materials containing segments of adhesion motifs, have only part of the cell surface attached to the materials

and interact neighboring cells. The remaining parts are often directly exposed to the culture media, unlike the tissue environment where every cell intimately interact with its neighbors and the ECM.

4. The transport phenomena of two-dimensional and three-dimensional are drastically different. In two-dimensional culture systems, cytokines, chemokines, and growth factors quickly diffuse in the media across the culture dish. Again, this sharply contrasts to the *in vivo* environment where chemical and biological gradients play a vital role in signal transduction, chemotaxis, cell–cell communication and development. Cells respond to local concentrations of a variety of molecules, which in traditional cell culture, are distributed homogenously. Yet, most cells, by virtue of being embedded in a tissue, live in an environment that contains a dynamic gradient of nutrients and secreted factors — a "spatial heterogeneity" that cannot be emulated using conventional two-dimensional tissue culture. Since many cell types secrete their own ECM, a synthetic scaffold can be used even if not initially coated with the particular ECM proteins.
5. Cells cultured on a two-dimensional Petri dish are not readily transportable, and it is difficult to move cells from one environment to another without incurring changes in the cell–material and cell–cell interactions. For example, enzymatic cell harvesting using trypsin or mechanical disruption using rubber policeman both may have adverse effects on cell–environment interactions. Culturing to on two-dimensional surfaces to cell confluence requires frequent passages, including more reagent costs and cell manipulation. In contrast, cells cultured in three-dimensions are more readily transportable without significantly harming cell–material and cell–cell interactions, thus providing a significantly new way to study cell biology.

D. Nanoscale Fibers vs. Microscale

In the last two decades, several biopolymers, such as PLLA, PLGA, PLLA–PLGA copolymers, and other biomaterials including alginate, agarose, and collagen gels have been developed to culture cells in three-dimensions.[2–6] These culture systems have significantly advanced our understanding of cell–material interactions and fostered a new field of tissue engineering. However, these biomaterials are often made of microfibers with diameters of 10 to 100 μm — drastically different in size, surface interaction, porosity, and concentration relative to the native ECM and cells interacting with it. Therefore, cells attached on microfibers are in fact two-dimensional, despite the various curvatures associated with the large diameter microfibers. In order to culture cells in a truly three-dimensional microenvironment, the fibers must be significantly smaller than cells so that the cells are surrounded by the scaffold, similar to the extracellular environment and native ECM.

We believe that the development of new biological materials, particularly those biologically inspired nanoscale scaffolds mimicking an *in vivo* environment that serve as permissive substrates for cell growth, differentiation, and biological function, is a key area of study. These materials will be useful not only in furthering our understanding of cell biology in a three-dimensional environment, but also in advancing medical technology, tissue engineering, regenerative biology, and medicine.

E. Ideal Synthetic Biological Scaffolds

The ideal biological scaffold and its building blocks should meet several criteria:

1. They are derived from chemically defined, synthetic sources which are present in native tissue,
2. They are amenable to design and modification to customize specific bioactive and functional requirements,

3. They allow cell attachment, migration, cell–cell, cell–substrate interactions, and recovery of cells from the scaffold,
4. They exhibit no cytotoxicity or biocompatibility problems while chemically compatible with aqueous solutions, cell culture and physiological conditions,
5. They are compatible with microscopy, molecular biology analysis, and flow cytometry,
6. They are sterile and stable enough for shelf life, transportation, bioproduction, and closed system cell therapy culture,
7. They are economically viable and scaleable material production, purification, and processing,
8. They exhibit a controlled rate of material biodegradation *in vivo* with nondetectable immune responses and inflammation,
9. They foster cell migration and angiogenesis to rapidly integrate with tissues in the body,
10. They are injectable along with cells, compatible with cell delivery and surgical tools.

II. SELF-ASSEMBLING PEPTIDES

A. Discovery and Development of Self-Assembling Peptides

1. Simple Repeating Units of Amino Acids Assemble into Nanofiber Scaffolds

The first molecule of this class of self-assembling peptides, EAK16-II, a 16 amino acid peptide, was found as a segment in a yeast protein, zuotin which was originally characterized by binding to left-handed Z-DNA.[15] Zuotin is a 433-residue protein with a domain consisting of 34 amino acid residues with alternating alanines and alternating charges of glutamates and lysines of remarkable regularity, AGARAEAEAKAKAEAEAKAKAESEAKANASAKAD.[15] We subsequently reported a class of biological materials made from self-assembling peptides.[16–22] This biological scaffold consists of greater than 99% water content (peptide content 1–10 mg/ml). They form scaffolds when the peptide solution is exposed to physiological media or salt solution.[23–26]

The scaffolds consist of alternating amino acids that contain 50% charged residues.[16–20,23–27] These peptides are characterized by their periodic repeats of alternating ionic hydrophilic and hydrophobic amino acids. Thus, the β-sheets have distinct polar and nonpolar surfaces.[16–20] A number of additional self-assembling peptides including RAD16-I and RAD16-II, in which arginine and aspartate residues substitute lysine and glutamate have been designed and characterized for salt facilitated scaffold formation.[16–20] Stable macroscopic matrix structures have been fabricated through the spontaneous self-assembly of aqueous peptide solutions introduced into physiological salt containing solutions. Several peptide scaffolds have been shown to support cell attachment of a variety of mammalian primary and tissue culture cells.[16–22]

2. Amenable to Design Incorporating Functional Motifs

Since these peptides are synthetic and molecular engineered, they can be modified through the incorporation of desired functional motifs for specific ECM–cell interactions. We have tailor-made several self-assembling peptides containing functional sequences incorporated into the scaffold and shown some important implications for three-dimensional cell culture and tissue engineering [unpublished results].

B. Structural Properties of Self-Assembling Peptides

In general, these self-assembling peptides form very stable β-sheet structures in water. Although, sometimes, they may not form long nanofibers, their β-sheet structure remains largely unaffected.[16,17]

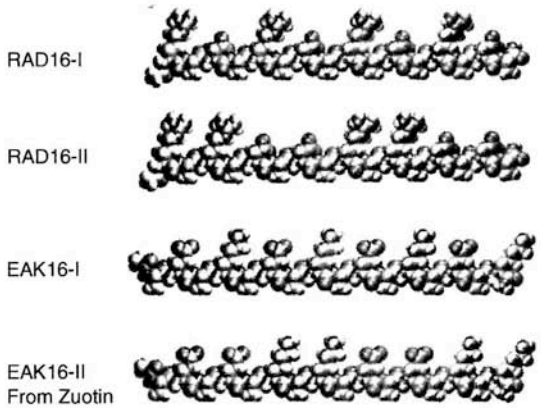


FIGURE 15.1 Molecular models of several self-assembling peptides, RAD16-I, RAD16-II, EAK16-I, and EAK16-II. Each molecule is ~5 nm in length with eight alanines on one side and four negative and four positive charge amino acids in an alternating arrangement on the other side.

