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Simplifying the Extracellular Matrix for 3-D Cell Culture and Tissue Engineering: A Pragmatic Approach

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Abstract The common technique of growing cells on tissue culture plastic (TCP) is gradually being supplanted by methods for culturing cells in two-dimensions (2-D) on matrices with more appropriate physical and biological properties or by encapsulation of cells in three-dimensions (3-D). The universal acceptance of the new 3-D paradigm is currently constrained by the lack of a biocompatible material in the marketplace that offers ease of use, experimental flexibility, and a seamless transition from in vitro to in vivo applications. In this Prospect, I argue that the standard for 3-D cell culture should be bio-inspired, biomimetic materials that can be used ''as is'' in drug discovery, toxicology, cell banking, and ultimately in medicine. Such biomaterials must therefore be highly reproducible, manufacturable, approvable, and affordable. To obtain integrated, functional, multicellular systems that recapitulate tissues and organs, the needs of the true end-users—physicians and patients—must dictate the key design criteria. Herein I describe the development of one such material that meets these requirements: a covalently crosslinked, biodegradable, simplified mimic of the extracellular matrix (ECM) that permits 3-D culture of cells in vitro and enables tissue formation in vivo. In contrast to materials that were designed for in vitro cell culture and then found unsuitable for clinical use, these semi-synthetic hyaluronan-derived materials were developed for in vivo tissue repair, and are now being re-engineered for in vitro applications in research. J. Cell. Biochem. 101: 1370–1383, 2007.

Key words: hyaluronan; in situ crosslinking; glycosaminoglycan; cell therapy; reparative medicine; growth factor delivery; ECM; hydrogel; HA; biodegradable scaffold

The fields of biomaterials, tissue engineering, and cell and developmental biology, are each approaching the destination of reparative medicine by different routes. However, unlike railroads built from opposite coasts, there is often little coordination or planning to assure that these trajectories will connect, that is, that the materials and biology will be directly translatable into clinically relevant products and protocols. Initially, the nascent field of biomaterials conscripted medically approved polymers, for example, polyesters such as Dacron® or polylactate and polyglycolate, for research and clinical uses. The clinical and research disciplines then diverged, with clinicians using what was available and approved, and researchers developing increasingly sophisticated, versatile, complex, and clever scaffold materials. Companies focused on "inert" scaffolds that were regulated as medical devices and thus more rapidly approved by the US Food and Drug Administration (FDA).

THE NEED FOR A USER-ORIENTED APPROACH

If we accept that tissue engineering is an applied science with the explicit, not implicit, goal of generating implantable tissues and organ constructs, then the best path to success may be a user-driven, or "marketing", approach. Indeed, the essence of engineering is to fabricate devices and systems with customer-dictated design criteria in mind. In contrast, most basic

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research laboratories at present adopt what one might call the "sales" approach—we develop materials and study biological and physical phenomena in order to expand our knowledge base. In effect, we "sell" our creative approaches to journals, to peers, and to granting agencies in order to support the educational experiences to our trainees and to enhance the reputations of our laboratories. This is the culture with which we are both familiar and comfortable, and it has resulted in enviable successes in basic research and applied technology.

However, in translating this technology to the marketplace, we invariably run up against the roadblocks of reality, including poor public acceptance, concern over ethical issues, untenable business models, lack of investor interest, difficulties in defining a path to regulatory approval, absence of reimbursement mechanisms, and difficulty with physician acceptance. This means that we, the tissue engineering research community, need to return to an engineering focus on user-driven design criteria. In order to get products to the marketplace, we must integrate research with marketing. To arrive at a desirable and needed product, we must understand what our customers, the physicians and patients, actually want—not what we think they need. With this end-user focus, it is feasible to chart a pathway to the goal: a profitable (but affordable), readily manufactured and FDA-approved clinical product that is sought by patients, accepted by physicians, and reimbursed by health care insurers. Only with this endpoint in mind can successful products be created that address and overcome these scientific, regulatory, educational, and financial hurdles.

TRANSLATING TISSUE ENGINEERING RESEARCH TO THE CLINIC

A May 2005 meeting of tissue engineering leaders and young investigators collected opinions regarding the next generation of tissue engineered products. Many observations relate directly to the user-driven marketing approach to develop FDA-approvable materials for three-dimensional (3-D) cell culture in order to move tissue engineered constructs from the bench to the clinic. For example, using a permissive matrix that promotes tissue remodeling is preferred to overengineering the final form of a complex tissue. Similarly, exact mimicry of the complexity of the native extracellular matrix (ECM) may be unnecessary, and a pragmatic biomimetic approach may be sufficient. That is, provide a simple device, and let biology do the heavy lifting. With the correct material, stromal regeneration may not require exogenous cells or growth factors [Freed et al., 2006; Ingber et al., 2006]. Nonetheless, the limiting step is therapeutic angiogenesis, and both microvascularization and macrovascularization are required to provide nutrients and oxygen in 3-D. The sequential presence of multiple growth factors is one way to achieve this outcome [Lee et al., 2000; Yancopoulos et al., 2000].

Building products for clinical use to meet patient needs when engineering a complex tissue is of paramount importance [Mikos et al., 2006], and there are numerous barriers in moving from the laboratory to the clinic [Mendelson and Schoen, 2006]. The lag in tissue engineering may be attributed to the necessity of employing clinically approved materials as scaffolds. Translating research into clinical applications requires two translational steps, in vitro fine-tuning of molecular and mechanical properties followed by in vivo optimization of safety and efficacy [Hunziker et al., 2006]. An awareness of the commercial constraints on the final product is essential: "If a tissue engineered technology . . . is to be successful . . . the clinical value must be accompanied by financial profit." (A. Ratcliffe, Synthosome, Inc. as quoted in [Hunziker et al., 2006]). Analogous to the marketing approach described herein, one must establish what features you *must* have, what you *should* have, and what you *might like* to have. The clinical and commercial failures of two tissue engineering companies demonstrate that ". . . the need is for improved product development, better management, and sounder business plans . . .", not more research. These failures were attributable to ". . . underpowered clinical trials, flawed business plans, ineffective marketing, and a Procrustean regulatory stance by the FDA." (M. Lysaght, Brown University, quoted in [Hunziker et al., 2006]).

