Appropriate Extracellular Matrix (ECM) Mimicking Surfaces Aid In The Establishment Of Serum-Free Cell Culture Media

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Abstract

Fetal bovine serum (FBS) is very commonly used in cell culture practices as a highly nutrient-rich supplement. The search for alternatives for FBS and the development of chemically defined media is gaining more attraction due to several ethical and scientific issues. FBS harvest may cause suffering to the calf fetus and compromise the reproducibility of cell culture experiments. Attachment factors are also provided by FBS. These attachment factors are absorbed onto the culture surface and support the binding of adherent cells. Therefore, the use of serum-free media often requires a specific surface coating in order to provide an environment in which adherent cells can bind. Specific culture surfaces play essential roles in promoting cell proliferation and the maintenance of cell function. This review discusses the concerns regarding FBS and alternatives for FBS, with a focus on cell culture surfaces that can aid in the development of serum-free media. Typically, mimicking the natural extracellular matrix (ECM) of the cell type of interest provides a suitable surface coating for *in vitro* culture. Mimicking ECM components can be done in various ways, such as coating culture dishes with ECM-derived proteins or decellularized ECM from tissues. More synthetic options are also available where specific cell-binding peptides or glycosaminoglycan chains are functionalized onto the culture surface or onto hydrogels. An accurate ECM mimicking surface can enhance the efficiency of serum-free media and thereby improve cell culture in a reproducible manner.

Layman's summary

Growing human or animal-derived cells and tissues in culture plates is an important tool to study the function of cells. A culture medium is supplied to provide nutrients needed for cell growth. Fetal bovine serum (FBS) is often added to the culture media because it is highly nutrient-rich, which supports the growth of most cell types. However, there are some serious ethical and scientific concerns about the use of FBS.

FBS is harvested from the blood of a fetal calf when a pregnant cow is sent to slaughter. The blood is often collected by a needle that is inserted into the heart of the fetus. There are several steps that can be taken to ensure that the fetus is unaware during the collection process, but there is no guarantee that these rules are always applied. The main scientific disadvantage of FBS is that the exact composition of FBS is unknown and very inconsistent. This leads to variability between batches, different effects on cells, and compromise the reproducibility of experiments. However, FBS is still the golden standard in cell culture since a serum-free alternative that works for most cell types is not available.

Many other animal- and human-derived products have been described as alternatives that can be collected in a more ethical manner. The most promising alternative is human platelet lysate, which has the ability to support the growth of several cell types. A disadvantage is, similar to FBS, variability between batches. Therefore, there is a need for chemically defined (CD) media that only contains supplements of known compositions. This increases specificity for specific cell types, and as a result, CD media has to be optimized for every cell type, which can be time-consuming and costly. Many cell types need to bind to a surface in order to survive and grow in culture plates. When FBS is added to the culture media, many factors adhere to the surface of the plate and allow cells to attach. In CD media, the plates need to be coated with specific attachment factors to allow cell binding.

In all organs, an extracellular matrix (ECM) is present. This is a non-cellular structure that has essential roles in the regulation of tissues. Mimicking the ECM of a cell type in a culture dish provides the correct environment for these cells to grow. This can be done by removing cells from tissues that are collected from humans or animals. This results in a structure composed of all the molecules present in the natural environment of the cells. An easier solution is to coat a culture plate with a specific protein that is abundant

in the natural ECM of the cell type. However, for some experiments, a more controlled environment is needed. Cell binding proteins contain specific peptide sequences that are responsible for the attachment to cells. These peptides can be linked to the surface of culture plates. The same can be done for glycosaminoglycan chains (GAGs), which are sugars that can interact with cell-binding proteins. These peptides or GAGs can also be linked to hydrogels that can mimic the natural elasticity of the ECM.

The use of an appropriate culture coating can enhance the efficiency of a CD medium. A recent study has shown that one serum-free media was able to support the growth of multiple cell types when the cells were cultured onto specific surfaces. Thus, mimicking ECM properties on a surface is important for the growth of cells in CD media and improves ethical and scientific issues in cell culture.

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1. Introduction

Cell and tissue culture are indispensable tools to research cell function in a well-defined environment. Research, diagnostic, biotechnology, and pharmaceutical industries all make use of cell culture methods. The composition of the cell culture media is essential for the reproducibility between experiments since differences in media can significantly influence cell behavior[1]. To maintain cells in culture for an extended period, the culture medium should supply sufficient components such as salts, nutrients, and a stable pH[2]. In most cases, an animal-derived serum is added to the media to provide the necessary components for cell proliferation and maintenance. Fetal bovine serum (FBS) was introduced to cell culture in 1958[3]. Since then, it has been widely used in *in vitro* and *ex vivo* cell and tissue cultures. When FBS is added, a complex mixture of growth factors, hormones, proteins, vitamins, transport proteins, trace elements, attachment factors, and spreading factors are provided to the culture media[4]. FBS is often preferred among several other kinds of animal or human sera, for which the main reason is the very low level of antibodies[5]. In combination with the high levels of growth factors, this allows for the growth and maintenance of most cell types. However, there are several ethical and scientific concerns for the use of FBS in culture media. The current way serum is collected may cause suffering for the unborn calf, and the differences in serum composition leads to variation between batches[6,7]. Both ethical and scientific problems are described in more detail below. The elimination of serum in culture media has proven to be challenging. A universal serum-free culture media has not yet been identified and seems unlikely due to the very specific needs per cell type. Lately, more insights have been gained on the importance of the correct use of cell culture coatings for the attachment of adherent cells. This review focuses on the most commonly used and promising forms of cell culture coatings and how these can optimize and aid in the generalization of a more universally applicable serum-free culture media.

2. Concerns regarding the use of FBS in culture media

2.1. Ethical concerns

The collection of FBS occurs after a pregnant cow is slaughtered. In most cases, it is not known beforehand if the cow is pregnant[6]. Approximately 8% of cows are pregnant when they are sent for slaughter. This is because cows and bulls roam free in many meat cattle herds[8]. The blood from a fetal calf is collected after the uterus of the slaughtered dam has been removed. Fetus bovine blood will be collected from fetuses if it is at the last two-thirds of gestation[8]. Blood collection is most often done by cardiac puncture where a syringe is inserted between the 4th and 5th rib into the heart[7,8]. Research shows that after 70% of gestation has passed, fetal calves may have the ability to be aware and experience pain[9]. Therefore, the fetuses at later stages of gestation could have the potential to be conscious and suffer pain and distress during the blood collection and the steps leading up to this, such as the cardiac puncture and the extraction from their mother's body[6]. However, the fetus cannot suffer if the EEG/ECoG has become flat before the procedure and stays flat during the entire process[10]. Steps can be taken to ensure that fetal brain function is severely impaired, resulting in the absence of awareness.

Several safeguards have been proposed by Van der Valk et al.[7] to make sure that the fetus is and stays unaware during the entire FBS collection process. The first safeguard is that after the dam has been slaughtered, the fetal blood collection may not begin until at least 5 minutes have passed. The next precaution is to restrict the fetus from breathing air after the removal of the uterus from the dam. Lastly, if the fetus is exposed to air before the 5-minute waiting time after the slaughter of the dam has passed, or

if the fetus breathes air, it has to be stunned. This can be done by a blow to the head using a blunt instrument or a captive bolt firearm.

The World Organization for Animal Health (OIE), the International Serum Industry Association (ISIA), and the American Veterinary Medical Association all endorse the rule that the fetus must remain inside the uterus for 15-20 minutes after the slaughter of the dam to ensure enough time for the fetus to have asphyxiated and lose consciousness[11]. The European Serum Products Association (ESPA) recommends a longer time of 30 minutes after the slaughter of the dam before the fetus should be removed from the uterus[11].

