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REVIEW

Three-dimensional polymeric systems for cancer cell studies

Feng Xu · Karen J. L. Burg

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Abstract Three-dimensional (3-D) culture of cancer cells and of normal mammalian cells in a polymeric matrix is generally a better alternate model for understanding the regulation of cancer cell proliferation and for evaluation of different anticancer drugs. A substantial amount of evidence demonstrates important differences in the behavior of cells grown in monolayer, i.e., two-dimensional (2-D), and in 3-D cultures. Cancer cells grown in 3-D culture are more resistant to cytotoxic agents than cells in 2-D culture; growth of cells in vitro in 3-D requires a suitable polymer that provides a structural scaffold for cell adhesion and growth. Many naturally derived polymers as well as synthetic polymers have been investigated as scaffolds. The aim of this review is to overview the polymeric materials of natural and synthetic origin that are of specific interest to 3-D cell cultures, and discuss the development of new polymers that should be specifically designed for 3-D culture applications.

Keywords Cancer cells · Cell culture · Polymer · Three-dimensional (3-D) · Two-dimensional (2-D)

F. Xu · K. J. L. Burg (✉)
Department of Bioengineering, Clemson University, Clemson, SC, 29634, USA
e-mail: kburg@clemson.edu

Introduction

Traditionally, most cell cultures are developed in 2-D environments. In mammalian tissue, cells connect not only to each other, but also to a support structure called the extracellular matrix (ECM). Natural tissues are comprised of functionally diverse cell types that are organized in spatially complex arrangements (Hutmacher 2002). ECM contains proteins, such as collagen, elastin and laminin, that give tissues their mechanical properties and help to facilitate communication between cells embedded within the matrix. Cells in the human body grow within an organized 3-D matrix, surrounded by other cells. The behavior of individual cells is controlled through their interactions with immediate neighbours and the ECM. Receptors on the surface of the cells, in particular a family of proteins called the integrins, anchor their bearers to the ECM, and also determine how the cells interpret biochemical signals from their immediate surroundings. The complex summation of these multiple signals determines whether a given cell undergoes differentiation, apoptosis, proliferation, or invasion. Given this complex mechanical and biochemical interaction, it is perhaps no surprise that biological subtleties are inadvertently omitted in 2-D cell culture systems (Smalley et al. 2006).

Indeed, many of these complex interactions are lost in 2-D culture. As a result, a growing number of studies report differences in phenotype, cellular signaling, cell migration, and drug responses when

the same cells are grown under 2-D versus 3-D culture conditions. One potential application of 3-D culture systems is to anticancer drug discovery, which has long been hampered by the lack of good preclinical models. Compounds with good antitumor activity in 2-D cell culture models often fail to translate into the clinic. Although many findings in cell and molecular biology have come from cells grown in monolayer, the limitations of 2-D cell culture are now becoming clear (Cukierman et al. 2001). Bissell and coworkers (1997) found that the antibody against β1-integrin completely changed the behavior of cancerous breast cells grown in 3-D culture. The cells seemed to become non-cancerous, losing their abnormal shapes and patterns of growth (Weaver et al. 1997). This result had never been observed in 2-D cultures. Since that time, Bissell and coworkers have demonstrated further important differences in the behavior of cells grown in 2-D and 3-D cultures (Gudjonsson et al. 2002; Nelson et al. 2005; Schmeichel et al. 2003; Wang et al. 1998; Weaver et al. 2002). Recently, 3-D cultures within a polymeric matrix (also termed "scaffold") were proposed as a better model to study complex biological processes than cells grown in monolayer (Abbott 2003). Cancer cells grown into a tissue-like structure in a polymeric matrix may closely mimic the biology of tumor development in vivo and hence can serve a better model system for preclinical evaluation of the cytotoxic effect of anticancer agents (Jacks et al. 2002).

Polymeric matrix composition and structures must be carefully considered when designing 3-D cell culture systems (Voytik-Harbin 2001). Many synthetic polymers such as polylactide (PL), polyglycolide (PG), poly (D,L-lactide-co-glycolide) (PLG), as well as natural polymers such as collagen, alginate, and chitosan, have been investigated as scaffold matrices. This review presents the need for 3-D cancer cell culture, current states of polymers used in 3-D culture, and recent advances in polymer research for 3-D cancer cell culture.