What product features should the research field of tissue engineering focus on? First, products and materials should, from the outset, be suitable for human use. Second, products should avoid or minimize the use of rare, expensive, unstable, hard to manufacture, or chemically ill-defined components. Third, products should be easy to use by the physician and

provide an obvious benefit to the patient. Fourth, products should be developed initially for an unmet need. Fifth, products should address market niches which are not crowded with competitors, that will support development costs, and that offer opportunities for off-label uses and follow-on products. Sixth, products should be capable of demonstrating readily defined clinical outcomes in a short timeframe. Finally, product development must anticipate the regulatory requirements and design for successful FDA action. Overall, the regulatory and financial burdens of drug-cell-device combination products may be addressed stepwise by first focusing on medical devices that allow self-repair. With these considerations, how can tissue-engineering research accelerate the appearance of products in our lives?

3-D CELL CULTURE: THE NEW PARADIGM, AND THE ONLY WAY FORWARD

Traditional cell culture methods on tissue culture plastic (TCP) do not represent an accurate model of the in vivo environment, and as described in the next section, a paradigm shift from two-dimensional (2-D) to 3-D techniques is in progress, as evidenced by the explosive growth in literature citations over the past two decades (Fig. 1). A number of laboratories and companies have begun to provide materials to energize this paradigm shift, and a variety of natural, synthetic, or semi-synthetic materials are now available. Comparisons of 3-D and 2-D methods indicate that cell behavior on plastic may be irrelevant to the actual in vivo conditions [Cukierman et al., 2001]. In organisms, a complex network of proteins and proteoglycans (PGs) constitute the ECM that surrounds every cell. Intricate processes such as cell proliferation, migration, differentiation, angiogenesis, and invasion are orchestrated by the ECM components and the signaling cascades [Geiger et al., 2001; Lutolf and Hubbell, 2005]. In addition, the ECM dictates the morphology and overall behavior of cells and, in turn, it is constantly shaped and molded by matrix-specific enzymes produced by cells.

Two important recent reviews highlight this paradigm shift. In the first, Bissell [2006] describes her experience over the past two decades in modeling the molecular mechanisms of breast cancer and cell invasion by using the normal mammary gland as a model system. She notes that "Half of the secret of the cell lies outside the cell." With a laminin-rich gel, 3-D cultured murine cells formed spherical acini which became hollow and could be induced to secrete the milk protein β-casein [Bissell et al., 2002]. In 3-D, the abnormal growth and proliferation of human breast cancer cells could be reverted to a normal phenotype by inhibition of β1 integrin signaling [Weaver et al., 1997]. Moreover, tissue geometry alone can determine the sites of mammary branching morphogenesis in organotypic cultures [Nelson et al., 2006]. Branching occurred only at the corners of the tubules and altering the geometry led to branching all around. She closes by citing an editorial comment on her work; since responses to chemotherapeutic agents were dependent on cell architecture and tissue polarity, "... all of a sudden, studying cancer cells in two dimensions appears to be quaint, if not archaic" [Jacks and Weinberg, 2002].

In the second review, Griffith and Swartz [2006] develop design principles for recreating the interwoven set of biochemical and

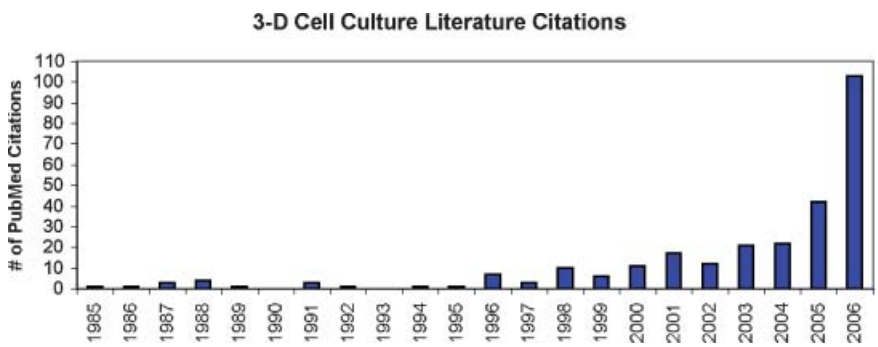

Fig. 1. Explosive growth in citations for 3-D cell culture. Pub Med citations from 1985 to 2006 (data collected by B. Pettys and J. A. Scott).

mechanical cues in the cellular microenvironment. "Designer 3-D gels" are now moving from the frontier to the mainstream in research, and offer the experimental flexibility to create molecular gradients in 3-D as well as integration of mechanical and chemical signaling. The authors stress the importance of having a molecular toolbox for 3-D research. "For most in vitro applications, it is not clear that the scaffold approaches that are derived from therapeutic tissue engineering currently offer strong advantages over readily accessible and widely used methods like spontaneous organization of cells within the ECM." In fact, the fabrication methods for preparing engineered microenvironments for the analysis of cell-biomaterial interactions in 2-D and 3-D is also an important frontier [Shin, 2007]. Since cells integrate signals and alter their responses, spatial organization in 3-D ECM is critical, and engineering in spatiotemporal control of GF presentation is required.