Even though regulations have been put in place, there is no guarantee that these rules are always applied. Especially since there is an increase in the use of FBS in cell culture, which has led to a major increase in the price for FBS in 2015, from around €80 to €1200 per 500 ml[6]. This can lead to an increase in wrongdoing, misconduct, and fraud, of which there have already been cases reported[12-14].

2.2. Scientific concerns

A major scientific disadvantage of FBS is the batch-to-batch variability[15-18]. The exact composition of FBS is unknown and very inconsistent. It has been estimated that human serum contains around 1800 proteins[19] originating from many different cell types and tissues. Further, it contains a wide variety of around 4000 metabolites[20]. It is likely that FBS contains a similar composition to support the growth of the fetus[1]. Many factors can influence the serum composition, such as the genetic diversity[21], animal diet[21], season[22], geographical location[23-25], and gestational age of the fetal calves[1]. But also whether the cows receive antibiotics or hormones can lead to variations in the serum[1]. These variations in composition and biological activity can cause changes in the effects on cells and compromise the reproducibility of experiments[26]. It can affect cell phenotype[24,27], proliferation[16-18], differentiation[28] and drug sensitivity[29,30].

In addition to the variability, FBS is not a good representation of the fluid that surrounds the cells in tissues. Most cells are in contact with the interstitial fluid that surrounds the organs, as opposed to direct contact with blood[1]. Some of the components that are highly enriched in serum are well regulated in organs[1]. For example, cerebrospinal fluid (CSF) contains a much lower protein concentration than serum[6,31], which eventually led to the first chemically defined culture system for primary neuronal cells[6].

Since serum is an animal-derived product, chances are that they are contaminated with fungi[16], bacteria[16], mycoplasma[32], viruses[25], or prions[33]. Around 10% of unfiltered FBS was shown to be contaminated with fungi and/or bacteria[16], and in 25-40% mycoplasma contamination was detected[32]. Mycoplasmas can be difficult to detect since some batches of contaminated serum may give a negative result when tested[34]. In recent years efforts have been made to prevent contamination by filtration, heat inactivation, and gamma irradiation. However, sterility cannot be guaranteed[21,32].

FBS is still the golden standard in many cell culture applications, even with the above-described disadvantages. It is taught in many cell culture trainings, and since it works for most cell lines, many researchers don't seem to realize the need to change it[6]. Unfortunately, there is no serum-free medium available that works for the majority of cell cultures, i.e. every medium seems cell type-specific, leading to an often time-consuming procedure when the decision is made to eliminate FBS in their culture media.

3. FBS alternatives

The search for alternatives to FBS has become an essential objective in many cell and tissue culture research fields as a consequence of the ethical and scientific complications. There have been many efforts that have

led to the development of different kinds of media, which can be classified by their components. Culture media can be protein-free, serum-free, or xeno-free, which means that they do not contain any products from another species. For human cells, this would mean that a xeno-free medium does not contain any animal-derived products but can still contain human-derived components[35]. For many cell studies, there is even a need for chemically defined (CD) media to eliminate the variability which the use of serum induces[35].

Many other animal-derived products have been described as alternatives, such as earthworm heat-inactivated coelomic fluid (HI-CF), sericin protein, bovine ocular fluid, bovine serum, and fish serum[36]. Although these are often byproducts of industry or can be more ethically sourced than FBS, disadvantages are that they have either low availability, high variability, or a lack of attachment/growth factors[36]. Supplements with the most potential for human *in vitro* research are human-derived products, of which the most promising one is described in more detail below.

3.1. Human platelet lysates (hPL)

Several human blood supplements have been described as an alternative to FBS, such as human serum, umbilical cord blood serum, and human platelet lysate (hPL)[6,35,37]. hPL seems to be the most promising due to higher levels of mitogens when compared to human serum and lower amounts of immunoglobulins and proteins (such as albumin) due to the washing procedure[38].

hPL is acquired from human blood platelet concentrates, usually by freeze/thawing[39,40]. Human donor platelet concentrates have a short shelf life span of maximum five days for transfusion to patients with platelet deficiencies[6]. However, after these five days, they are still suitable for cell culture purposes, making them very accessible. It has been estimated that around 50-60% of human platelet concentrates expire before they can be used for transfusions[6]. Therefore, it would be convenient to use them, so they don't go to waste. Platelet concentrates are obtained from certified blood donation centers and are clinically tested[6]. The high quality needs to be guaranteed since they are typically collected to be used in patients. Pooled buffy coats can be used for the generation of platelet concentrates, or they can be derived from apheresis[41]. Since hPL has not undergone a clotting process, anticoagulants need to be added to the culture media, such as heparin, to prevent gelling[42]. Platelet lysates do not necessarily have to be of human origin, as platelet lysates of bovine blood have also been successful as FBS replacement[43].

In 1980 hPL was already introduced in cell culture media with growth-promoting properties for different cells[44,45]. Many thrombocytic growth factors are supplemented when hPL is added to the culture media[38]. This provides a xeno-free culture system for human cells[38]. hPL supports the growth of several different cell types coming from human, rat, mouse, monkey, Syrian hamster, and Chinese hamster origins[45]. In humans, it has been shown to induce proliferation in adherent kidney cells as well as anchorage-independent cell lines, including Raji lymphoma cells[38]. In addition, it improved the growth of chondrocytes in combination with human serum[44]. hPL has even promoted the expansion of mesenchymal stromal/stem cells (MSCs)[42], and a processed form of hPL demonstrated a similar growth rate and viability to FBS in human corneal keratinocyte cell lines indicating that hPL could be used for corneal graft storage[6,46]. These studies demonstrate the broad applicability of hPL. Further, hPL contains attachment factors such as fibronectin, vitronectin, sCD40L, soluble vascular cell adhesion protein 1 (sVCAM), and soluble intercellular adhesion molecule 1 (sICAM-1), which allow for the attachment of most cell types[40,41,47]. The formation of a platelet lysate gel can also support cell attachment in 2-dimensional (2D) or 3-dimensional (3D) cultures by allowing a clotting process[48].

So, platelet lysates seem to be a good solution to the ethical issues of FBS. The availability is also high, with expired human blood platelet concentrates and animal blood from slaughterhouses, providing an economic benefit. The use of hPL is becoming increasingly more accepted, which led to hPL products becoming

commercially available[6]. However, similar to FBS, there is some variability between individual batches. The composition of hPL can be influenced by age, blood group, gender, platelet counts, and the hPL isolation process[49]. Variability in the isolation process can come from differences in the filtering procedure, addition of heparin or other anticoagulants, and storage typology[37]. Variability between batches can be reduced by pooling different donations. However, this also increases the risk of pathogen contamination[49]. Fortunately, potential contamination can be abolished by using UVA light to inactivate viruses, bacteria, and leukocytes, generating a safe inactivated hPL[50].

3.2. Chemically defined (CD) media

Chemically defined media only contains supplements of known compositions. These supplements, such as growth factors, were often obtained from human or animal origins. However, nowadays, it is possible to produce many proteins with the use of recombinant technologies, making it possible to develop completely xeno-free CD media[37]. The use of CD media can be advantageous since it often increases the selectivity for specific cell lines and types, with the aim to imitate its natural environment as well as possible in a reproducible manner. Practically every cell line has its own media supplement requirements. As a result, it seems unlikely that a universal CD media will be developed. The number of generated cell lines grows rapidly every year, leading to an increase in the need for cell-specific media[51]. Non-specific CD media often does not perform as well as FBS supplemented media which really demonstrates the need to optimize the CD media per cell line/type[52]. This is a disadvantage of using CD media, as it can be time-consuming and costly to optimize the correct conditions. A range of biochemical supplements of high quality are needed, and commercial availability cannot always be guaranteed[6]. The threshold for switching to CD medium can therefore be high, especially when the current FBS-containing medium works well[35]. FBS-free components can be more expensive than FBS itself, which is an inconvenience for many researchers. However, it should be noted that the costs of FBS are rising as the global demand for FBS is increasing[6].