Significance of 3-D cancer cell culture

Two-dimensional in vitro cell cultures have been applied in cancer research for a few decades. However, 2-D cell cultures cannot completely simulate the in vivo cellular environment, due to the important differences in the cellular surroundings. Monolayer cultures generally consist of cells growing in a nutrient-rich liquid environment with plenty of oxygen, which form a nearly homogeneous colony. In vivo, a mature tumor with extensive vasculature has a very complex structure, generally consisting of regions of regularly dividing, hypoxic, and necrotic cells (Friedrich 2003).

Though animal models give a more accurate morphological representation of tumor development, they are considerably less amenable to large-scale molecular biology research. A 3-D cell culture system, therefore, combines the virtues of animal models and monolayer cell cultures. Three-dimensional in vitro models allow cells to develop into structures similar to those in living organisms and thus enable researchers both to perform genetic manipulations and to observe some of the biological changes. It is recognized that 3-D cell culture systems better reflect the in vivo behavior for most cell types (Kunz-Schughart et al. 2004).

Natural and synthetic polymers used as 3-D cell culture matrices

Polymers are a class of that are distinguished by repetition. In general, polymers can be classified into two groups, naturally occurring and synthetic. Cells are seeded into a 3-D polymeric matrix and, following their adhesion, spreading, proliferation, and differentiation, they develop into a tissue-like structure (Sachlos et al. 2003). One of the essential requirements for promoting 3-D cell growth in vitro is the design of a suitable polymeric matrix scaffold that provides a structural template for cell adhesion and growth. Providing an appropriate environment is no easy matter. An ideal degradable matrix should degrade at a rate matching the rate of extracellular matrix deposition until no residual polymer remains (Vunjak-Novakovic et al. 1998).

Natural polymers include collagen (Liao et al. 2004), chitosan (Madihally et al. 1999), and alginate (Alsberg et al. 2003; Shea et al. 1994). Hydrogels, such as alginate, are cross-linked macromolecular networks formed by hydrophilic polymers swollen in water or biological fluids (Peppas et al. 1996). Their 3-D networks can retain large volumes of water in the

cross-linked structures. The extent of swelling and the content of water retained depend on two factors: the hydrophilicity of the polymer chains and the cross-linking density.

Synthetic polymers that have been investigated as scaffold matrices include polylactide, polyglycolide and their copolymers (Mikos et al. 1993; Mooney 1999). PL and PLG are the biocompatible and biodegradable polymers most widely used in the fabrication of different scaffold forms (Murphy et al. 2002). PL and PLG have better mechanical strength but less cell affinity than natural polymers. One way to address this problem is to modify the polymer surface to improve cell adhesion. Surface modification of PL/PLG has been accomplished by either blending them with a hydrophilic polymer or by coating the scaffolds with an extracellular matrix (Chen et al. 2003).

Recent studies have demonstrated the significance of polymer characteristics on cell adhesion and proliferation (Cui et al. 2003; Yang et al. 2002; Zhu et al. 2002). Self-assembly is an important concept in ECM function. Many ECM molecules can self-assemble into larger units, as in the case of collagen and laminin. Self-assembly allows molecular units to form larger structures, such as basement membrane, which present an array of sites for cell binding. Enlightened by this concept, some researchers are now using nanoscale structured hydrogels, fashioned through the self-assembly of peptides, for 3-D cell culture, (Jayawarna et al. 2006; Semino et al. 2003).