So where do the scaffolds and matrices for 3-D cell culture come from? Many biointeractive hydrogels have been developed for tissue engineering [Lee and Mooney, 2001; Sakiyama-Elbert and Hubbell, 2001], tissue repair, and release of drugs and growth factors [Langer, 2000; Lee et al., 2000] over the past two decades. Photopolymerized poly(ethylene glycol) diacrylate (PEGDA) has been an important research tool for preparing "bio-neutral", synthetic, degradable, and readily customized matrices for tissue engineering and drug delivery [Anseth et al., 2002; Nguyen and West, 2002]. Multiarmed PEG derivatives with matrix metalloprotease (MMP)-degradable peptide linkages provide specific microenvironments engineered to inform morphogenesis [Lutolf and Hubbell, 2005] and cell-invasion characteristics [Lutolf et al., 2003]. Another material, PuraMatrix™ is synthetic, peptide-based and forms fibrous scaffolds that can be used for 3-D cell embedding or surface plating [Zhang et al., 1995; Holmes et al., 2000; Semino et al., 2003]. While its composition allows in vivo research by significantly reducing immunogenicity, handling the material is technically difficult. While each of these materials can provide experimental control and generate elegant data on cell behavior in vitro and in animal models, there remain serious concerns for obtaining regulatory approval for use of these materials in the clinic.

ECM scaffolds derived from natural sources address, in some regards, the issues of biological recognition, presentation of receptor-binding ligands, cell-induced proteolytic degradation and remodeling [Lutolf and Hubbell, 2005]. One such material, PureCol™ (formerly Vitrogen®, 99.9% pure type I collagen), was tested for tissue engineering and is widely used as a coating material for medical devices and other applications [Schor et al., 1982; Weinberg and Bell, 1986]. A different ECM product, Matrigel™, is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains proteins, glycoproteins, and growth factors that are normally found in connective tissues [Kleinman et al., 1982]. Matrigel™ was successfully utilized for a variety of applications such as cell growth and differentiation, angiogenesis, and invasion assays, and was shown to promote a natural cell morphology and behavior [Terranova et al., 1986; Li et al., 1987]. Matrigel™ has been extensively used for acinus formation with human breast MCF-10A cells, and MDCK cells [Debnath et al., 2003; Debnath and Brugge, 2005] also form acini in Matrigel. However, concerns related to limited availability, pathogen transmission, immunogenicity, and animal tumor origin mean that Matrigel™ will never become a clinical material. As a result, the search for a practical, approvable, versatile synthetic ECM for 3-D cell culture, and tissue engineering has occupied a number of labs, including our own.

MARKET-DRIVEN RESEARCH: AN ACCIDENTAL TOURIST

I arrived in the arenas of biomaterials and tissue engineering completely by accident. For some 20 years doing organic synthesis and natural products of small lipid molecules, my "experimental animals" were caterpillars and termites. When a company offered to support a student to work on the chemical modification of hyaluronic acid (HA)—a large, amorphorous, water-soluble polysaccharide—I initially refused. But, funding was funding, so I accepted the challenge of learning unfamiliar chemistry and biology. Thus, from the outset, I was an outsider, a novice with no canonical training in polysaccharide chemistry or biology, no knowledge of biomaterials or tissue engineering, and no preconceived notions of how research should be done in this arena. This has been both a

liability and (in hindsight) an asset. Several years into our HA project, events in the 1990s lured me into technology commercialization and the biotechnology industry. My professorial suspicion of business turned to admiration, and an image of how to link an academic career with entrepreneurism slowly took shape.

Our naiveté allowed us to dream. We based our research efforts on clinically relevant goals derived from discussions with physicians, polymer chemists, biologists, and engineers. After succeeding in developing a new chemical modification of HA [Pouyani and Prestwich, 1994], we needed a plan driven by clinical utility. Upon arriving in Utah, I was first enticed by the late Dr. Steven Gray to develop an injectable HA material for vocal fold repair. Steve treated public speakers and professional singers worldwide and wanted a better material than fat or suspensions of Teflon particles to treat vocal insufficiency and repair damaged vocal folds. Together we looked at the biological ECM, a complex network of covalent and non-covalent interactions among glycosaminoglycans (GAGs), proteins, and PGs, and sought a simple solution to create ECM-mimetic materials based on chemically modified HA. Once we had our first success, the word spread on campus, and physicians in rhinology, otology, dermatology, abdominal surgery, cardiovascular surgery, and orthopedics came to us with patient-driven clinical problems that could be addressed if we developed the right materials. What emerged were perhaps unrealistically ambitious long-term goals:

- Prevent postsurgical adhesion formation in ENT, abdominal, and orthopedic surgery.
- Accelerate acute and chronic wound healing without scarring.
- Repair bone and cartilage defects.
- Engineer functional liver, kidney, and cardiovascular tissues.
- Deliver growth factors for angiogenesis, cell differentiation, and wound repair.
- Improve models for preclinical evaluation of drug metabolism, safety, and efficacy.

As described below, we ultimately developed a set of biomaterials that were highly reproducible, manufacturable, approvable, and affordable. These materials were modeled after the ECM, but simplified and practical to optimize the experimental versatility for the user. The resulting covalently crosslinked, biodegradable, simplified, synthetic extracellular matrices, or sECMs, were first developed to promote tissue formation and promote scar-free healing in vivo. After establishing biocompatibility in culture, we immediately tested materials in animal models. Some materials which succeeded in vivo failed to promote growth or showed cytotoxicity during cell encapsulation, such as our polyethylene glycol dialdehyde crosslinked hydrazide derivatives of HA [Kirker et al., 2002]. These materials were subsequently modified by introducing the thiol-modification as described below, and then optimized for tissue engineering applications. Little time and effort was expended on materials that were effective in vitro but had little possibility of approval for reparative medicine. While skipping much of the traditional hypothesis-driven research phase left many unanswered questions, with clinically usable materials in hand we could seek answers to biologically relevant questions.