The first synthetic CD medium was developed in 1950 and called Medium 199[53]. It was shortly followed by others such as Eagle's Minimal Essential Medium (MEM)[54], Dulbecco's Modified MEM (DMEM)[55], and Ham's Nutrient Mixture F-12[56]. Medium 199 contains base elements such as electrolytes, glucose, amino acids, vitamins, and trace elements. The other synthetic media are often enriched with certain ingredients or supplemented with other components. For example, DMEM is enriched in amino acids, and Ham's F-12 mixture is supplemented with certain vitamins, inorganic salts, and other components. Ham's F-12 mixture combined with DMEM in a 50:50 (v/v) ratio is the basal medium for most CD serum-free cell culture media[57,58]. This media mixture combines the highly enriched Ham's F-12 components with the high amino acid content of DMEM[6]. The media has to be supplemented with insulin, transferrin, and selenium (ITS)[59] and is often enhanced with the addition of growth factors, trace elements, and hormones. Insulin is the most frequently used hormone in cell culture because it is essential for cellular glucose transport[60]. Transferrin is a protein that is important for the transport of iron into the cells[61]. The last component of ITS is selenium, a trace element that functions as a cofactor of selenium-dependent enzymes that protects against oxidative stress[62]. The supplementation of additional growth factors, trace elements, and hormones are often beneficial for cell-specific growth. Additionally, other supplements which are non-essential for most cells can be added, such as protease inhibitors, shear force protectors, proteins, lipids, antibiotics, and attachment factors[35].

Several serum-free and CD media have been developed for specific cell types (see, for instance, https://fcs-free.org/). But, most cell types need to attach to a specific substrate in order to proliferate in culture dishes. FBS contains a wide variety of attachment factors, and most cell types can bind to these factors *in vitro*[4]. When cells are grown on tissue culture polystyrene (the most common cell culture dish) without FBS, there

is a clear defect in cell attachment to the surface[63-66]. Coating the dishes with a suitable substrate when the cells are grown in a CD media is therefore essential for cell attachment, survival, and proliferation.

3.3. Adaptation to a serum-free culture media

Over the last few years, many components have been identified which are essential for the culture of cells within serum-free culture media. It is important to give the cells enough time to adapt to their new serum-free culture media. Often the change of a serum-containing medium to a serum-free medium is so significant that the cells cannot endure it[67]. Therefore, a gradual adaptation process to sequentially lower serum concentrations should be helpful[35]. It is also good to keep in mind that the cells which you want to adapt should be healthy with viability over 90% and in the logarithmic phase of growth[35].

Many cell lines can be cultured in serum-free media, and the numbers are rapidly growing[35]. When switching to a serum-free medium, it is recommended to check if there is already a medium described for the cell type of interest before a lot of time and money is invested in developing one. Serum-free media formulations can be found through a literature study or by checking an online serum-free media database, such as https://fcs-free.org/[6]. Next, the focus will be on the role of the ECM and cell culture coatings on the development of serum-free culture media.

4. The extracellular matrix (ECM)

The extracellular matrix is a non-cellular structure present in all organs and tissues (Figure 1). It plays important roles in cell signaling and forms the physical environment around cells. It initiates essential biochemical and biomechanical signals required for tissue homeostasis by regulating tissue movements, growth, survival, differentiation, and morphogenesis[68,69]. In addition, the ECM can induce signaling by serving as a source and reservoir of signaling molecules, such as cytokines[68]. Each tissue has a unique composition of the ECM components water, proteins, and polysaccharides. The unique physical characteristics can be observed by fiber size, pore size, stiffness, elasticity, and ligand density[68]. Organizational ECM properties of the lung can thereby vary tremendously from the ECM properties of the bone[69]. The topological, biochemical, and physical compositions of the ECM are very heterogeneous, also within a tissue. Enzymatic and non-enzymatic mechanisms and post-translational modifications are constantly remodeling the ECM, generating a highly dynamic structure[69,70].

The ECM contains two domains: the basement membrane and the interstitial matrix[68,70]. The basement membrane consists mostly of core proteins which are organized into condensed, flat networks of interconnected ECM molecules. These sheet-like structures consist predominantly of collagen IV, laminins, and proteoglycans[68]. The basement membrane surrounds the organs and underlies epithelia. Within the interstitial matrix, collagens, fibronectin, tenascin, elastin, and laminin make up the characteristic fibrous network[68]. Proteoglycans and water support their interstitial spaces.

The ECM has the ability to bind growth factors and interact with cell-surface receptors, thereby inducing signal transduction and regulating gene transcription within cells[69]. This can direct the morphology and physiological functions of tissues. Cells can bind to the ECM by different receptors such as discoidin domain receptors, syndecans, and integrins[69]. The stability, quantity, and distribution of ECM-cell adhesions can vary between tissues, developmental stages, and even neighboring cells[68]. Integrins mediate the most common ECM-cell adhesions and link the ECM to the internal cell cytoskeleton[68,70]. The cells use these adhesions to bind directly to fibrous proteins or glycoproteins in the interstitial matrix or the basement membrane[68]. Integrin-ligand interactions also induce signal transduction pathways in the cells. There is an interrelated relationship between signaling through integrins, signaling through receptors for soluble

ligands, and cell adhesion through integrins. Integrin-ligand interactions result in effects on gene transcription and cell survival, proliferation, and motility[70]. Cells that do not adhere to the ECM through integrins have a deficiency of responding to growth factors, indicating how important adhesion is on cell survival and proliferation[70]. Therefore, in serum-free or CD media, in which no attachment factors are present, it is essential to provide an ECM mimicking surface for the maintenance of cells. Without a suitable culture substrate or coating, the cells will not receive the correct signals for their survival.

Proteoglycans and fibrous proteins, such as collagens, laminins, and fibronectin, are the two main classes of macromolecules that compose the ECM and are described in more detail below.

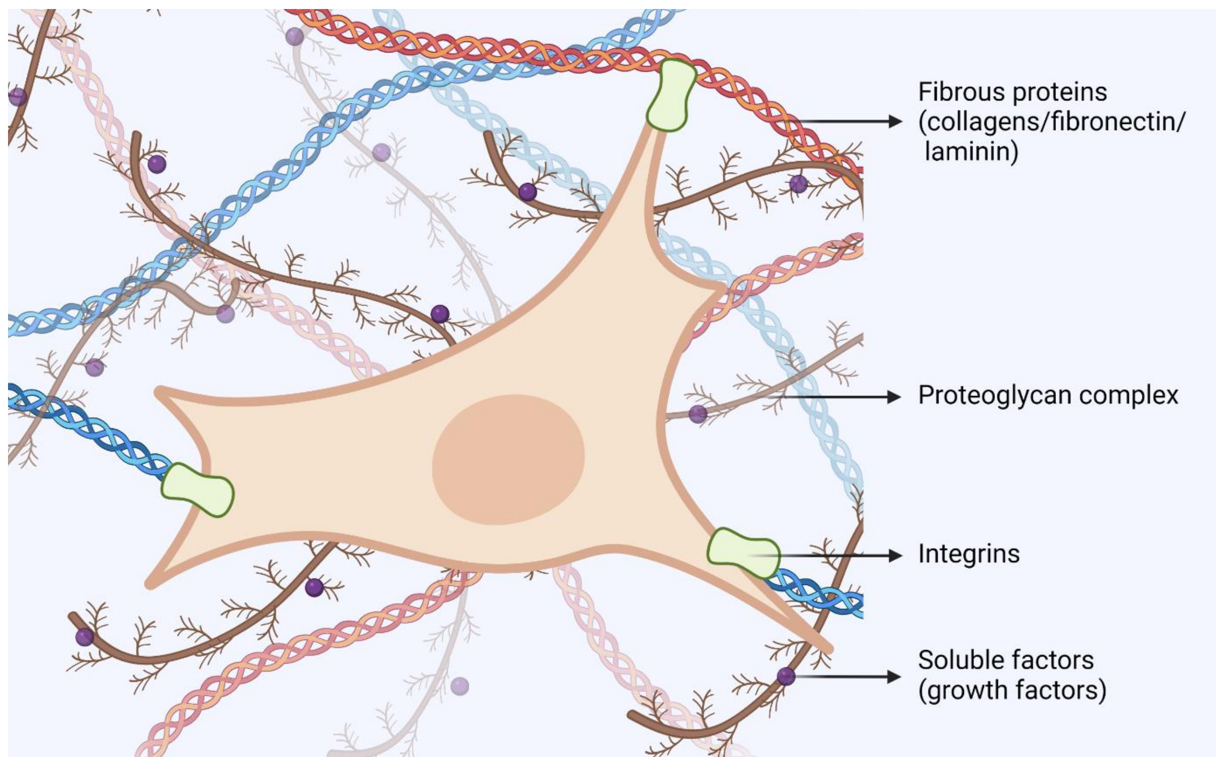

Figure 1: The main components of the extracellular matrix (including fibrous proteins, proteoglycans, and soluble (growth) factors) and the manner by which cells bind to the ECM through integrins. Created with BioRender.com.