One of the possible reasons is their unique structure. The alternating alanine residues in PuraMatrix are similar to silk fibroin such that the alanines can pack into interdigital hydrophobic interactions (Figure 15.1). The ionic complementary sides have been classified into several moduli (Modulus I, Modulus II, Modulus III, Modulus IV, etc., and mixtures thereof). This classification scheme is based on the hydrophilic surface of the molecules that have alternating positively and negatively charged amino acids, alternating by one residue, two residues, three residues, and so on. For example, charge arrangements for Modulus I, Modulus II, Modulus III, and Modulus IV are $-+-+-+-+$, $--++--++$, $---+++$, and $----++++$, respectively. These well-defined sequences allow the peptides to undergo ordered self-assembly, resembling some situations found in well-studied polymer assemblies.

III. PEPTIDE NANOFIBER SCAFFOLDS

A. EAK16-II

The EAK16-II, AEAEAKAKAEAEAKAK, is the first member in the self-assembling peptide family. EAK16-II was the first peptide to be characterized in detail[16–18,28] and has also been shown to retain β-sheet structure for extended periods of time. (One sample was stable for over 10 years; Zhang, unpublished results). An EAK membrane-like scaffold was first discovered in the tissue culture media where PC12 cells were used to test for EAK16-II cytotoxicity. The EAK scaffold showed no apparent toxicity; instead, the PC12 cells were found to attach onto the membranous materials where EAK16-II was added and not in the dishes where EAK8, a single unit of AEAEAKAK, was used.[16] Furthermore, the EAK16-II nanofibers were found to support neurite outgrowth in nerve growth factor (NGF) treated PC-12 cells and synapse formation in primary rat hippocampal neurons.[16] The membranous material was examined under scanning electron microscopy (SEM) to reveal a well-ordered nanofiber structure (Figure 15.2). Later, using atomic force microscopy (AFM), the nanofiber structure was confirmed.[29]

B. RADA16

We then designed several peptides altering the amino acid sequences containing RAD motif. RADA16-I, RADARADARADARADARADA, and RAD16-II, RARADADARARADADADA, were studied (Figure 15.3). These peptides have motif RAD that is similar to the ubiquitous integrin

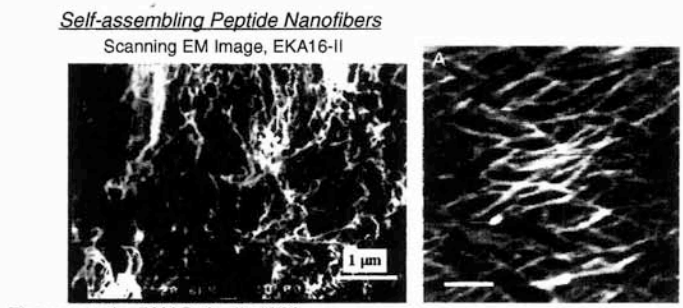


FIGURE 15.2 EAK16-II nanofibers. EAK16-II nanofiber formation was demonstrated using (SEM)[16] and AFM.[29] The nanofibers are ~10 to 20 in diameter with structural regularity due to the repeating peptide sequences and amphiphilic nature of the peptides. The scale bar in AFM is 200 nm.

receptor binding site RGD. Although it is not known if these RAD repeats in the PuraMatrix scaffold behave similarly to RGD motifs, they have been studied in the context of cell attachment across a number of cell lines.[18,19] These peptides form well-ordered nanofibers, similar to EAK16. Interestingly, RADARGDARADARGDA, and RADA8, RADARADA, did not form stable β-sheets or nanofibers when studied under the identical conditions as RAD16. These observations suggest that the formation of stable β-sheet is important not only for nanofiber, but also for scaffold formation.

Furthermore, increasing concentration of RADA8 added into RAD16-I inhibited the long and well-ordered RADA16 nanofiber formation [Zhang, unpublished results]. Other variations of RAD16 including RAD16-IV and DAR16-IV have also been studied.[30–33] DAR16 has an identical composition to RADA16-I but a different sequence arrangement. When it formed a β-sheet, the nanofibers are formed but upon heating, the DAR16 transformed into an α-helical structure and nanofibers were no longer observed [Zhao, et al., unpublished results]. These results indicate that simple properties, such as length of the peptide and order of amino acids within that peptide, determine its ability to form and maintain stable β-sheet nanofibers.

C. KFE8 AND KLD12

Self-assembling peptides less than 16 residues have also been studied including KFE8 and KLD12 (Figure 15.4).[20,23–27,34] Many self-assembling peptides that form scaffolds have

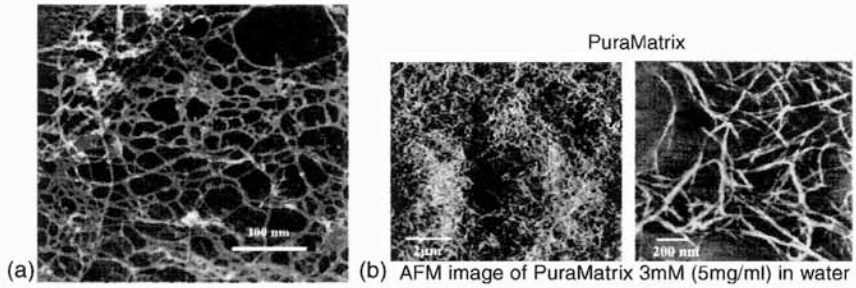


FIGURE 15.3 PuraMatrix. (a) Transmission electron microscopy (TEM) and (b) AFM. Note the nanofiber scale fiber with pores ranging from 5 to 200 nm, the right pore size for biomolecular diffusion. This is in sharp contrast to the microfibers of traditional polymer scaffolds, where the fiber diameter is ~1 to 50 μm and the pores range from 10 to 200 μm.[2–4]