DEVELOPMENT OF THE sECM TECHNOLOGY FOR 3-D CELL CULTURE

The native ECM is a heterogeneous collection of covalent and non-covalent molecular interactions comprised primarily of proteins and GAGs. Covalent bonds connect chondroitin sulfate (CS), heparan sulfate (HS), and other sulfated GAGs to core proteins to give PGs. Non-covalent interactions include electrostatic associations with ions, hydration of the polysaccharide chains, binding of link modules of PGs to HA, and triple helix formation to generate collagen fibrils. HA, a non-sulfated GAG present in all connective tissue as a major constituent of the ECM, has key roles in morphogenesis [Toole, 2001] and is a versatile starting material for preparing biodegradable biomaterials [Shu and Prestwich, 2004; Prestwich and Kuo, 2007]. Recent reviews detail the uses of chemically modified HA for drug delivery [Luo and Prestwich, 2001] and as biomaterials for tissue engineering [Shu and Prestwich, 2004; Allison and Grande-Allen, 2006]. More recently, a covalently crosslinked, sECM [Shu et al., 2004b; Shu et al., 2006] has been developed as a consistent, experimentally controllable material for research in cell biology, toxicology, drug discovery, and tissue engineering [Prestwich et al., 2007]. These synthetic analogs of the

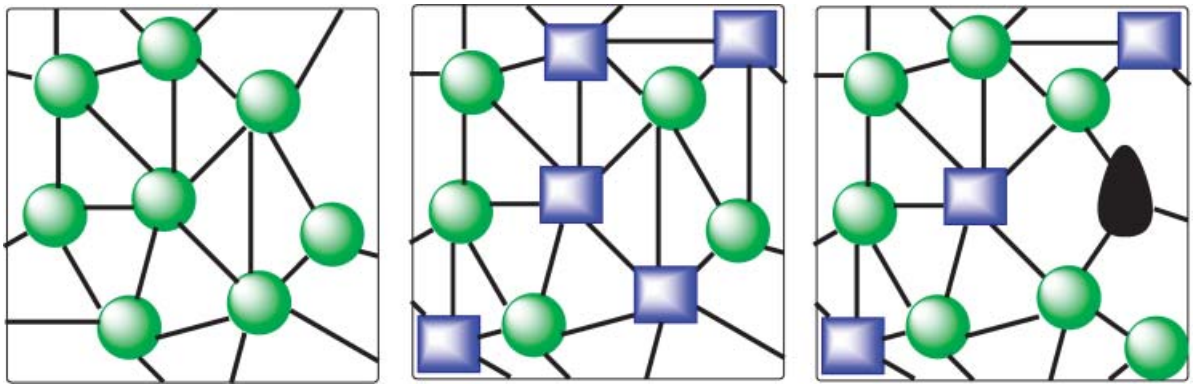

Fig. 2. Three simplified sECMs for 3-D cell culture. **Left panel**, non-cell adhesive crosslinked HA derivative suitable for incorporation of peptides and ECM proteins; **middle panel**, general use crosslinked HA-gelatin derivatives; **right panel**, HSPG-mimetic sECM, including a low percentage of crosslinked heparin for growth factor delivery. *Key*: green circle, thiol-modified HA; blue circle, thiol-modified gelatin; black diamond, thiol-modified heparin; and black lines, PEGDA crosslinkers.

natural ECMs were developed as 3-D scaffolds in an effort to meet our long-term goals and to provide in vivo-like microenvironments for cell culture and reparative medicine.

Figure 2 shows a schematic diagram of the three minimal sECMs that were designed to meet the needs of most in vivo applications as well as to provide flexibility for basic research applications. First, crosslinking of a thiol-modified HA derivative known as CMHA-S with PEGDA affords a hydrogel that has demonstrated preclinical potential in wound repair adhesion prevention [Liu et al., 2007b], covalent incorporation of Cys-containing polypeptides [Ghosh et al., 2006], and non-covalent incorporation of native ECM proteins [Mehra et al., 2006]. In addition, Cys-containing RGD peptides [Shu et al., 2004a] or a mixture of three Cys-containing recombinant domains of human fibronectin [Ghosh et al., 2006] were covalently incorporated to promote cell attachment and growth. Second, co-crosslinking thiol-modified HA derivatives with thiol-modified gelatin (Gtn-DTPH) produces a hydrogel that may be formed in situ in the presence of cells or tissues to provide an injectable cell-seeded hydrogel for 3-D cell culture [Shu et al., 2006]. This sECM was able to grow 1-cm diameter healthy fibrous tissue when seeded with murine NIH 3T3 fibroblasts and grown subcutaneously in nude mice for 8 weeks [Shu et al., 2006]. Third, co-crosslinking of thiol-modified HA with a small amount of a thiol-modified derivative of heparin incorporates immobilized heparin into the sECM, thus mimicking the heparan sulfate proteoglycans (HSPGs) of native ECMs [Cai et al., 2005]. This HSPG-mimetic sECM allowed controlled-release, localized delivery of one or more growth factors, including bFGF, VEGF, angiopoetin-1, and KGF [Cai et al., 2005; Peattie et al., 2006; Pike et al., 2006; Riley et al., 2006]. Moreover, the HSPG-mimicking sECMs resulted in a larger number of interconnected vascular networks, in contrast with the leaky angiogenic response to single growth factors provided without the immobilized heparin for spatiotemporal control.