4.1. Proteoglycans

Within the ECM, proteoglycans form a hydrated gel inside the extracellular interstitial space of a tissue[69]. It has hydrating, binding, and force-resistant properties but also functions as a buffer. Proteoglycans have a specific protein core that is covalently linked to glycosaminoglycan chains (GAGs) composed of repeating disaccharide units, forming unbranched polysaccharide chains[69]. An important property of proteoglycans is their high hydrophilicity. As a result, they form a hydrogel by adopting highly extended conformations. The hydrogel functions to withstand high compressive forces[69]. Proteoglycans can be classified into three families: small leucine-rich proteoglycans (SLRPs), modular proteoglycans, and cell-surface proteoglycans.

SLRPs can activate various signaling pathways by binding to and activation of receptors such as insulin-like growth factor 1 receptor (IGFIR), epidermal growth factor receptor (EGFR), and low-density lipoprotein-receptor related protein 1 (LRP1)[69,70]. Additionally, SLRPs can bind to and activate transforming growth factor-beta (TGF-β) and regulate inflammatory response reaction[69,70]. Modular proteoglycans are involved in regulating cell adhesion, migration, and proliferation. When modular proteoglycans are present in the basement membrane, they can function both as pro- and anti-angiogenic factors[69]. Cell-surface proteoglycans facilitate ligand interactions with signaling receptors and thereby act as co-receptors[69,70]. Examples of this are glypicans which are tethered to the plasma membrane, and syndecans which have a

small cytoplasmic and a transmembrane domain. It has been shown that these proteins can interact with a variety of growth factors and their receptors to modulate the response to growth-factor-induced signaling[70].

4.2. Collagen

Within the interstitial ECM, collagen is the most common fibrous protein. Collagens provide tensile strength, regulate cell adhesion (by binding to integrins) and migration, and direct tissue development. Twenty-eight different collagens have been identified in humans, most of which form a triple-stranded helix[69]. This helix has the ability to assemble into supramolecular complexes and thereby forms the main structural element of the ECM. Normally within a tissue, a heterogeneous mix of different collagen types are present. However, one type often predominates[69]. Collagen is arranged into fibrils in tissues that have to resist shear, tensile or pressure forces, such as bone, cartilage, tendons, and skin. Only collagen types I, II, III, V, and XI have the ability to self-assemble into fibrils[70]. Other collagens, such as types IV, VIII, and X, form networks, including the basement membrane (predominantly collagen IV)[70]. Additionally, some collagens associate with fibril surfaces, form periodic beaded structures or are transmembrane proteins[70].

4.3. Laminin

Laminin is an essential component of the basement membrane and is composed of three disulfide-linked chains, α, β and γ[70]. Laminin has a variety of homologs per chain. All laminin chains contain small globular domains and common epidermal growth factor-like repeats. These properties are involved in chain polymerization and contain the nidogen-binding site, respectively. Laminin gets linked to collagen type IV by nidogen. In addition, the α chains contain many binding sites for integrins in the C-terminal globular domain[70].

Almost all epithelial, cardiac muscle, smooth muscle, skeletal muscle, nerves, endothelial, neuroretina, and bone marrow cells synthesize laminin[70]. Laminins can aid cells in adhesion, migration, and differentiation, mostly through integrins. The main role of laminin appears to be enhancing the interaction between the extracellular matrix and cells. However, different laminins can induce specific functions in cells. Laminin 1 in epithelial cells induces differentiation, whereas laminin 2 promotes neurite outgrowth in neural cells, and laminin 5 seems to be mostly involved in cell adhesion and migration[70].

4.4. Fibronectin and vitronectin

Fibronectin and vitronectin are glycoproteins that have essential roles in mediating cell attachment and are involved in regulating the organization of the interstitial ECM[69,71]. Several functional domains are present in the proteins, such as the RGD (Arg-Gly-Asp) sequence that is recognized by cell surface integrins[37,71]. When the fibrous protein fibronectin is in its native structure, the cell-binding domains are hidden. They become exposed when the protein binds to a surface with adequate charge and wettability[37]. Fibronectin can be stretched by cellular traction forces and has binding sites to other fibronectin dimers, collagen, heparin, and integrin receptors[69].

4.5. Other ECM proteins

The ECM contains many other proteins, including elastin and Tenascin. Elastin is another important fiber protein and has the ability to associate with collagen. The main function of elastin fibers is to recoil tissues that frequently undergo stretch[69]. Tenascin proteins have the ability to interact with many extracellular matrix proteins. They can bind to cell surface receptors such as integrins, annexin II, a transmembrane chondroitin sulfate proteoglycan, and cell adhesion molecules of the Ig superfamily. Tenascin-cytotactin, a

member of the tenascin family, also interacts with fibronectin and lecticans, which is a family of extracellular chondroitin sulfate proteoglycans[70].

Since many distinct molecules are present in the ECM, it is difficult to reproduce such an environment in an *in vitro* cell culture system. A suitable culture coating should provide appropriate materials to which the cells can bind. For that reason, the natural ECM environment of the cell should be taken into consideration. Usually, not every factor of the natural ECM has to be present in the culture coating, but it is often beneficial to use the most abundant component(s) of the ECM. This is described in more detail below.

5. Cell culture coatings to promote cell growth

As stated above, the ECM plays crucial roles in cellular activities such as adhesion, survival, proliferation, differentiation, and migration. The most commonly used culture dishes contain a tissue culture polystyrene (TCPS) surface. A protein layer quickly absorbs to the TCPS surface when FBS is supplemented to the culture media, and this includes several cell attachment proteins[72,73]. These proteins, such as fibronectin and vitronectin, are important for the initial cell attachment and enable various cell types to produce their own ECM, like fibroblasts, osteoblasts, and chondroblasts[37]. Without the addition of specific cell culture coating in serum-free culture media, this often results in no or impaired cell attachment to TCPS[63-66]. A questionable strategy to culture cells in serum-free conditions is to do a pre-incubation step with FBS[74]. This will provide the cells with the necessary attachment elements. Therefore, alternative options are needed for appropriate cell adhesion in completely xeno- or serum-free conditions. The most promising surface coatings are described below, and an overview is presented in Table 1.

Table 1: Different surface coatings available for serum-free or xeno-free *in vitro* cell culture.