Specifications of polymers commonly used in 3-D cancer cell cultures

Collagen type I

Collagen is the major component of mammalian connective tissue, and is found in every major tissue that requires strength and flexibility, such as tendons and skin. Collagen is secreted by chondrocytes, fibroblasts, and other cell types. Fourteen types of collagens have been identified to date, where the most abundant is type I. Because of its abundance and its unique physical and biological properties, type I collagen has been used extensively in the formulation of biomedical materials (Pachence 1996). Each type of collagen contains the same basic macromolecular unit: an α-helical chain formed by the interaction of three polypeptides. These polypeptides chains are approximately 1,050 amino acids in length and are specific to each type of collagen. Following secretion into the ECM, molecules of type I collagen organize themselves into larger fibrils of 10 to 300 nm diameter. These fibrils are stabilized by crosslinks, which connect lysine residues within or between adjacent collagen molecules. In some tissue, these fibrils become further organized, forming larger collagen fibers several micrometers in diameter. Fibrillar collagen interacts with cells through the integrin receptors located on cell surfaces. Cell differentiation and migration during development are influenced by fibrillar collagen (Pachence 1996).

When collagen is implanted in vivo, it is degradated by collagenases, frequently eliciting a host immune response if the collagen is from an animal source. Chemical modification of collagen (e.g., cross-linking) makes it less susceptible to enzymatic degradation and also can reduce its immunogenicity (Weadock et al. 1984).

Collagen matrices may be designed with a porous structure, thereby providing three critical functions for the scaffold. First, pores and channels can allow the migration or entry of cultured cells into the scaffold. Second, the porous structure can give the scaffold an enormous surface area for cells to adhere to and interact with the scaffold. Third, the porous structure can allow nutrients to diffuse into the scaffold to support the growth of the seeded cells (Dagalaskis et al. 1980). Three-dimensional type I collagen cell culture systems are able to support short- and long-term growth of various cell types, including cancer cell lines, endothelial cells, endometrial cells, hepatocytes, osteoblasts, and fibroblasts, and to sustain or even enhance cell differentiation in vitro (Themistocleous et al. 2004). Some recent applications of type I collagen to 3-D cell cultures are now described.

Since the available 2-D cultures systems could not provide sufficient information to assess the morphologic evidence of bone reaction to cancer cells, Koutsilieris and coworkers developed a 3-D type I collagen hydrogel system that allows co-culture of human osteoblasts (MG-63) with cancer cells, such as MCF-7, MDA-AB-231 or ZR-75 breast cancer cells, PC-3 prostate cancer cells, KLE endometrial cancer cells, and Calu-1 lung cancer cells (Mitsiades et al.

2000). The 3-D system was prepared by mixing MG-63 cells with type I collagen in 24-well plates, followed by inoculation with cancer cells and maintenance using standard cell culture procedures. After one week of culture, the hydrogel matrix was fixed with formalin and embedded in paraffin. The inoculation of PC-3 cells in this collagen matrix produced a blastic reaction, documented by an increased number of MG-63 cells and increased density of type I collagen. The human KLE cells produced no reactions, while MCF-7, ZR-75 and Calu-1 produced local degradation of the collagen matrix. Adriamycin induced apoptosis in prostate cancer cells and estrogen receptor negative (ER-) MDA-MB-231 breast cancer cells, but the adriamycin-induced apoptosis was inhibited by co-culture with osteoblast-like cells (MG-63). Therefore, the authors concluded that the 3-D culture system was a useful in vitro model, allowing the analysis of local mediators of osteolytic and osteoblastic reactions to bone metastases and treatment response.

Three-dimensional culture systems that simulate the tumor extracellular microenvironment may be appropriate to test cancer cell potential for invasion and tumor cell sensitivity to anticancer drugs. Koutsilieris and coworkers embedded and grew human PC-3 prostate cancer cells, A549 colon cancer cells, HT-29 lung cancer cells, MCF-7, and MDA-MB231 breast cancer cells in a collagen hydrogel surrounded by a fibrin clot (Doillon et al. 2004). Cisplatin, doxorubicin, paclitaxel and 5-fluorouracil were compared for their ability to inhibit tumor cell proliferation and colony formation in vitro. The results demonstrated that all cells, except MDA-MB231, formed colonies in collagen. PC-3, A549 and HT-29 cells massively invaded the fibrin, forming migratory fronts. Cell colonies were also formed in the fibrin, apart from the migratory fronts. HT-29 cells were the most aggressive in this regard. PC-3 cells were primarily sensitive to cisplatin and doxorubicin. A549 cells displayed the greatest potential for invasion and were sensitive mostly to cisplatin. HT-29 cells were sensitive to fluorouracil and doxorubicin. MCF-7 showed sensitivity to all anticancer regimens tested, while MDA-MB231 cells were particularly sensitive to doxorubicin. The authors recommended that 3-D collagen cell culture systems be used to study cancer cell invasion potential and cancer cell relative sensitivity/resistance to anticancer drugs.