These sECMs have been used in animal models for the engineering and repair of both hard and soft tissues in vivo and to support cell attachment, growth, and proliferation in 3-D. Successful in vivo tissue engineering and repair applications have included restoration of viscoelasticity and repair of biopsied vocal folds in rabbits [Duflo et al., 2006], accelerated cortical bone defect repair in rats [Liu et al., 2006a], osteochondral defect repair in rabbits [Liu et al., 2006b], and both re-epithelialization and re-vascularization of wounds in diabetic mice by sustained release of bFGF [Liu et al., 2007a]. In the rabbit cartilage injury model, the in situ crosslinkable, injectable sECM was used to deliver and retain autologous bone marrow-derived stromal cells [Liu et al., 2006b]. The sECM can also be used as an injectable cell delivery vehicle to produce uniformly sized, orthotopic human xenografted cancers in nude mice using human breast, colon, and ovarian cancer cell lines, even those which are poorly tumorigenic [Liu et al., 2007c]. These new

sECM materials are now available for research use as the Extracel™ product line.

From 2-D to 2.5-D to 3-D

Much of what is called 3-D cell culture is in fact 2-D cell culture on a 3-D scaffold. This applies to PLGA scaffolds, non-woven meshes of HA esters, microfibers fabricated from polyurethanes, spun nanofibers of synthetic polymers, and macroporous sponges prepared from any synthetic or natural polymer. The cells attach and spread in 2-D on either a rigid or compliant surface, and may (or may not) degrade the material, secrete a new ECM, and remodel the scaffold into a 3-D tissue construct. I refer to this as 2.5-D cell culture, to distinguish these approaches from techniques and materials designed for bonafide 3-D cell culture by cell encapsulation, thereby giving the cell the ability to move in 3-D, not simply along a more complex 2-D surface. Figure 3 illustrates the formation of the Extracel™ sECM either for 2.5-D culture on a sponge, or for 3-D culture by cell encapsulation using the in situ crosslinkability of the hydrogel components.

Frequently, 2.5-D techniques are used for initial evaluation of new matrices and compositions to determine suitability for a given cell type. For example, human dermal fibroblasts (HDFs) were cultured on sECM hydrogels of different compositions using HA-DTPH, CS-DTPH, and Gtn-DTPH crosslinked with PEGDA [Prestwich et al., 2007]. While few HDFs attached to the HA- and CS-only hydrogel surfaces, HDFs attached and spread on the surface of sECM hydrogels containing 5 and 20% gelatin. ECM microarrays have also been employed to screen for optimal compositions for probing differentiation conditions for a specific cell type [Flaim et al., 2005].

Monolithic or Living?

Materials for cell culture and tissue engineering can be classified as either *monolithic* or *living* [Prestwich and Kuo, 2007]. In *monolithic* materials, the final form of the material cannot form new chemical bonds in the presence of cells or tissues, and can only be manipulated by fabrication into different physical forms. These forms include films, woven and non-woven meshes, fleeces, and electrospun fibers. All 2-D and some 2.5-D cell culture is conducted on such monolithic materials. For example, a non-woven mesh of PLGA would be considered a monolithic material for 2.5-D cell culture, as would crosslinkable HA materials that have been electrospun and coated with fibronectin [Ji et al., 2006].

In contrast, *living* materials can form new covalent bonds or can change physical form in the presence of cells or tissues, thereby allowing encapsulation in vivo or in vitro. Some 2.5-D cell culture, such as that with variable composition, thickness, and compliance sECM hydrogels, uses living materials. All 3-D encapsulation

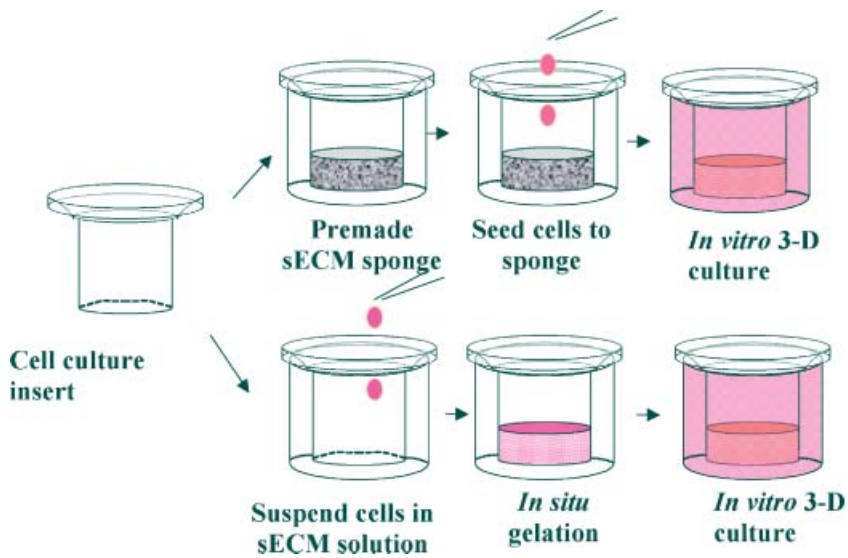

Fig. 3. Two modes of 3-D cell culture, shown in tissue culture inserts. **Top**, cells and medium added to preformed porous sECM sponge; **bottom**, cells encapsulated by in situ crosslinking of sECM.

techniques—Matrigel®, Purecol™, Puramatrix™, alginate, and Extracel™—use living materials, in which either a temperature change, peptide self-assemby, ionic crosslinking, or covalent crosslinking leads from a liquid form to a hydrogel form. The in situ-crosslinkable sECMs for cell delivery such as Extracel™ [Prestwich et al., 2007] are living materials with maximal experimental flexibility, as discussed below. Other living materials include shape controlled, harvestable cell-seedable hydrogels prepared from PEGDA or methyacrylated HA that can be micromolded and photocrosslinked [Arcante et al., 2006; Yeh et al., 2006].