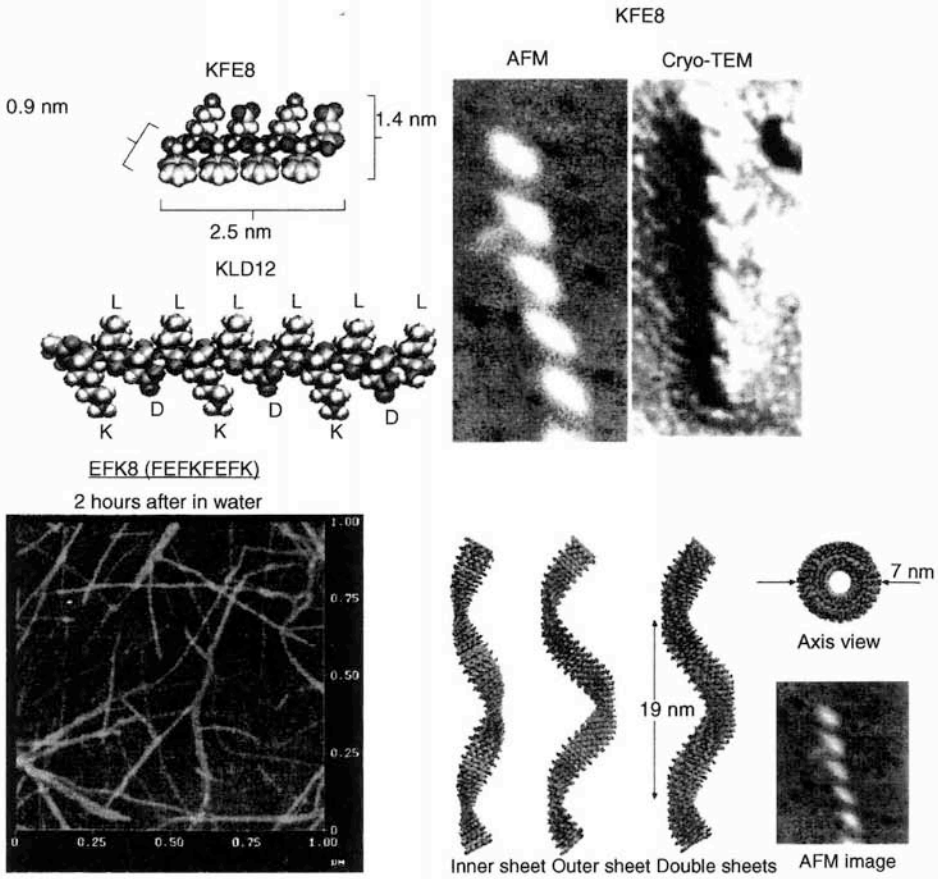


FIGURE 15.4 Molecular model of KFE8 and KLD12 and AFM/TEM image of KFE8. These peptides undergo self-assembly to form left-handed nanofibers with a diameter of approximately 7 nm as single fiber and thicker when bundle with other fibers. After 2 h, the nanofiber length increases. The left-handedness is unmistakable from the detail AFM (left) and Quick-freeze/deep-etch TEM (right) images.[27] Molecular simulation of KFE8 in left-handed double helix. The simulation was carried out using CHARMM program (updated 2002). There are an inner helix and an outer helix with hydrophobic Phe inside away from water and hydrophilic Lys and Glu exposed to water. The diameter is 7 nm and the pitch is 19 nm as reproducibly observed experimentally by AFM.[27]

been reported and the numbers are increasing.[31–33] We now understand that the formation of the scaffold and its mechanical properties are influenced by several factors, one of which is the level of hydrophobicity.[20,23–27,34] That is, in addition to the ionic complementary interactions, the extent of the hydrophobic residues, Ala, Val, Ile, Leu, Tyr, Phe, Trp (or single letter code, A, V, I, L, Y, P, W) can significantly influence the mechanical properties of the scaffolds and the speed of their self-assembly. The higher the content of hydrophobicity, the shorter the length required, the easier the scaffold formation is, and the better their mechanical strength and gelation properties.[20,23–27,34] Interestingly, different cells may behave differently in different peptide scaffolds depending on the culture conditions. This again demonstrates the importance of tailor-making scaffolds for different tissue cells microenvironment.

IV. PURAMATRIX *IN VITRO* CELL CULTURE EXAMPLES

PuraMatrix has been used to culture diverse types of tissue cells including stem and progenitor cells, as well as differentiated cell types and organotypic tissue slices.[15–22,35] A subset of cell types cultured in PuraMatrix is exemplified below (Table 15.1).

A. HEPATOCYTES

Hepatocytes have been cultured on PuraMatrix and Matrigel where they attach, proliferate, exhibited proper spheroid morphology, and created tight junctions. On the other hand, hepatocytes cultured on collagen coated Petri dish did not survive long-term, and did not attach when cultured on plastic dish alone.

Hepatocyte morphology was evaluated at Day 2 and Day 5 (Figure 15.5). Cytochrome p450 1A1 was evaluated as follows on Day 10. Substrate 8 μm 7-ethoxyresorufin metabolized by cells for 30 min at 37°C to resorufin conjugate. Fluorescence was measured at 530 nm excitation and 590 nm emission. Acceptance criteria requires hepatocytes cultured on the Matrigel substrate is twice the

TABLE 15.1
A Variety of Tissue Cells and Tissues Cultured on PuraMatrix

Mouse fibroblast	Bovine calf and adult chondrocytes
Chicken embryo fibroblast	Bovine endothelial cells
Chinese hamster ovary	Rat adult liver progenitor cells
Rat pheochromocytoma	Rat cardiac myocytes
Rat neural stem cells	Rat hippocampal neural tissue slice
Mouse embryonic stem (ES) cells	Mouse neural colony stem cells
Mouse cerebellum granule cells	Mouse and rat hippocampal cells
Bovine osteoblasts	Hamster pancreas cells
Human cervical carcinoma	Human osteosarcoma
Human hepatocellular carcinoma	Human neuroblastoma
Human embryonic kidney	Human foreskin fibroblast
Human epidermal keratinocytes	Human neural stem cells
Human hepatocytes	Human ES cells

These cells include stable cell lines, primary isolated cells from animals, progenitor, and stem cells.