Culture surface coatings	Animal- or human-component-free	Examples of the culture surface coatings	Suitable for specific cell types
Decellularized ECM	No	- Matrigel - Decellularized tissues from humans or animals - Decellularized ECM from cells grown on culture plates	- murine myoblast cell line C2C12[75] - fetal rat brain cells[76] - primary MSCs[77]
ECM proteins	Possible	- Collagens - Laminins - Fibronectin - Vitronectin - Etc.	- Limbal epithelial progenitor cells[78] - Renal cancer 786-O and HKCSC cell lines[79] - human-induced hepatocyte-like cells[80]
ECM peptides	Yes	- RGD peptide - TAT peptide - IKVAV peptide - YIGSR peptide - Laminin E8 peptides - Etc.	- endothelial colony-forming cells[81] - human-induced pluripotent stem cells (hiPSCs)[82] - human embryonic stem cells (hESCs)[82,83]
ECM GAGs	Possible	- Chondroitin sulfates - Heparin/heparin sulfates - Hyaluronan - Keratan sulfate	- Human fibroblast cells[84] - breast cancer MDA-MB-231 and MDA-MB-468 cell lines[85] - Neuronal cells[86]

Hydrogels	Possible	Natural: - Collagens - Gelatin - Fibrin - Etc.	Synthetic: - Polyethylene glycol (PEG) - Polyacrylamide (PAAm) - Polyvinyl alcohol (PVA) - Polylactic acid (PLA) - Etc.	- hiPSCs[87] - hESCs[88] - human umbilical vein endothelial cells (HUVECs)[89] - human adipose tissue-derived stromal cells (hASCs)[89]

5.1. Decellularized ECM

For *in vitro* cell culture, it would be beneficial to mimic the native ECM properties since many ECM molecules, and the collection of soluble factors it contains, have a significant influence on cell behavior. Constructs that are composed of cellular fractions and their corresponding ECM can be constructed from tissues in living bodies or by *in vitro* cell culture.

Matrigel is a commercially available gel that can be used as an *in vitro* basement membrane model. It is made from Engelbreth-Holm-Swarm (EHS) murine sarcoma cells. The tumor tissues within these mice have an abundance of basement membrane components that can be extracted in a gel form, such as laminins, collagen IV, entactin, and the perlecan proteoglycan[90]. The advantage of matrigel is that a wide variety of cells have good survival and proliferation abilities when grown on this substrate[90]. However, matrigel also has some ethical and scientific disadvantages. The preparation of matrigel requires the induction of tumor growth in mice, and subsequently, the mice need to be sacrificed to extract the tumor tissues[91]. This could lead to discomfort or pain in the mice. Additionally, like FBS, matrigel is an ill-defined product with batch-to-batch variabilities[92]. For some cells, ECM derived from normal human tissues is required[93]. Especially since it only recapitulates basement membrane characteristics, matrigel might not be sufficient in providing the correct ECM properties for some cell types.

Another strategy to obtain decellularized ECM components is from human or animal tissues. The cell growth and differentiation capacities of murine myoblast cell line C2C12 were compared on different kinds of cell culture coatings. For cell attachment, decellularized muscle ECM from mouse or rat tissues was used or single protein coatings with fibronectin or collagen I (described further in section 5.2). Interestingly the proliferation of cells grown on the ECM from mouse or rat tissues was slower, but the differentiation potential towards myotubes was increased[75].

When certain cells are grown on culture plates *in vitro*, they start to secrete their own ECM components. In connective tissues, the ECM is largely secreted by fibroblast cells, although many other cell types also have the ability to produce ECM molecules[94]. The secreted ECM on a plate can also be decellularized and used as an ECM substrate. The ECM structure can be maintained in the original dish or can be collected and transferred to different dishes/substrates without losing its characteristics[37].

An example of ECM secreting cells when grown on culture dishes are bovine corneal endothelial cells. This ECM structure was decellularized and used as a culture coating for fetal rat brain cells[76]. A variety of surface coatings such as fibronectin, collagen IV, and pol-L-lysine were capable of binding fetal rat brain cells, but only on the ECM substrate they were able to survive in serum-free culture media. The addition of serum actually showed a deficiency in attachment. In another study, primary MSCs proliferated faster in serum-free media when they were plated on bone marrow ECM than on uncoated dishes[77]. Cells were also able to keep their differentiation potential when cultured in serum-free media on bone marrow (BM)-ECM. The generation of BM-ECM was done by culturing BM-MSCs in culture dishes. Unfortunately, in both studies, culturing the cells in dishes to generate the ECM was done in FBS containing culture media.

The decellularization process can also influence the ECM properties. The use of enzymes such as proteases or trypsin can damage ECM components, and the lysis of cells using detergents can contaminate the ECM

with intracellular components. Therefore, the decellularization protocol based on cold EDTA is recommended. This removes intact cells from the ECM without the use of enzymes or detergents[95].

So, decellularized ECM can, for many cell types, provide an accurate micro environment for cell growth, survival, and differentiation. However, the generation of ECM substrates is often not done under xeno-free or serum-free conditions[75-77]. The use of human or animal-derived tissues for the generation of decellularized EMC can also lead to variability between experiments.

5.2. ECM proteins

Precoating TCPS surfaces with purified adhesive proteins is one of the easiest ways to improve cell attachment in cell culture. Most proteins can be isolated from animal or human plasma, but they can often also be synthesized by recombinant technologies. Different proteins of the basement membrane or interstitial matrix can be used, such as fibronectin, vitronectin, laminin types, collagen types, and gelatin. Which protein should be used is dependent on the cell type. Usually, a protein that is abundant in the natural ECM of the cell works best. When working with stem cells, specific protein coatings can also influence differentiation capacities[96].

Fibronectin and vitronectin are both glycoproteins that contain RGD sequences recognized by cell surface integrins. The chemistry of the surface of the culture plate used to coat with adhesive proteins influences the amount and conformation of the absorbed proteins. Different proteins have the ability to bind to hydrophilic or hydrophobic surfaces[97]. Hydrophilic substrates are more efficient in binding vitronectin from serum than hydrophobic substrates, and the increase in protein attachment also leads to enhanced cell attachment[98]. Pure solutions of fibronectin and vitronectin were actually able to bind both hydrophilic and hydrophobic regions, indicating that the differences in adsorption from serum is a competitive process[99]. However, vitronectin was not able to bind to uncharged hydrophilic surfaces[100]. The cell-binding function of vitronectin does not vary with the adsorption onto hydrophilic or hydrophobic surfaces[101]. Fibronectin, on the other hand, has a deficiency in cell-binding when absorbed onto hydrophobic surfaces[102], caused by a more elongated structure on hydrophilic surfaces in contrast to a more compact structure on hydrophobic surfaces[103]. The elongated form of fibronectin allows for better cell-binding by an increase in accessibility to cell-binding regions such as RGD sequences. So, the chemistry of the culture dish should also be taken into consideration when coating the dish with cell-binding proteins.

Another ECM protein that can be used for cell surface coating is laminin. Limbal epithelial progenitor cells show increased attachment and migration on recombinant laminin-511 and -521 coated plates than on uncoated culture plates in xeno-free conditions[78]. Laminin-511 and -521 coatings also increased cell proliferation compared to the uncoated plates. However, laminin-332 was also capable of increasing cell attachment and migration, but it decreased cell proliferation. This shows how important it is to properly optimize the correct cell culture coatings and look at cellular effects beyond initial cell attachment.

Laminin can also be used to generate more 3D cell culture conditions. *In vitro*, 3D cell cultures mimic *in vivo* tumor characteristics more closely than cells grown in 2D. Therefore, Maliszewska-Olejniczak et al.[79] screened 13 different culture media for the growth of renal cancer cell lines in 3D structures. They show that the 786-O and HKCSC cell lines grow well in 3D structures with specific xeno-free media. Importantly, the addition of laminin-coated plates stabilizes the 3D structures and provides a useful tool for renal cancer biology research, such as drug toxicity screening.

Likewise, other ECM proteins can be used for 3D cultures. In another study[80], microcarrier culture is used to achieve high density, large-scale expansion of human-induced hepatocyte-like cells (hiHep) in a CD media. Different types of microcarriers and basic culture conditions to optimize the process were explored.

They found that fibronectin coating on cytodex 3 microcarriers, which are already gelatin-coated, promotes hiHep attachment and proliferation. These optimizations for hepatocyte serum-free microcarrier culture are an important step forward in the creation of bio-artificial livers. This study also indicates that the use of two or more different proteins as coatings might enhance cell proliferation and attachment when compared to one.