Flexible Experimental Control Parameters

Experimentally variable composition. A single sECM composition is not optimal for every cell type for in vitro or in vivo applications. In a poignant demonstration of this fact, an ECM microarray was used to probe cellular differentiation [Flaim et al., 2005]. Using a robotic printer, a matrix of 32 different combinations of five ECM proteins (collagen I, III, IV, laminin, and Fn) embedded in polyacrylamide background was prepared. ECM combinations were identified that synergistically affected primary rat hepatocyte function and murine ES cell differentiation. This 2.5-D screen was then followed by 3-D culture in photopolymerized PEG hydrogels to assess hepatocellular function of differentiated dipotential mouse embryonic liver cells.

In our experience, stem cells and HA-rich environments prefer a lower gelatin percentage in the gelatin-DTPH: CMHA-S mixture. For liver stem cells and hepatoblasts, self-replication occurs optimally on crosslinked CMHA-S hydrogels and in the presence of minimal quantities of Type III collagen. In contrast, stem cells and hepatoblasts will expand in HA hydrogels containing Type IV collagen and differentiate in the presence of type I collagen or any materials containing significant amounts of Type I collagen [Reid et al., 2007]. In vivo, repair of biopsied rabbit vocal folds (an HA-rich environment) was examined using gelatin-DTPH:CMHA-S ratios from 0:100 to 50:50, and an optimum of 5:95 was observed [Duflo et al., 2006]. In contrast, higher gelatin percentages are better for differentiated cells, in that these compositions facilitate migration and remodeling in 3-D. With MDCK cells, for example, higher gelatin percentages favor cell polarization and acinus formation.

Experimentally variable compliance. Fibroblasts, neurons, myocytes, chondrocytes, and other cell types all require adherence to the ECM to form tissues and exhibit the correct morphological and biochemical phenotypes. However, each cell type requires a matrix with a different stiffness, and cells respond most appropriately to the stiffness of their native tissue source. Intracellular changes in molecular signaling are transmitted transcellularly by contractile forces via adhesion complexes and the actin–myosin cytoskeleton [Discher et al., 2005]. Matrix elasticity also directs stem cell lineage specification [Engler et al., 2006]. In collagen I-coated polyacrylamide crosslinked gels, mesenchymal stem cells (MSCs) will commit to a neurogenic lineage on soft matrices (0.1–1 kPa), to myogenic fates on a muscle-like stiffness (8–17 kPa), and to osteogenic fates on matrices mimicking collagenous bone (25–40 kPa). Migration in 2-D is governed by a balance between counteracting tractile and adhesion forces. In 3-D, migration of tumor cells was found to be governed by matrix stiffness and cell-matrix adhesion using DU-145 prostate carcincoma cells and HT-1080 fibrosarcoma cells in Matrigel™ [Zaman et al., 2006].

Adult HDFs adapt to covalent fibronectin domain-modified sECMs similar to Extracel™ as a function of the different viscoelastic properties of the hydrogel. Using crosslinker concentrations of 4.5, 1.5, and 0.5% PEGDA, shear storage moduli of 4270, 550, and 95 Pa were obtained. The HDFs modified their mechanical response to match substrate stiffness; cells on stiffer substrates had higher moduli as measured by AFM, and exhibited a more stretched and organized actin cytoskeleton that those on softer substrates [Ghosh et al., 2007]. In vivo, gel compliance (soft vs. rigid) also plays a critical role; thus, cartilage repair and soft tissues are best engineered in hydrogels [Liu et al., 2006b] while sponges appear more suited to bone repair [Liu et al., 2006a].

Advantages of biodegradable materials in vitro and in vivo. Having established the optimal composition and compliance, the ability to use a material for cell expansion ex vivo or tissue repair in vivo is facilitated if the degradation rate can be controlled. Most monolithic materials, for example, the polylactides and

polyglycolides, undergo bulk erosion by slow ester hydrolysis. This means the polymer, not the biology, controls the rate of degradation. Other polymers, for example, polyacrylamides and alginates, are not metabolized by endogenous enzymes, leading to undesirably long retention times in vivo. Ideally, the biology of the tissue growth should dictate the rate of degradation, with the material acting only as a temporary and biologically realistic scaffold. The sECMs do precisely that: as the cells proliferate and differentiate, the sECM is degraded by MMPs and GAG hydrolases concomitant with the cells secreting their own native ECM during tissue remodeling. In vitro, true 3-D cell culture also requires synchronous degradation and remodeling to best mimic biology. Moreover, for cell expansion and recovery, one must be able to harvest cells from a 3-D construct or from the surfaces of meshwork of a 2.5-D construct. This is easier when the matrix can be enzymatically or non-enzymatically removed under mild conditions that do not damage the cells or tissue constructs.

Practical Considerations

In addition to the ability to control composition, compliance, and degradation, the preparation, handling and availability of materials becomes paramount when moving from the research laboratory scale to a commercial process or a clinical product. Table I summarizes the features desired for a 3-D cell culture material. While the important parameters may vary for each application, we incorporated the following features into the sECM materials developed at the University of Utah.

Multiple physical forms. The sECMs are uniquely flexible for fabrication into hydrogel films, tubes, porous sponges, nanofibers, and other forms. For example, the sECMs can be centrifugally cast into tubes [Mironov et al., 2005] or used for bioprinting of cell aggregates [Mironov et al., 2007]. The flexibility of physical forms allows the same compositions to be explored in a variety of in vitro and in vivo formats that enable the separation of compositional effects from mechanical cues.

Batch to batch consistency. Unlike other commonly used materials for 3-D cell culture, the sECM technology provides a consistent manufactured product with negligible batch-to-batch variability. Since the sECM is not extracted from murine tumors, it contains no undesired growth factors or undefined components. The non-animal sourcing and reproducibility of composition are essential features for achieving FDA approval for a biomaterial.