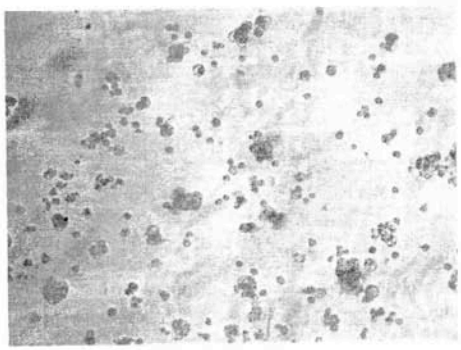


FIGURE 15.5 Primary rat hepatocytes cultured on PuraMatrix at Day 5. Cells begin to form clusters.

level of hepatocytes on the collagen I substrate. Hepatocytes on PuraMatrix grew much better than collagen I, thus suggesting PuraMatrix is a suitable scaffold for hepatocytes culture *in vitro*.

B. Adult Liver Progenitor Cells

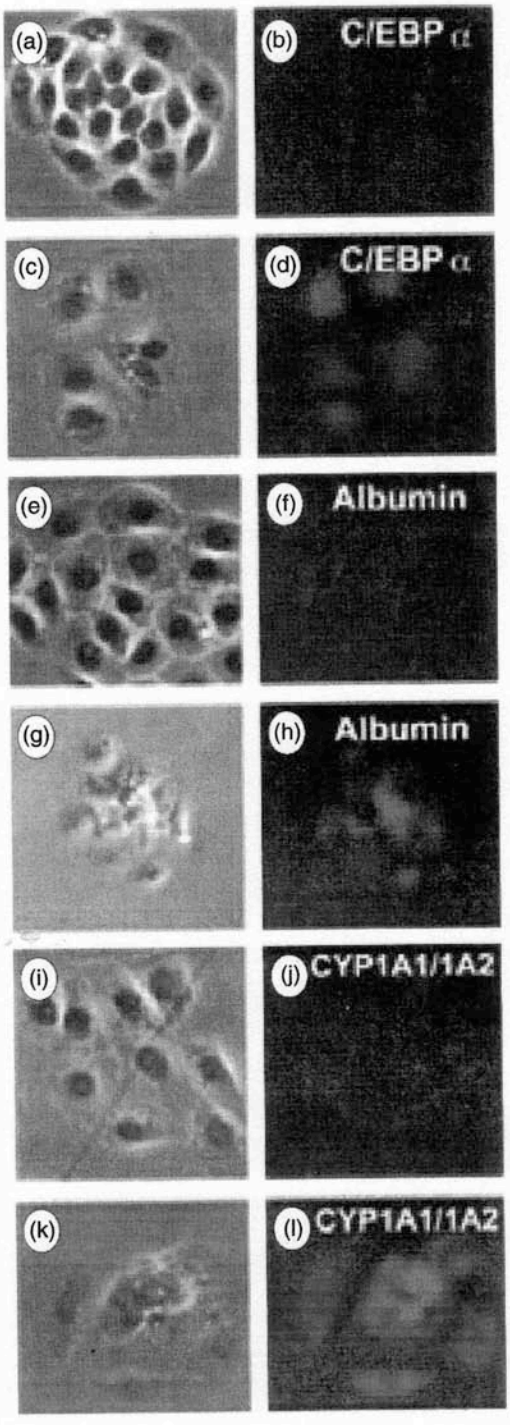
A hepatocyte progenitor cell line has also been cultured using three-dimensional PuraMatrix. These cells exhibited nonexponential cell kinetics, acquired spheroidal morphology, and produces progeny cells with mature hepatocyte properties.[21] The differentiated progeny cells display increased expression of albumin and several other indicators of hepatocyte maturation, including binucleation, upregulation of transcription factor C/EBPα, and expression of cytochrome P450's CYP1A1, CYP1A2, and CYP2E1 (Figure 15.6). In contrast, markers of hepatocyte progenitors, α-fetoprotein and CK8, are unchanged. These relationships suggest production of transition spheroidal units comprised of asymmetrically cycling adult progenitor cells and their differentiating progeny. All three cytochrome p450 enzyme activities are 3-methylcholanthrene-inducible in such spheroids. These results demonstrated the ability of a designed biological scaffold to provide a microenvironment in which adult progenitor cells regain their intrinsic ability to continuously produce differentiating progeny cells. This three-dimensional nanofiber scaffold may provide a physiological approach for biomedical applications and pharmaceutic high throughput drug screening tools with more reliable content outcome.[21]

C. Chondrocytes Form Molded Cartilage in Cell Culture

In choosing a scaffold for cartilage repair, it is important to identify a material that can maintain a normal rate of proliferation of differentiated chondrocytes and a high rate of chondrocyte synthesis of specific ECM macromolecules, including Type II collagen and GAGs, until repair evolves into a steady state tissue maintenance. Kisiday et al. first used RADA16 scaffold, but it did not give the optimal results because it is mechanically rather weak. We then designed the KLD12 (****n-KLDLKLDLKLDL-c) peptide scaffold reasoning that because leucine is more hydrophobic than alanine, the leucines would probably pack more tightly in the nanofibers in aqueous conditions and thus provide a higher mechanical strength.[20,25,26]

In Alan Grodzinsky's laboratory, Kisiday et al. used the self-assembling peptide KLD12 scaffold as a model for cartilage repair and developed a method to encapsulate chondrocytes within the scaffold. During 4 weeks of culture *in vitro*, chondrocytes seeded within the peptide scaffold developed a cartilage-like ECM, rich in proteoglycans and Type II collagen, indicative of a stable chondrocyte phenotype (Figure 15.7). Time dependent accumulation of this ECM was paralleled by increases in material stiffness, indicative of deposition of mechanically functional newly formed tissue. The content of viable differentiated chondrocytes within the peptide scaffold increased at a rate that was fourfold higher than that of parallel, chondrocyte seeded agarose culture, a well-defined reference chondrocyte culture system. These results demonstrate the potential of a tailor-made peptide scaffold as a scaffold for the synthesis and accumulation of a true cartilage-like ECM in a three-dimensional cell culture for cartilage tissue repair. The peptide KLD12 used in this study represents a designed, self-assembling peptide made through molecular engineering, which can be modified to suit specific cell and tissue application interests.[20]

FIGURE 15.6 Promotion of hepatocyte differentiation by PuraMatrix. Lig-8 hepatic progenitor cells were cultured in two-dimensional adherent culture or in three-dimensional PuraMatrix. Spheroids were isolated and analyzed approximately 16 h after transfer to adherent culture. **a**, **e**, **i**, adherent cell colonies (phase contrast); **b**, **f**, **j**, respective *in situ* immunofluorescence for adherent colonies with anti-C/EBPα, antialbumin, and anti-CYP1A1/1A2 antibodies; **c**, **g**, **k**, isolated scaffold spheroids (phase contrast; note binucleated cells); **d**, **h**, l, respective *in situ* immunofluorescence for isolated scaffold spheroids with anti-C/EBPα, antialbumin, and anti-CYP1A1/1A2 antibodies.