These studies demonstrate the wide applicability of ECM protein coatings to support serum- or xeno-free culture conditions. However, sometimes the complexity of decellularized ECM is necessary for optimal growth or differentiation conditions, which cannot be provided by a single protein surface coating for instance. As described in section 5.1, C2C12 myoblasts differentiated more efficiently on an ECM substrate than on collagen I or fibronectin[75].

5.3. ECM peptides

Chemically modified synthetic surfaces are being used in order to provide a more controlled environment, with higher storage stability and lower batch-to-batch variability. Coating a surface with full-length proteins does not allow for the regulation of accessibility of cell-binding domains[37]. As shortly described before in section 4.4, fibronectin and vitronectin contain RGD peptides. RGD is the most common peptide to bind cells through integrin receptors. Other cell-binding peptides include IKVAV and YIGSR, which are peptides isolated from laminins[104]. Techniques that covalently immobilize cell-binding motifs on a substrate have been developed to increase the accessibility of cell-binding peptides. Additionally, the surface is completely synthetic, eliminating the need for proteins from human or animal origins[37]. This is especially beneficial for cells that are going to be used for clinical applications, such as stem cell treatments.

Soylemez et al.[105] studied cell adhesion properties of different cell lines on RGD and TAT peptide modified gold film coated indium tin oxide (gold/ITO) surfaces. RGD sequences have already been identified with the ability to bind cells. However, this was the first study to look at the cell-binding ability of TAT sequences, which have previously been identified as a cell-penetrating peptide[106]. Using the self-assembled monolayers (SAMs) technique, cysteine-containing RGD and TAT peptides were assembled on the gold/ITO surface. The peptide-bound surfaces were tested for adhesion by several cell lines, such as human cervical carcinoma (HeLa), monkey kidney epithelial (Vero), human glioblastoma (U87-MG), and human immortalized skin keratinocyte (HaCaT). Either TAT or RGD peptides were able to improve adhesion in all cell lines except for HeLa. So, this study showed that cell-binding peptides are able to bind to a variety of cell types and identified a new cell-binding peptide called TAT. However, this study was performed in a standard FBS-containing medium, resulting in the need for additional experiments to check if it holds true in serum-free or xeno-free cultures for all of the used cell types.

In multiple studies, the use of ECM peptides has been shown to work in serum-free conditions. For example, the expansion on human peripheral blood endothelial progenitor cells to obtain endothelial colony-forming cells (ECFCs) has been optimized by using a fluorophore-tagged (TAMRA) RGD peptide linked on polystyrene culture surfaces. RGD-TAMRA surfaces were able to more efficiently promote ECFC adhesion, spreading, and clonal expansion when compared to collagen-coated plates, which were often used to obtain ECFCs, in xeno-free culture conditions[81].

5.4. ECM GAGs

GAGs are large unbranched polysaccharides often linked to proteins to form a proteoglycan. GAGs are involved in a wide variety of extra- and intracellular functions and are able to interact with ECM proteins such as fibronectin and laminin, which are able to promote cell adhesion[107]. For these reasons, GAGs are being investigated as surface coatings and 3D scaffolds for tissue engineering applications[37]. Chondroitin

sulfates (CS) are the most common GAGs found in the human body[108] and are therefore often used as a cell-binding substrate. GAGs are also known to bind various soluble growth factors, with heparin often being the preferred GAG[109]. Linking GAGs to culture surfaces might therefore be useful to bind and concentrate growth factors on the surface, avoiding the need for extra high concentrations of growth factors in the culture media.

Oxidation of the uronic acid component of GAG (aGAG) can be used to immobilize GAGs on amino-functionalized surfaces. Since oxidation changes the molecular structure of GAGs, it was investigated if the bioactivity of GAGs is altered by analyzing the direct interaction of cells with the aGAG-modified surfaces. Human fibroblast cells were able to bind and spread on aGAG surfaces with increased specificity towards sulfated aGAGs, in serum-free medium[84].

GAGs also play important roles in the development of cancer by affecting adhesion, growth, and migration of the cancer cells. The effect of GAG immobilized surfaces on cancer cells was evaluated by the use of two breast cancer cell lines: MDA-MB-231 and MDA-MB-468. The GAGs were chemically thiolated (tGAG) to immobilize them on vinyl-terminated, self-assembled monolayers prepared on glass surfaces. Results showed that tGAG surfaces were able to bind more cells than vinyl-coated glass, and the presence of FBS decreased adhesion abilities (except on heparin-coated surfaces). In addition, this study also shows that GAGs with higher sulfation levels have increased cell adhesion abilities. However, outgrow studies showed that cell migration was enhanced on hyaluronan-coated surfaces, which is a nonsulfated GAG[85].

Proteoglycans containing CS (CSPGs) are inhibitors of neuronal regeneration at scar sites. However, during other physiological stages, some CSPGs are involved in neuronal growth. In the central nervous system, sulfation patterns of CS chains influence their interaction with various growth factors. Thiolated CS GAGs with differentially sulfated CS chains were immobilized on poly-L-lysine coated substrates and investigated for neuronal navigation. Results showed that neurons avoided the SC sulfation variant C but preferred the SC sulfation variants A, B, and E. Biomaterials with immobilized GAG chains could be useful for central nervous system injuries where directional growth of neurons is essential for recovery[86].

5.5. Hydrogels

Cells in their natural environment usually receive signals in all three dimensions, whereas most typical culture systems are in 2D. Culture systems that mimic the natural ECM in a more accurate way can be obtained by hydrogels, which support cell adhesion, mimic elements of native ECMs, and have mechanics similar to soft tissues. A hydrogel consists mostly of water (around 95% of the volume) but exhibits solid-like material properties[110]. Especially for stem cells, hydrogels can provide an accurate environment to support proliferation while maintaining pluripotency[111].

Hydrogels can be formed as 2D films, where cells are placed on top of the substrate, or as a 3D matrix, where cells are encapsulated within the gel. Cells on 2D surfaces are less constrained than in 3D structures and might provide a very suitable environment for epithelial and endothelial cells[112]. 3D hydrogels model the natural environment of many other tissues better, leading to a more realistic cellular response[112]. Nevertheless, 2D hydrogels provide a greater level of control over essential environmental factors such as elasticity and cell adhesion than conventional polystyrene culture surfaces can[112].

Hydrogels are formed by the transition of liquid precursor solutions into a solid substance. To assemble the hydrogel components, they need to be crosslinked, either physically or chemically[112]. Most hydrogels based on proteins or peptides are formed by physical crosslinking through self-assembly, such as collagen I or peptides that can assemble into β-sheets[113]. In addition, different kinds of chemical crosslinking can be used, such as chain-growth polymerizations and step-growth polymerizations[112]. It is important to check

the compatibility of the crosslinking procedure with the cells of interest, keep the polymerization times short and use non-toxic initiators[112].

Hydrogels can be broadly classified into two groups, those which are made of natural or synthetic materials[112]. In the previous chapters, we already described two kinds of natural hydrogels, matrigel and surface coatings of certain ECM proteins. Proteins such as gelatin, fibrin, and certain collagens are able to make stable hydrogels on their own[112]. Other ECM proteins such as laminin and fibronectin can be joined into a larger ECM matrix or chemically modified to form a gel. Natural hydrogels have the advantage that they are often highly biocompatible, but the material costs can be inconvenient[110].