Ease of use at physiological temperature and pH. The sECMs can be seeded with cells and then gelled within 5–30 min at ambient or body temperature and pH 7.4. This obviates the need to conduct complex manipulations with ice-cold syringes or extreme pH adjustments to control rates of gelation, both of which can compromise cell viability in vitro or in vivo. Ease of use is of paramount importance to achieving physician acceptance and compliance.

Resistance to contraction and expansion. The crosslinked sECMs exhibit minimal swelling or contraction as ionic strength changes within the physiological range. Unlike collagen gels, no contraction occurs during triple-helical fibril formation or when cells are activated by growth factors [Mehra et al., 2006]. However, as the materials degrade by cell-secreted enzymatic hydrolysis, swelling increases as the materials are resorbed.

TABLE I. Features Desired for Optimal 3-D Cell Culture Material

Design feature	Rationale
Chemically-defined scaffold	Reproducibility, regulatory approval
Controllable composition	Optimize for cell type and cell fate
Controllable compliance	Optimize for cell type and cell fate
Transparent	Ease of visualization of encapsulated cells
Consistent	Approval by regulatory agencies
Multiple physical forms	Flexibility of gels, sponges, meshes, electrospun nanofibers
Minimal swelling or contracture	Avoid volume changes leading to problems in vivo
Easy to handle at room temperature and under physiological conditions	Increase experimenter and physician comfort with use
Inexpensive	Increase experimental and clinical uses
Biodegradable	Cells remodel and body resorbs sECM
Scaffold disassembles under mild conditions	Permits easy cell expansion and re-harvest
Scaffold protects cells during cryopreservation	Cell viability on storage and recovery
Spatiotemporal control of soluble factor release	Control of cell growth and differentiation

Competitive cost. The sECM materials are affordable to maximize use in the marketplace. By reaching expanding market niches currently unable to afford existing 3-D products, the range of uses will expand. This creates a positive feedback spiral, with expanding applications driving increased sales and increased acceptance in the marketplace. With the end-user focus I have advocated, the economics was factored into the technology from the outset.

Transparency. For cell visualization during 2.5-D or 3-D culture, using optical, fluorescence, or confocal microscopy, the sECMs are transparent at visible and ultraviolet (>256 nm) wavelengths. Many materials are colloidal, opaque, or translucent, making visualization of cells problematic.

High throughput screening (HTS) compatibility. Flexible experimental parameters, consistency, multiple physical forms, ease of handling under cell-friendly conditions, resistance to contraction, cost, and transparency are all features that are important in developing a material for HTS. For contract services, in-house pharma studies of drug efficacy, toxicology screening with multiple cell lines, toxicogenomic profiling, and proteomic studies of drug-pathway interactions, the sECMs offer many opportunities not accessible with existing 3-D products [Prestwich et al., 2007].

Translational potential. For tissue engineering, biomaterials themselves are not the answer; they are a means to an end. Nonetheless, from a commercial perspective it is easier to develop a tissue engineered construct or to conduct cell therapy with a biomaterial that has a demonstrable clinical benefit by itself. This is clearly not the case with many polyesters, alginate, "smart gels" like poly(NIPAMM), or chitosan. In contrast, structurally robust biomaterials that can be machined, spun, woven, or molded and then provide mechanical support, for example, Dacron, polyethylene, Teflon, will continue to have a role as cell-biomaterial constructs enter clinical use. A clear need still exists for engineered, bioresorbable, and mechanically robust biomaterials with practical in vivo utility for repair of elastic and stiff tissues.

USER-DRIVEN APPLICATIONS FOR 3-D CELL CULTURE

The uses of sECMs to promote both in vitro and in vivo growth of healthy cellularized 3-D tissues have been summarized above. Beyond the long-range tissue engineering applications, this technology has immediate potential in stem cell biology, high content cell-based screening, proteomics, toxicology, and drug discovery in both academic and pharmaceutical laboratories. Three specific examples are highlighted below.

Toxicology Models

Currently, it costs some $1.2 billion over 12–15 years to bring a new chemical entity (NCE) from the laboratory bench to the bedside [DiMasi, 2003]. About one-fifth of all drug candidates currently fail in Phase III human clinical trials due to hepatotoxicity, after $100–500 million may have been spent. Reducing failure at this stage could substantially lower the overall costs of drug discovery [Li, 2004]. "The holy grail of the [pharmaceutical] industry is to be able to predict [drug] toxicity from a cell culture" [Friedman, 2004]. Current methods for identifying hepatotoxic drugs are far from achieving this goal. Measuring cytotoxicity in 2-D cultured hepatocytes can predict clinical acute hepatotoxicity [Dambach et al., 2005], but this does not take into account the many drugs (~40%) which fail because they are metabolized in vivo to toxic species. This idiosyncratic toxicity cannot be adequately detected until Phase III clinical trials [Dambach et al., 2005], and sometimes not even then. Cytochrome P450 enzymes play essential roles in both bioactivation and bioconjugate formation, with CYP 3A4 being a key player for over 50% of all approved therapeutic agents [Guengerich, 2006].

The sECM technology offers opportunities for in vitro and in vivo liver toxicology models by culturing human liver cells—from the immature hepatic stem cells to mature hepatocytes—in 3-D. The key feature for the sECMs is that different materials can be employed to recapitulate the cellular microenvironment experienced along the maturational lineages [Reid et al., 2007]. The ability to grow metabolically competent engineered liver tissue in 3-D is an important "growth industry", and the sECM technology is unique in enabling both in vitro toxicological studies and in vivo liver regeneration possibilities [Prestwich et al., 2007].