(a)
(b) C/EBP α
(c)
(d) C/EBP α
(e)
(f) Albumin
(g)
(h) Albumin
(i)
(j) CYP1A1/1A2
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(l) CYP1A1/1A2

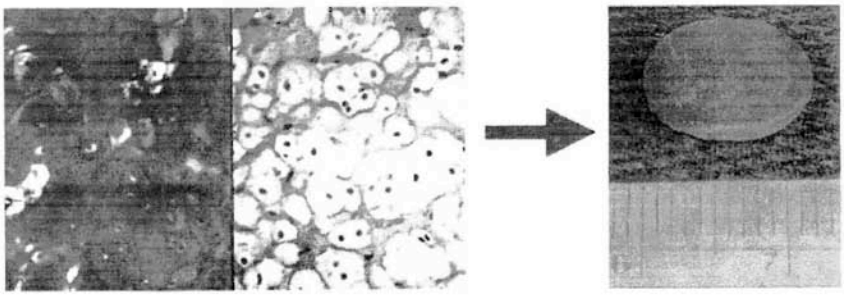

FIGURE 15.7 Peptide KLD12 (KLDLKLDLKLDL) chondrocytes in the peptide scaffold and cartilage. The chondrocytes stained with toluidine blue (TB) showing abundant glycosaminoglycans (GAG) production (left panel) and antibody to Type II collagen demonstrating abundant Type II collagen production (right panel). A piece of premolded cartilage with encapsulated chondrocytes in the peptide nanofiber scaffold. The cartilage formed over a 3 to 4 weeks period after the initial seeding of the chondrocytes.[20]

D. Extensive Neurite Outgrowth and Active Synapse Formation on PuraMatrix

PuraMatrix serves as a substrate and portable membrane media to support both neuronal attachment and differentiation in the form of extensive neurite outgrowth. Functional synapse formation also occurs between attached neurons when the cells are grown on PuraMatrix (Figure 15.8).[19] Neurite outgrowth from primary neuronal cultures was also tested by using several nerve cell types, including primary dissociated neurons from the mouse cerebellum and rat hippocampus. Cerebellar granule neurons undergo postnatal development and are morphologically distinguishable from other cerebellar cells. PuraMatrix scaffolds support extensive neurite out-growth from cerebellar granule neurons prepared from 7 days old mice and the neurites were readily visualized in two different focal planes, suggesting that the neurites closely follow the contours of the matrices. The primary cerebellar neuronal cultures on the PuraMatrix scaffolds were maintained for up to 4 weeks. Dissociated mouse and rat hippocampal neurons also attach and project neurites on the scaffolds (Table 15.2).

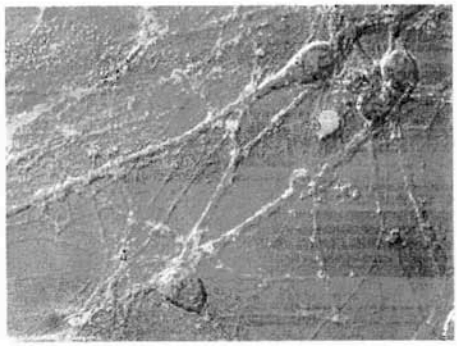

FIGURE 15.8 Active synapses on the peptide surface. Primary rat hippocampal neurons form active synapses on peptide scaffolds. The confocal images shown bright discrete green dot labeling indicative of synaptically active membranes after incubation of neurons with the fluorescent lipophilic probe FM-143. FM-143 can selectively trace synaptic vesicle turnover during the process of synaptic transmission. The active synapses on the peptide scaffold are fully functional, indicating that the PuraMatrix is a permissible substrate for neurite outgrowth and active synapse formation.[19]

TABLE 15.2
Neurtite Outgrowth on PuraMatrix Membrane Surface

Cell Type	Length of Process (μm)	Cell Sources
NGF treated rat PC12	400–500	Cultured cell line
NGF preprimed PC12	400–500	Cultured cell line
Human SY5Y neuroblastoma	400–500	Cultured cell line
Mouse cerebellar granule neurons	200–300	Primary cells[a]
Mouse hippocampal neurons	100–200	Primary cells[a]
Rat hippocampal neurons	200–300	Primary cells[b]

Cells were seeded onto PuraMatrix scaffold coating. The cell bearing PuraMatrix was transferred to dishes with fresh medium. Maximum neurite length was estimated visually with scale bars three to seven days after cell attachment for primary cultures and 10 to 14 days after matrix attachment for the cultured cell lines.

[a] Seven day old mouse.

[b] One day old rat.

E. Organotypic Hippocampal Tissue Culture in PuraMatrix

PuraMatrix has also been used to isolate and expand self-renewing neural cells *ex vivo*. Neurogenesis occurs in restricted areas of postnatal mammalian brain including the dentate gyrus and subvetricular zone (SVZ).[22] PuraMatrix has been used to entrap migrating neural cells (potential neuroprogenitors) from postnatal hippocampal organotypic cultures in three-dimensional PuraMatrix (Figure 15.9). In those experiments, brain tissues from the rat neonatal hippocampus were laid on top of preformed PuraMatrix and cultured for up to 2 weeks. Within a few hours, cell division activity was observed only at the interface zone.[22] The migrating neural cells with mitotic activity from the dentate gyrus, CA1, CA2, and CA3 regions of the postnatal rat hippocampus were greatly enriched in organotypic culture and they were entrapped in PuraMatrix. After a few hours, the cells exhibited high proliferating activity, as measured by incorporation of $BrdU^+$ cells at the "interface zone" between the tissue slice and the culture surface. Using PuraMatrix for neural progenitor cell isolation and expansion *in vitro* may thus have applications in developing new strategies and for cell-based therapies in regenerative medicine.