There are many different synthetic polymers that can be used to create hydrogels. They usually have certain characteristics in common such as a polymer backbone, biocompatibility, and the incorporation of functional groups that can be modified to adjust certain features (adhesion to cells or proteins)[112]. Some examples of synthetic hydrogels are those made of polyethylene glycol (PEG), polyacrylamide (PAAm), polyvinyl alcohol (PVA), polylactic acid (PLA), and many more. Synthetic polymers can easily be tuned by controlling crosslinking, are inexpensive, and are very reproducible, which are some very important advantages[110]. However, they lack biological moieties, which can be a disadvantage when mimicking natural ECM environments[110]. To improve functionality, many synthetic hydrogels are optimized by the incorporation of ECM protein or cell-binding peptides (RGD) into the gel[110,112]. Many other factors should also be taken into consideration when selecting a hydrogel, such as stability, biophysical properties (elasticity), and cell-binding capacity[112].

Hydrogels have been especially useful in the culture of stem cells. In a study by Sung et al.[87], multiple xeno-free culture media were tested in combination with the use of several biomaterials to generate human-induced pluripotent stem cells (hiPSCs). They found that cells were able to reprogram into hiPSC at high efficiencies when cultured on laminin-511, -521, and Synthemax II (RGD peptide) coated dishes. Additionally, hydrogels of poly(vinyl alcohol-co-vinyl acetate-co-itaconic acid) (PVI) engrafted with vitronectin oligopeptides with appropriate elasticity were able to reprogram the cells very efficiently into hiPSC.

Another study[88] explored the optimal hydrogel conditions for the xeno-free culture of human embryonic stem cells (hESCs) and hiPSCs. They used poly(vinyl alcohol-co-itaconic acid) (PVA) hydrogels engrafted with oligopeptides of bone sialoprotein, vitronectin, and heparin-binding domains. They also designed several structures of the engrafted oligopeptides in formations of single-chain with or without joint-segment, a dual chain with joint-segment, and a branched-type chain. The hydrogels with dual chain or joint-segment vitronectin-derived oligopeptides with an elasticity of 25kPa supported the long-term culture of both hESCs and hiPSCs while keeping their pluripotency characteristics.

5.6. ScreenMATRIX as a useful tool to find the correct ECM conditions

Recently, Thamm et al.[114] described a new cell growth and adhesion screening tool with 96 combinations of GAGs and biomimetic peptides, called screenMATRIX. The development of such a large-scale screening tool indicates the importance of optimizing the correct ECM mimicking components per cell type. The GAGs or GAG analogs included in the screening tool are dextran sulfate, heparin, chondroitin sulfate, and dermatan sulfate. The dextran sulfate is a GAG-mimetic, whereas the other GAGs are animal-derived. The biomimetic peptides, which are combined with one of the GAGs, are derived from fibronectin, laminin, vitronectin, or collagen. Additionally, RGD, bone-related ECM, proteoglycan, and several other peptides are used. In some conditions, two biomimetic peptides are even combined with a GAG. The matrices are formed

by the interaction of repetitive lysine/alanine peptides, which are conjugated to a four-arm polyethylene glycol (starPEG), with the sulfated GAGs and biomimetic peptides.

In their paper, they describe that 65 surfaces of the screenMATRIX were not able to support the growth of hBM-MSCs in serum-free conditions. The surface coating composed of dextran sulfate and bone-related ECM peptide 1 resulted in the highest growth rate and was therefore chosen to continue with. The subsequent experiments showed that this surface was able to support long-term cell culturing while maintaining typical MSC characteristics, such as differentiation and immunomodulatory capacity, cell morphology, and expression of stemness markers. In another study[115] screenMATRIX was used to determine the best culture substrate for human adipose stem cells. They found that the different GAGs did not influence the adhesion capacity in serum-free medium, but the different peptides did.

ScreenMATRIX is commercially available at denovoMATRIX (https://denovomatrix.com, Germany), but the procedure that was used to coat or link the combinations of GAGs and biomimetic peptides onto culture plates is not described. However, in the Thamm et al. paper, they state that it was based on the noncovalent thin layer coating technology that was previously published[116]. If certain cells of interest grow well on one or multiple coating conditions from the screenMATRIX, then this paper might provide more insights into the recreation of this specific surface. Alternatively, the preferred coating conditions, or a combination of multiple coatings, can be ordered from denovoMATRIX. The advantages of the screenMATRIX are that it is partly xeno-free (depending on which GAG is used) and very easy to use. It has the ability to screen many combinations of cell-binding combinations at once, making it relatively simple to optimize cell-type-specific surfaces. However, information about the exact formulations of the commercially available screenMATRIX is limited, which makes it complicated to recreate.

6. Organ-on-a-chip systems

Recently, serum-free organ systems are being developed to investigate organ interconnectivity and functionality *in vitro*. Organ systems with serum-containing media have been developed before. However, the use of serum and its ill-defined components limits the reliability of the assay. The use of a serum-free organ system can optimize current drug toxicology studies. Unfortunately, it is difficult to maintain the functionality of multiple cell types for longer periods in the same serum-free medium.

A low-cost gravity-driven flow system has been described for the maintenance of human cardiac, skeletal muscle, liver, and neuronal cells within a common serum-free medium[117]. The cell types had distinct origins of primary cells, cell lines, and cells derived from hiPSCs. This multi-organ system uses electrical and mechanical readouts to assess functional changes in the human tissues in response to five well-defined drugs. Cardiac cells were evaluated by measuring cardiac beat frequency, skeletal muscle cells by contractibility, liver cells by albumin and urea production, and neuronal cells by electrophysiology. Additionally, the cell viability of cardiac, skeletal muscle and liver cells was evaluated through an MTT assay, and cell morphology was used to evaluate the viability of neuronal cells. The cell types were cultured in separate chambers while being connected with one or two other compartments through microfluidic channels, allowing the exchange of metabolites and signaling molecules. Results showed that the four different cell types were able to survive and keep their functionality over a culture period of 14 days. Toxicology effects of the five tested drugs correlated well with the effects previously reported in the literature.

The various cell lines were grown in their own culture media and substrates before being transferred to the co-culture system. The human hepatocellular carcinoma HepG2/G3 cell line was used for the liver cell type. They were cultured in an FBS-containing medium, and four days before the transfer to the co-culture, they

were plated on collagen I coated glass coverslips. Cardiac cells were generated from hiPSCs, and seven days before transfer to the co-culture, they were plated on fibronectin-coated SiO_2 surfaces or cantilevers. Human skeletal muscle stem/progenitor cells were differentiated into myofibers over a period of around 22 days. During the entire differentiation process, cells were cultured on N-1(3-(trimethoxysilyl) propyl) diethylenetriamine (DETA) modified coverslips. Neuronal cells were obtained from the differentiation of a human spinal cord stem cell line. The differentiation process was done on poly-D-lysine/fibronectin-coated culture dishes. After differentiation, they were replated onto DETA-coated coverslips. So, every cell line is grown onto specific culture substrates, both before and after the transfer to the co-culture system. The same research group has been optimizing this system further and is able to elongate the culture period to 28 days. This provides evidence that multiple cell types have the ability to survive long-term in a common serum-free media[118].

Another serum-free three organ system has been described containing cardiac, skeletal muscle, and liver cells. In this system, circulating THP-1 immune cells were added to generate an immune system-on-a-chip[119]. In this process, primary hepatocytes were cultured on collagen I, iPSC-derived cardiomyocytes were plated on fibronectin-coated cantilevers, and skeletal muscle myoblasts were cultured on patterned cantilevers. The system had a culture period of 7 days and had the ability to mimic immune responses to tissue-specific damage.

So, different cell types are able to survive for a long period in a common serum-free culture medium. This is a good indication that as long as cells are grown on an appropriate substrate, a more universally applicable serum-free medium can be generated. In addition, the development of these systems is a great example of *in vitro* cell culture models that, in the future, can be used instead of animal models to determine the direct immunological or toxicology effects of biological therapeutics.