Proteomics and Chemogenomics

Cell-biomaterial interactions have not been extensively investigated by transcriptomic or

proteomic studies. Most comparisons have been performed in 2-D on non-physiological substrata, as recently reviewed [Gallagher et al., 2006]. A comparison of 2-D TCP with culture on a collagen gel was performed using human fetal lung fibroblasts [Klapperich and Bertozzi, 2004]. Genes involved in cell signaling, ECM remodeling, inflammation, angiogenesis, and hypoxia were selectively activated in cells in 2.5-D on collagen-GAG mesh. In principle, the sECM technology provides a "tabula rasa"—a low background, all covalent, tunable substratum—which allows many nuances of protein and gene expression to be gleaned as the composition and compliance are varied in a physiologically meaningful context.

Engineered Human Xenografts

Current animal xenograft models used to evaluate new anticancer therapies are limited to "good take" cell lines, fail to mimic normal human disease, and poorly predict clinical outcomes [Sausville and Burger, 2006]. The injectable, in situ crosslinkable sECM has now been used to deliver and grow cancer cells in vivo [Liu et al., 2007c] in a technique we call "tumor engineering." The sECM was seeded with breast, colon, and ovarian cancer cells prior to gelation and injected subcutaneously into mammary fat pads, subserosally in colons, and intracapsularly in ovaries, respectively. Two cell lines were use for each cancer, and results were compared with orthotopic injection of cells in serum-free medium. Overall, orthotopic delivery of cancer cells in sECM hydrogels showed: (i) increased incidence of cancer formation and reduced variability in tumor size; (ii) enhanced growth of organ-specific cancers with good tumor-tissue integration; (iii) improved vascularization and reduced necrosis in the tumor; (iv) reduced cancer seeding on adjacent tissues or organs; and (v) better overall health of the animals. Thus, engineered tumors—injectable, orthotopic, xenografted cancers—offer a new tool to study cancer biology, invasion and metastasis, and to investigate new therapeutic and diagnostic protocols.

BUSINESS MODELS AND CLINICAL REALITIES

For market acceptance and approval, whether in veterinary practice or in human medicine, new materials that have a beneficial effect by themselves will be more likely to achieve FDA approval than materials that require cells to show a benefit. The cost of bringing a medical device to market, whether by a 510(k) equivalence claim or by the more rigorous pre-market approval (PMA) route, is faster and more straightforward than trying to bring a cell-material combination to market.

Cell therapies, aside from the complex cell sourcing issues, are presently limited by three problems: cell delivery, cell retention, and cell integration (= engraftment). A biomaterial is required that provides the relevant sequence of signals for expansion, differentiation, and self-organization into a tissue. More importantly, cells should be able to remodel the biomaterial scaffolding into a natural ECM on demand. Materials from the 20th century—PLGA, alginates and chitosans, or polyethylene meshes, Dacron, or Teflon—will simply not be the materials of the future. In the past, to paraphrase a former Secretary of Defense, cell delivery and tissue repair were forced to go with the scaffolds that we had, not with the scaffolds that we wanted. The next generation scaffolds for 3-D cell growth and including tissue engineered constructs, such as the flexible Extrace (TM) platform, should be designed with the marketplace in mind. That marketplace is extending and improving the quality of life, both for humankind and for our animal companions.

For economic viability, and accelerated market acceptance, it is prudent to design for dual-use or multiple-use applications of the same product. For example, the consumer market can compliment and in some cases provide a financial driver for the important critical care human needs. For example, in wound healing one could envision band-aid type products for road rash, veterinary products for injuries to horse shins or dog bites on other dogs. Compassionate use materials for military and trauma needs include large surgical reconstructions for victims of bombings, falls, or vehicular accidents.

CONCLUSIONS

We have reached the end of the utility of TCP as a predictive research tool in biology. In the past, its lack of biological correctness has been overshadowed by its virtues: generality for most cell types, ubiquitous commercial availability, simplicity of use, ease of shipping and long shelf

life, and relatively low cost. The dominance of TCP was driven by sales, not marketing, considerations. These days are numbered. At present there is no accepted "TCP equivalent" of the 3-D marketplace, and the transition to 3-D cell culture will be driven by availability of versatile research tools that meet the criteria outlined herein. Most importantly, no 3-D cell culture product can hope to become the "standard of use" unless it can lead seamlessly to an FDA-approvable, tissue engineered construct. With the shift of emphasis at the National Institutes of Health and in university laboratories and hospitals to engaging in translational research, identifying a 3-D cell culture material with clinical potential would seem to have high importance.

A new generation of biomaterial scaffolds will be required to accomplish the promise of functional tissue engineering, defined as the combination of cells, an ECM equivalent, and appropriate signaling factors, to generate a complex, vascularized living tissue. If tissue engineering is to succeed clinically, a philosophical and operational paradigm shift may be necessary. Is our primary goal limited to probing cell biology in model systems, or are we committed to a concerted effort to recapitulate developmental biology in order to develop clinically useful products? Given that we aspire to clinical products, is our goal to conduct ex vivo tissue engineering by the conventional definition using cells, materials, soluble factors, and bioreactors? Or is our goal that of what one might call "assisted regenerative medicine"—the use of materials alone, whether of synthetic or natural origin, or materials with cells for in vivo tissue repair? These decisions will heavily influence the way in which the research will be conducted, and should not be postponed until after the research is essentially completed.

Finally, I reiterate my underlying theme in this Prospect. To conduct translational research, commercialization is sine qua non. No product reaches a patient unless it has been the focus of an intense research and development effort by a for-profit company. Successful products must be focused on the unmet needs of patients, who are the ultimate customers of our research efforts. I believe that it is both our obligation and our responsibility to adjust our research priorities to meet the needs of our customers.

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