Wu and coworker cultured SH-SY5Y human neuroblastoma cells in 3-D collagen hydrogels (Desai et al. 2006). Their results showed differences between 2-D and 3-D resting membrane potential development profiles upon differentiation. In response to a high K^+ depolarization, 3-D cells were less responsive in terms of increase in intracellular Ca^{2+}, in comparison to 2-D cells, supporting the hypothesis that 2-D cell calcium dynamics may be exaggerated. In general, this study confirms the depolarization-induced differences in intracellular calcium release when cultured using a 2-D vs. a 3-D matrix.

Chitosan

Chitosan is a biosynthetic polysaccharide, which corresponds to the deacetylated form of chitin. Chitin, a structural and protective material, is found in the shells of crustacean and mollusks, in the backbone of squids, and the cuticle of insects, and is an important constituent of the exoskeleton (Baxter et al. 1992). Chitin is also present in the algae commonly known as marine diatoms, in protozoa, and in the cell walls of several fungal species (Wu et al. 2005). Chitin is one of the most abundant organic materials, second only to cellulose in the amount produced annually by biosynthesis. As a natural polymer, chitosan is biodegradable due to its β-1, four glycosidic linkages, which are susceptible to the lysozymes present in the human body. Chitosan is therefore a potential candidate for tissue engineering application.

Chitin is a copolymer of N-acetyl-glucosamine and N-glucosamine units, distributed randomly or in block throughout the biopolymer chain—the distribution depending on the processing method used to derive the biopolymer. When the number of N-acetyl-glucosamine units exceeds 50%, the biopolymer is termed chitin, whereas the term chitosan is used to describe an N-acetyl-glucosamine unit content less than 50%. Chitosan chains are strongly hydrogen-bonded, making the biopolymer insoluble in common solvents. Chitosan is more prevalent because of its solubility in dilute acids—rendering chitosan more

accessible for chemical reactions and a preferred choice for utilization (No et al. 2000).

In order to develop and standardize polymeric scaffolds of chitosan for 3-D growth of cancer cell lines, chitosan scaffolds of varying degrees of deacetylation were prepared for 3-D growth of MCF-7 breast cancer cell lines by Panda and coworkers (Dhiman et al. 2004). With cells growing in the near tissue-like morphology and structure of a polymeric scaffold, the chitosan scaffolds could be used for cytotoxic evaluation of anticancer drugs. It was expected that, due to the 3-D nature of cell growth in chitosan scaffolds, the cytotoxicity of anticancer drugs in vitro would be very similar to that observed in in vivo tissue conditions. It was observed that chitosan with a high degree of deacetylation promoted better cell growth. MCF-7 cell growth was comparable to that observed in the tissue culture flasks, with the added advantages of 3-D growth. Metabolic activities of the MCF-7 cell lines, i.e., glucose uptake and lactic acid production, were comparable to those observed in cell culture with standard tissue culture flasks. Growth of anchorage-dependent cell lines such as MCF-7 on chitosan matrices provided a better alternative to static culture experiments for the evaluation of anticancer drug response. It is expected that by using cell culture models on polymer scaffolds, the anticancer activities in terms of dose and action will be better understood than when cells are propagated under 2-D growth conditions. Such models can also be used to more effectively screen the activities of new drugs for anticancer effects. The ease of availability, low cost, simple preparation, and excellent biocompatibility of chitosan thus make it a very attractive substratum for cell culture in vitro.