7. Discussion

The previous chapters describe the variety of coatings and substrates that have been used to optimize cell adhesion and growth in serum-free or xeno-free cultures. The ECM contains many different molecules, and the combination of components differs between tissues[68,69]. Additionally, within a group of proteins, such as laminins, a different type can induce very distinct cellular responses. For example, laminin 1 induces epithelial cell differentiation, laminin 2 in neural cells promotes neurite outgrowth, and laminin 5 is involved in cell adhesion and migration[70]. This can make the search for a suitable culture surface or coating in serum-free media challenging. It is therefore important to look at the ECM molecules that are abundant in the natural ECM environment of the cell type of interest. Further, the aim of the cell culture system should be taken into consideration. The chosen ECM molecules can be essential to induce, for example, cell growth or differentiation.

Usually, it is complicated to use all the molecules which are present in the natural ECM environment of the cell. It can be done by using decellularized tissues from human or animal origins. The disadvantage of this is high batch-to-batch variabilities, ethical concerns regarding the use of animal products, and the risk of contaminations. The commercially available matrigel is a decellularized ECM product, which originates from EHS murine sarcoma cells and has the ability to support many cell types[90,92,93]. However, matrigel mainly contains components from the basement membrane and might therefore not be able to accurately mimic the properties of interstitial ECM, such as for stromal cells[120]. Alternatively, some cell types can be grown onto culture plates where they eventually start to secrete their own ECM components that subsequently can be decellularized. A disadvantage of this technique is that the cells are typically grown in FBS-containing media[76,77], likely due to the presence of attachment factors which the cells need to bind to the polystyrene

surface. It is possible that after the decellularization process, FBS components still remain in the ECM structure. These components could affect the cells which are grown onto the ECM surface. Additionally, it increases the risk of contamination. Therefore, the use of animal- or human-derived components should be avoided whenever possible. In a study by Chaturvedi et al.[75], it was shown that C2C12 myoblast cells were able to bind, grow and differentiate on etched but not untreated glass in serum-free medium. Etched glass is a surface able to quickly adsorb amino acid polymers and proteins. This probably allows the initial cell-binding before the cells start secreting their own ECM components. Fibronectin and perlecan proteins were already deposited on the substrate 3.5 hours after plating. After 3 days also collagen I and IV were present below the cell layer. This is a good indication that, in some cases, the generation of a xeno-free decellularized ECM structure is possible, but this needs to be explored further.

Fortunately, most cells can also be grown onto culture surfaces that are coated with only one type of ECM molecules. Many ECM molecules can be derived from human or animal origins but can also often be synthesized by recombinant technologies, providing a xeno-free culture coating. This coating has lower batch-to-batch variability than decellularized ECM structures and is often still very biocompatible. Typically, some ECM molecules are abundant in a tissue, and these molecules often make the best surface coating for a specific cell type. To create more control over the accessibility of specific cell-binding domains, culture surfaces can also be linked to synthetic peptides. This creates a surface that has high storage stability and low batch-to-batch variability[37]. Specific cell-binding peptides can be adjusted to create optimal binding activity specific for certain cell types.

For various cells, especially stem cells, it is essential to mimic the natural ECM more accurately. Hydrogels can have elasticities similar to soft tissues and thereby induce mechanical signals in the cells which are not generated by most single ECM protein or peptide coatings[110]. 3D hydrogels model the natural ECM environment of many cells in the most accurate way[112]. However, 2D hydrogels are more easily applicable, and the elasticity provides a suitable environment for most cells. The addition of certain cell-binding peptides can increase the biocompatibility to a greater extend[112]. Hydrogels can be made from a variety of natural and synthetic components[112]. There is no standard hydrogel that is most commonly used in cell culture systems since almost all hydrogels are easily adjustable for specific preferences.

Synthetic ECM peptide surfaces seem to be the most reliable for use in clinical applications, with advantages such as low batch-to-batch variability, minimal risk of contamination, relatively low prices and, high storage stability. Additionally, they contain cell-binding activity and are animal and human component free. ECM peptides can also be incorporated into hydrogels when a specific elasticity is required. Therefore, synthetic ECM peptide linked surfaces or hydrogels are often very suitable for cell culture. Due to the increase in demand, they have become commercially available, for example at Corning® (PureCoat™ ECM Mimetic Cultureware Flasks and Synthemax® Vitronectin Substrate). It can be challenging to find the correct peptides for cell adhesion, screening tools such as sreenMATRIX can aid in this process. However, for some cell types future research is still needed to optimize cell growth on synthetic surfaces in prolonged cultures[121]. When synthetic peptides do not provide accurate ECM properties, then the use of full-length ECM proteins or GAGs are recommended. Many studies have used full-length ECM proteins and GAGs with positive results, also in long-term cultures. Decellularized tissues give the highest batch-to-batch variabilities and are often not human- or animal-component-free. Therefore these should be used as the final option.

It is often thought that the CD media needs to be optimized per cell line to provide the correct culture conditions. However, the organ-on-a-chip systems described above show that a universal serum-free medium is able to support multiple cell types in prolonged cultures of 7, 14, or 28 days[117-119]. The cells are grown onto specific surfaces per cell type. This is a good indication that the exact components of the CD

medium might be less important when the cells are bound to appropriate surfaces. Accurate cell-binding will lead to the induction of essential signals that are needed for cell survival and proliferation. Therefore, the use of an appropriate culture surface is necessary for the enhancement of CD media.

8. Conclusions and recommendations

The use of FBS in cell culture systems is due to ethical as well as scientific reasons undesirable. Harvesting FBS from bovine fetuses can lead to the unnecessary suffering of the fetus[6]. Furthermore, the ill-defined components and batch-to-batch variability of FBS result in variations among cell culture data, within and between research groups[15-18]. Therefore, especially in clinical applications, which often use stem cell cultures, there is an increase in demand for serum-free culture media.

The elimination of FBS in cell and tissue culture should be encouraged among researchers whenever possible. In the last couple of years, a wide collection of commercially available serum-free and/or xeno-free culture media has been generated. Many substitutes for FBS have been described, with hPL being the most promising alternative for human cultures. It has the capacity to support many different cell types, just like FBS[45]. Unfortunately, hPL is also an ill-defined product with batch-to-batch variabilities[49]. For that reason, CD media is often needed, depending on the aim of the cell culture experiment. No universal CD medium is available yet. In fact, it has to be optimized for every cell line.

Before a CD medium is optimized for a specific cell line, it is recommended to use the fcs-free.org database to enquire if a CD medium is already described, which is suitable for the cell line of interest. Apart from an appropriate CD medium, the use of a suitable culture substrate is essential. A correct cell culture surface is suitable for cell attachment and is often able to bind growth factors, promoting proliferation. Generally, a substrate that closely mimics one or multiple components in the natural ECM environment of the cell type of interest works the most efficiently.

Cell culture surfaces can be coated with decellularized ECM from human or animal tissues, or ECM-derived proteins, such as fibronectin or collagen. Alternatively, specific cell-binding peptides or GAGs can be functionalized onto hydrogels or the cell culture plate. Each of these cell culture substrates has its own advantages and disadvantages. Some are very easily applicable (coating with an ECM-derived protein), whereas others might need some more preparation time (decellularized ECM). Further, synthetic coatings often have very low variability between batches, whereas ECM proteins might reproduce a natural environment more efficiently. These considerations should be taken into account when choosing an appropriate cell culture surface. Additionally, it is recommended to use a screening tool such as screenMATRIX when the culture surface still needs to be optimized. This provides a quick and easy way to compare 96 different surface coatings to find the best possible option for your cell type of interest. To eliminate variations between experiments as much as possible, it is recommended to use synthetic surfaces since they contain the lowest batch-to-batch variabilities.

Even though many studies still use serum or other animal-derived components in their culture conditions, a variety of different experimental systems have been developed for the optimization of serum-free cultures. Good examples are the organ-on-a-chip systems described in section 6. They showed that a common serum-free medium is able to support multiple cell types for a long time when cultured onto appropriate surfaces. So, the use of an accurate ECM mimicking surface coating leads to the enhancement of CD media and thereby improves cell culture techniques, with fewer ethical and scientific concerns.

9. References

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