F. Osteoblasts

Osteoblasts (ATCC MT3T3) have been maintained with increase cell density successfully in PuraMatrix up to 1 month (Figure 15.10). These cells not only proliferated but also formed gap junctions connecting osteoblasts in a manner that is also found in native bone tissues.

V. Standard *In Vitro* Toxicology and Biocompatibility Studies

In addition to over 10 years of cell culture studies in our laboratory and elsewhere, standard *in vitro* toxicology studies have been completed, including EN/ISO tests for cytotoxicity and hemocompatibility. The tests below were completed at a Federal Drug Agency (FDA) certified toxicology testing company (Toxikon Corp, Bedford, MA) using established standards of measure on the commercially available PuraMatrix (RADI-16; Table 15.3).

A. Cytotoxicity

The agar diffusion test (ISO 10993-5) measures a material's effect on cell cultures, which are extremely sensitive to minute quantities of diffusible chemicals and readily

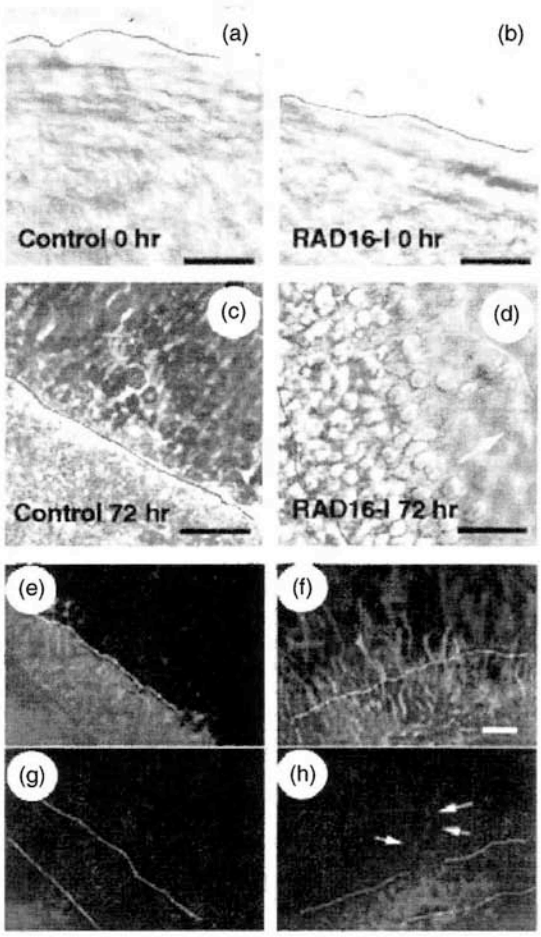

FIGURE 15.9 Hippocampal organotypic slice cultures cultured on peptide scaffolds develop extended tissue scaffolds.[22] Hippocampal slices were cultured organotypically either on control membrane or on RAD16-I peptide scaffolds layers (~500 μm thick). A time lapse experiment was carried out to follow up the tissue scaffold growth from the perimeter of the dentate gyrus region. (a) time 0 (0 H) of control slice culture; (b) time 0 (0 H) of scaffold slice culture; (c) 72 h of control slice culture; (d) 72 h of scaffold slice culture. The red line indicates the original border of the tissue slice and the yellow in d the extended tissue scaffold. The yellow arrow in d indicates the direction of tissue scaffold growth and extension. Black bars indicate 100 μm. (e) 72 h control slice culture immunostained for GFAP (glia cell marker); (f) 72 h scaffold slice culture immunostained for GFAP; (g) same optical layer as e immunostained for NeuN (neuron marker); h) same optical layer as f immunostained for NeuN (red). Red lines in e and f and yellow in g and h indicates the original perimeter of the tissue slice. The white line in e and g indicates the extended tissue scaffold in the control cultures. The white line in f and h is use to compare the over extension obtained on peptide scaffolds cultures. Yellow arrows in h indicate neuron cells staining with NeuN migrating into scaffold slice cultures. The white bar in f is 100 μm.

display characteristic signs of toxicity in the presence of potentially harmful diffusible substances. The biological reactivity of a mammalian monolayer, L929 mouse fibroblast cell culture, in response to the test article was determined as "no reactivity" in tests.

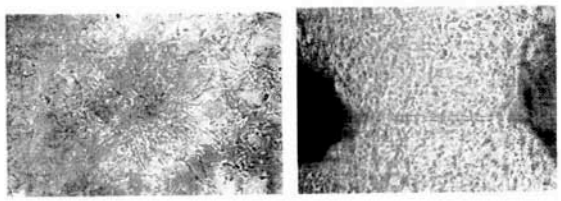

FIGURE 15.10 Osteoblasts in PuraMatrix. Cells form clusters (left panel) and junctions connected by cells (right panel) (image courtesy of 3DMatrix).

B. Hemolysis

The hemolysis test (direct contact, ISO-10993-4) measures the ability of a material to cause red blood cells to rupture. This test is derived from well-established National Institute of Health (NIH) protocols and is performed in triplicate. This test uses rabbit blood in direct contact with the test material and the degree of hemolysis is measured spectrophotometrically. PuraMatrix tested "nonhemolytic" in tests carried out by an independent FDA certified testing agency.

C. Coagulation Prothrombin Time

Prothrombin time (human plasma, ISO 10993-4) measures the effect of a test article extract on human blood coagulation time. This assay has become a suitable clinical means of determining the presence and functioning ability of prothrombin in the process of coagulation. PuraMatrix did not have an adverse effect on prothrombin coagulation time of human plasma in tests carried out by an independent FDA certified testing agency.

VI. *IN VIVO* BIOCOMPATIBILITY AND TOXICOLOGY STUDIES

Several kinds of biocompatibility and toxicity examinations of PuraMatrix have been carried out in numerous animal models, including a number of standard FDA/ISO toxicology and *in vivo* implant studies, to assess the safety of PuraMatrix. PuraMatrix has passed every one. These tests point to the biocompatibility of this particular peptide material, but more long-term studies are required for the commercialization of therapeutic applications (Table 15.4). These tests were outsourced and conducted under GLP conditions by Toxikon Corporation, an FDA certified facility in Bedford, Mass.

TABLE 15.3
***In Vivo* Biocompatibility and Toxicology Tests**

Test	Result[a]
Cytotoxicity:	
Agar diffusion ISO 10993-5	Noncytotoxic
Hemolysis:	
Direct contact (ISO 10993-4)	Nonhemolytic
Prothrombin time assay:	
Human plasma (ISO 10993-4)	No adverse effect on prothrombin coagulation

[a] These tests are contracted to the FDA certified commercial toxicology test facility (Toxikon Corp, Bedford, MA).