Because 3-D culture of MCF-7 cells on chitosan matrices provided a better understanding of the cell's carbohydrate metabolism, Panda and coworkers further studied the cytotoxic effect of anticancer drug and, more importantly, the kinetics and uptake of cathepsin D in breast cancer cells (Dhiman et al. 2005). On chitosan scaffolds, the MCF-7 cells produced more lactic acid in an amount similar to that observed for tumor cells in vivo, suggesting that their metabolism more closely resembled that observed in tissues. Tamoxifen-induced cell growth retardation was low in 3-D culture in comparison to the tissue culture growth at a similar drug concentration. It was also observed that cathepsin D uptake was inhibited by tamoxifen in the 3-D culture of MCF-7 cells. As cathepsin D was an autocrine mitogen for breast cancer cells, it can be concluded that one of the ways by which tamoxifen works on growth arrest of estrogen positive cancer cells is by the inhibition of cathepsin D uptake during cell growth. Chitosan offers many more advantages, like low immunogenicity, biodegradability and biocompatibility, allowing it to be used as 3-D scaffold for tissue engineering purposes (Nettles et al. 2002). Thus such 3-D tissue growth models can be used not only for evaluating the anticancer activities of new drugs but also for providing information about the regulation of both autocrine and paracrine growth factors that control cancer cell growth.

Alginate

Alginates are produced by brown seaweeds, and are linear unbranched polymers containing β-(1–4)-linked D-mannuronic acid (M) and α-(1–4)-linked L-guluronic acid (G) residues. Although these residues are epimers, they possess very different conformations. Alginates are not random copolymers but, according to the source of algae, consist of blocks of similar and strictly alternating residues (i.e., MMMMMM, GGGGGG and GMGMGMGM), each of which has a different conformational preference and behavior. Alginates may be prepared with a wide range of weight average molecular weights (50–100,000 residues) to suit the specific application (Draget et al. 1997).

The primary function of alginates is as thermally stable cold setting gelling agents in the presence of calcium ions. Such gels can be heat-treated without melting, although they may eventually degrade. Alginate's solubility and water-holding capacity depend on pH, molecular weight, ionic strength and the nature of the ions present. Generally alginates show high water absorption and may be used as low viscosity emulsifiers and shear-thinning thickeners. As a macromolecule of natural origin, alginate has been used as a scaffold material (Hutmacher et al. 2001). Zhang and coworkers suspended cells in a 1.5% w/v sterilized sodium alginate and dispensed the solution into a 100 mM $CaCl_2$ solution to form calcium alginate hydrogel beads (Zhang et al. 2005).

The hydrogel beads were incubated with 0.1% w/v poly-L-lysine to form alginate-poly-L-lysine-alginate (APA) membranes around the surface. The membrane-enclosed hydrogel beads were further suspended in 55 mM sodium citrate to liquefy the alginate hydrogel core. MCF-7 cells in the resulting APA microcapsules began to aggregate after 24 h and formed a single multicellular tumor spheroid up to 150 μm diameter after 5 days of cultivation. Similar to monolayer culture, the cell viability of the multicellular tumor spheroid was reduced after treatment with anticancer drugs. However, the inhibition rate of cell viability in the spheroid was much lower than that in monolayer culture. The spheroid was more resistant to anticancer drugs than cells in a monolayer culture. These results suggested that multicellular tumor spheroids have the potential to be rapid and valid in vitro models, useful in the screening of chemotherapeutic drugs and mimicking in vivo cell growth patterns.

PLGA

Poly (α-hydroxy acid) is a general name for a class of polymer including polylactide (PL), polyglycolide (PG), and copolymer PLG. These polymers are currently the most widely investigated and most commonly used synthetic biodegradable polymers in the biomaterials fields. One of the important reasons for their wide use is that the constituent units of these polymers are derived from natural metabolites (Athanasiou et al. 1998). Lactic acid is a chiral molecule, existing in two stereoisometric forms: D-L and L.

Therefore, it has three corresponding polymers: two stereoregular polymers, poly (D-L) and poly (L), and the racemic form poly (D,L-L). Both poly (D-L) (PDL) and poly (L) (PL) are crystalline polymers, while poly (D, L-L) is an amorphous polymer. PL is the most commonly used form, as opposed to PDL, because the degradation product L-lactic acid is the natural occurring stereoisomer of lactic acid. As an absorbable polymer, PL has satisfactory in vitro biocompatibility (Ashammakhi et al. 1997). It is essentially non-toxic and elicits only a mild inflammatory response. The hydrolysis product L-lactic acid is a normal intermediate of carbohydrate metabolism and will not accumulate in vital organs. PL has been proposed and successfully applied to the reconstruction of bone, articular defects, suture materials, drug carriers, and fixation devices. PL degrades slowly in vivo (Tsuji et al. 2005).

Polyglycolide is a highly crystalline linear aliphatic polyester. It has been used in suture materials and bone fixation devices. PG degrades relatively quickly compared to PL. Therefore PG is frequently used as co-monomer to modify the degradation rate of the copolymer (Athanasiou et al. 1996; Avgoustakis et al. 2004). When used in tissue engineering as a scaffold, poly (α-hydroxy acids) is processed into 3-D scaffolds. There have been many processing methods developed to prepare PL, PG, and their copolymer scaffolds. One of the commonly used methods is termed the solvent-casting/particulate-leaching technique.

Sahoo and coworkers designed and evaluated biodegradable porous polymeric microparticles as scaffolds for cell growth (Sahoo et al. 2005). Their hypothesis was that microparticles with optimized composition and properties would have better cell adhesion and hence facilitate cell growth into a tissue-like structure. The solvent-evaporation method was modified using sucrose as an additive to form large porous microparticles of PLG and PL polymer. Microparticles containing hydrophilic polymers (poly (vinyl alcohol) and chitosan) incorporated in their internal matrix structure were also formulated. Different formulations of microparticles were evaluated for physical properties, cell adhesion, and cell growth in culture.

PL microparticles containing poly (vinyl alcohol) (PVA) in the matrix structure (PL–PVA) and treated with serum prior to cell seeding demonstrated better cell adhesion and cell growth than other formulations of microparticles. Cells grew into aggregates, engulfing microparticles completely with time, and forming a 3-D tissue-like structure. Cell density (cells per mg of microparticles) was achieved in 9 days of culture, which resulted in a 7-fold increase from the initial seeding cell density. The improved cell growth on PL–PVA microparticles appears to be due to the PVA associated with the internal matrix structure of microparticles. These microparticles demonstrated better wetting and cell affinity in culture; they may be used to grow cancer cells in vitro and provide a model system for preclinical evaluation of the cytotoxic effect of anticancer agents (Sahoo et al. 2005).

Significance of normal cell culture in 3-D polymeric systems

Although this paper is focused on the growth of malignant cancer cells in 3-D culture systems with polymeric scaffolds, it is worth mentioning explicitly that the growth and differentiation of normal cells is also improved in a 3-D culture environment (Battle et al. 1999). Hepatocyte transplantation is an effective treatment for patients suffering from hepatic failure. However, one of the major problems has been that a large number of transplanted hepatocytes died within a few days. A 3-D PL scaffold is a good support material for the cultivation of fetal liver cells, and may be used as a vehicle for in vivo transplantation (Jiang et al. 2004). Studies of primary hepatocyte cultures on woven and non-woven poly(ethylene terephthalate) fibers showed that cell cultures in 3-D exhibited clear differences in morphology and phenotype compared to those observed in 2-D culture (Leong et al. 2002). Semino and coworkers studied the capacity of a 3-D peptide hydrogel culture system to promote functional hepatocyte differentiation of an expandable clonal epithelial stem cell line (Lig-8). In conventional dish culture, Lig-8 cells divide exponentially, expressing the primitive hepatocyte marker α-fetoprotein. However, after culturing Lig-8 cells in hydrogels, the cells acquire spheroid morphologies, cycle with non-exponential cell kinetics, produce progeny cells, and express mature hepatocyte differentiation markers (Semino et al. 2002). Frisk and coworkers found it possible to perform medium- to long-term cultivation of cells in a controlled 3-D environment, a concept which opens the possibilities of performing studies of normal cells in a more physiological environment compared to traditional 2-D cultures on flat substrates (Frisk et al. 2005). Skeletal muscle cells show distinctly different characters in 2-D vs. 3-D culture. Cells from the skeletal muscle cell line, C2C12, differentiate in 3-D type I collagen gel cultures, but do not differentiate in 2-D cultures on type I collagen coated dishes (Tanaka et al. 2002).

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