TABLE 15.4
PuraMatrix Used in *In Vivo* Animal Studies

Test	Result
ADME tox: 14 day implant	No problematic organ accumulation, excretion. 14C Radiolabeled PuraMatrix 10% excreted by day 14
Rabbit muscle implant: 14 day (ISO 10993-6)	Nontoxic score across 13 categories of reactions
Rabbit pyrogenicity: 24 h (ISO-10993-11)	No effect on animals
Intracutaneous implant: 72 h (ISO 10993-12)	Primary irritation index = 0.0 "Negligible" No febrile reaction from any animal over 24 h after intravenous injection of dilute solution
CNS lesions in hamsters: 2–60 days	Reduced scarring and reinnervation across the severed optic tract caudal to the lesion only in PuraMatrix treated animals[35]

Moreover, previously studies of animal reactions[19,35] described lack of immunogenicity and inflammation in rat, rabbit, goat, and hamster models.

A. ADME AND BIODEGRADABILITY

In order to measure the local and whole animal distribution of PuraMatrix, and given that previous experiments were unable to raise antibodies to it, we generated a carbon radioisotope labeled version of ^{14}C-PuraMatrix. The radioisotope ^{14}C was internally labeled at the third alanine site (Acetyl-(RADA)-(R-[^{14}C(U)-Ala]-D-A)-$(RADA)_2$-$CONH_2$) as opposed to a labeled acetyl group, which could be cleaved off, in order to better characterize the adsorption, degradation, metabolism, and excretion (ADME) of the PuraMatrix material *in vivo*.

The radioisotope labeled ^{14}C-PuraMatrix was then implanted into Sprague Dawley® rats in a femur defect model. Urine and stool samples were collected and the radioactivity was counted.

B. RABBIT MUSCLE IMPLANT (2 WEEKS)

This test assesses the local effects of material on contact with living tissue. Test article was implanted into paravertebral muscle of three New Zealand white rabbits, with negative control (GelFoam) implanted in the contralateral muscle of each animal. Healing was allowed for 2 weeks. Animals were sacrificed and implants excised. Excised implants were examined macroscopically with a magnifying lens and fixed in formalin. Histologic slides of hematoxylin and eosin (H&E) and Mason's Trichrome stained sections were prepared, studied microscopically by a board certified veterinary pathologist, and evaluated on a scale of 0 to 3.

PuraMatrix implants retained the initial implant volume, collagen fibers, and vascularization of the injection site (Figure 15.11). PuraMatrix was rated nontoxic (0.13) on a scale ranging from nontoxic (<1), slightly toxic (1 to <2), mildly toxic (2 to <3), moderately toxic (3 to >4), to severely toxic (<4), in tests carried out by an independent FDA certified testing agency.

C. INTRACUTANEOUS REACTIVITY

Irritation reactivity tests assess the localized reaction of tissues, including breached tissue and blood contact, to device materials or extracts. A 0.5% w/v peptide PuraMatrix solution was extracted in NaCl and CSO at a ratio of 200 mg/1.0 ml at 70 ± 2°C for 24 h. Control extracts were prepared, in a similar manner to the test article. Three rabbits were injected intracutaneously, using one side of the

FIGURE 15.11 PuraMatrix creates a permissive environment for tissue bulking (2 weeks postinjection).

animal for the test article extracts and the other side for the control extracts, at 0.2 ml per site. The injected sites were examined at 24, 48, and 72 h post inoculation for gross evidence of tissue reaction such as erythema, edema, and necrosis. A primary irritation index of 0.5 or less will be considered a negligible irritant, where 0.5 to less than 2 are slight irritants, 2 to less than 5 are moderate irritants, and greater than 5 are severe irritants. The test sites injected with PuraMatrix did not exhibit any signs of erythema or edema over the 72 h observation point. The primary irritation index for the material is 0.0 in tests carried out by an independent FDA certified testing agency.

D. Rabbit Pyrogen

The purpose of the test was to detect the risk of a patient to a febrile reaction as a result of the administration of the test article extract. The test article was prepared by mixing 9 ml of the test article with 9 ml of 0.9% USP sodium chloride for injection (NaCl) and the resulting gel extracted at a ration of 0.2 gm/ml. The test article extract was administered by intravenous injection at 10 ml/kg. The rectal temperatures of the injected rabbits were compared with the temperature of a control rabbit similarly injected with 0.9% USP Sodium Chloride for Injection (NaCl). The baseline temperature of the rabbits, determined no more than 30 min prior to injection of the test article extract, were used to exclude rabbits whose body temperatures vary by more than 1°C from each other and whose temperatures are greater than 39.8°C. Body temperatures were recorded at 30 min intervals between one and 3 h subsequent to injection. If no rabbit exhibited a rise in temperature of 0.5°C or more above its baseline temperature, then the product met the requirements for the absence of pyrogens (Table 15.4). None of the animals injected with the test article extract exhibited signs of pyrogenic response, in tests carried out by an independent FDA certified testing agency.

VII. FUTURE PERSPECTIVES

A. Compatible with Bioproduction and Clinical Applications

PuraMatrix fulfills both traditional biological materials and a nanoporous/nanofiber hydrogel to enable proper three-dimensional cell growth and the creation of microenvironments surrounding cell colonies. For cells that prefer culture on a two-dimensional surface, better results were obtained by culturing cells on PuraMatrix floating sheets, allowing a three-dimensional nutrient bath within the two-dimensional growth context.

PuraMatrix mimics important aspects of the *in vivo* environment, while eliminating complicating variables traditionally experienced from animal derived materials. Unlike most

TABLE 15.5
PuraMatrix Attributes and Advantages Comparison Table

Attributes	PuraMatrix Synthetic ECM	Natural ECM	Synthetic Scaffolds	PuraMatrix Advantage
Scaffold Properties				**Defined, consistent bare ECM analog**
Composition	RAD_{16} 16 peptide monomer in 0.5 to 1.0% w/v patented worldwide	Collagen, matrigel, cadaver tissue, basement membrane	PLA, PLGA, carbon fiber, calcium phosphate	Animal free, reproducible cell culture and cell signaling
Fiber size & sequence	7 to 10 nm diameter Bare ECM free of unwanted signals — add components as needed	5 to 10 nm diameter complex undefined protein sequences	10,000 to 100,000 nm looks 2D relative to cell	Approximates *in vivo* ECM nanoscale without the complexity
Pore size	50 to 400 nm	50 to 400 nm	20,000 to 1×10^6 nm	Encapsulates like ECM
Water content	99.5 to 99.9%	80 to 97%	60 to 80%	Better hydration and nutrient diffusion
Mechanical strength	Low to medium, cells can migrate within it	Low to medium	Medium to high	More rapid cell migration, ingrowth
Physical loading	Able to simulate physiological loading and flow	Depends on material	Often brittle, shift load from cells	Create physiologically relevant microenvironment
Cell Encapsulation and Handling				**Stable, Injectable, Customizable**
Scaffold formation	Fibers and gel form around cells with simple addition of culture media or injection *in vivo*	Require refrigeration or complex processing	Preformed scaffolds tough to seed with cells	Superior encapsulation allows cells to create own microenvironments and surrounding ECM
Combination with bioactives	Bioactives, ECM proteins can be added for tailored reproducible 3D cultures	Inconsistent levels of proteins and GF	Yes, but not true microenvironments	Enables consistent, defined ECM microenvironments
Allows cell attachment, migration, angiogenesis	Yes, enables anchorage dependent cell culture	Yes	Some	Allows cell–cell interactions, migration and invasion assays
Sterile	Gamma irradiation or pregelled filter sterilization *in situ*	No, destroys material and proteins	No gamma, often limited to ethylene oxide	Able to use gold standard sterilization, and filter sterilize *in situ if* needed
Injectable	Yes, will not swell beyond injected volume. Compatible with cardiovascular catheters	Yes, when chilled	Not injectable	Injectable along with cells, yet gel forms upon introduction *in vivo*

Clinical cell culture "closed system"	Sterility (gamma irradiation) and injectability enables closed system culture for bioproduction and clinical cell expansion	Animal components discouraged by FDA	Often hard to sterilize via gamma irradiation	Gold standard sterility, combined with injectability and easy handling
Stable	Shelf stable at room temperature and across broad temperature range for at least 2 years.	Natural product requires refrigeration with short shelf life	Shelf stable only while dry	Ideal stability independent of water content
Molding, coating, sheeting	Amenable to casting, coating, layering, 3D printing	Often monolayer only or limited sheets	Fixed shapes can be cut to shape	Simple to configure as needed
Cell Recovery and Analysis				**Research and Clinical Compatibility**
Microscopy	Transparent	Often cloudy	Often opaque	Easy visualization
Cell passaging harvesting, separation	Spin cells out, wash, replate, or re-encapsulate	Trypsin, collagenase	Hard to harvest cells without disruption	Easy cell harvesting with high cell viability
Molecular biology	Protein monomer has simple fingerprint to distinguish	Variation and noisy background data	Not compatible	Straightforward westerns, southerns, northerns
Flow cytometry	Compatible without separation	Must separate	Not compatible	Compatible
Closed system cell harvesting	Compatible	Depends on material	Not compatible	Requirement for clinical and bioproduction apps
Biocompatibility				**Superior *in vivo*, rigorously tested**
Nonimmunogenic	No discernable antibodies, foreign body response, chronic inflammation	More immune response and inflammation	Foreign body response, scarring, acidic breakdown	Superior in direct comparison studies
Biodegradeable	Yes, rapid due to low material/water ratio	Yes, but heavy lymphcyte activity	Yes, but illicits foreign body reaction.	More rapid, but rate can be varied & controlled
Swelling/absorption	Nonswelling upon injection	Nonswelling	Often swell, causing irritation	Consistent control of injected volume
Ingrowth, angiogenesis	Weak gel & cell migration allow rapid ingrowth	Undefined cell signaling	Large structure inhibits ingrowth	More rapid ingrowth

other scaffolds, PuraMatrix can be sterilized through UV radiation or filtration, and has proven itself shelf stable at room temperature for over 10 years. PuraMatrix not only lowers the handling, processing, transportation, and inventory costs, but also meets the stringent bioproduction requirements through its synthetic composition, free of the undesired factors and adventitious agents from animal product. PuraMatrix not only provides the three-dimensional context for cell research, but they are forward compatible with bioproduction and clinical requirements necessary for eventual tissue engineering and stem cell-based therapies.

B. Synthetic Origin, Clinical-Grade Quality, Clinical Delivery

Because PuraMatrix is synthetic and sterile, it is thus suitable for bioproduction, and can be readily in this and clinical settings, unlike many animal derived materials (bovine, pig or others) that lack the consistent quality control, thus complicating clinical reproducibility and risking the introduction of undesirable contaminants (cell signaling factors, prions, adventitious agents, etc.). PuraMatrix is currently manufactured in large quantities, some under good manufacturing processes (GMP) grade processes and commercially available. Additionally, PuraMatrix can be used for closed, sterile system culture *in vitro* and can also be injected *in vivo* without eliciting immune responses, unlike collagens and alginates. Upon injection or pipetting into solution, the PuraMatrix volume does not swell, retaining a consistent volume since it already has more than 99% water content.

C. Tailor-Made PuraMatrix

In order to fabricate tailor-made PuraMatrix, it is crucial to understand the finest detail of peptide and protein structures, and their influence on the nanofiber structural formation and stability. Since there is a vast array of possibilities to form countless structures, a firm understanding of all available amino acids, their properties, and the peptide and protein secondary structures is a prerequisite for further progress in the fabrication of peptide and protein materials.[36,37] We are moving in that direction, which will further accelerate new scaffold development.[38–42]

To date, PuraMatrix has been used in diverse cell and tissue systems from a variety of sources. This demonstrates a promising prospect in further improvement for specific needs since tissues are known to reside in different microenvironments. The PuraMatrix used thus far are general peptide nanofiber scaffolds and not tailor-made for specific tissue environment. We are designing second generation PuraMatrix scaffolds and to show that these tailor-made PuraMatrix incorporating specific functional motifs will perform as superior scaffolds in specific applications. They may not only create a fine-tuned microenvironment for three-dimensional tissue cell cultures, but also may enhance cell–materials' interactions, cell proliferation, migration, differentiation, and the performance of their biological function. The ultimate goal is to produce tailor-made scaffolds for particular tissue engineering and for regenerative and reparative medical therapies.

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