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An Overview of Tissue Engineering as an Alternative for Toxicity Assessment

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Abstract - Tissue engineering is a multidisciplinary field that combines aspects of biology, material sciences, engineering and medicine - the ultimate goal being able to fabricate replacement tissues and/or organs for an ageing population. However, parallel to this milestone, is the exploitation of the biomimetic constructs as feasible alternatives to *in vivo/ex vivo* toxicity testing models due to their accurate representation of innate tissue and organs. Herein, we summarise a range of concepts within tissue engineering with a particular emphasis on biological material selection and implications to animal testing.

INTRODUCTION

Tissue engineering and scaffolds

Tissue engineering, a term proposed at a National Science Foundation workshop in 1988, described the subject area as "an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue function"[1]. In light of this, many consider the repair or replacement of diseased and damaged tissues/organs - being able to create autologous engineered transplant material and/or procure direct tissue replacements on demand - as the "gold standard" of tissue engineering. Yet, an important parallel aspect of the applied technology is the ability to develop accurate representation of tissues and organs that can be used for drug development and toxicology studies[2]. Accordingly, the field has now been defined as "the application of biological, chemical, and engineering principles towards the repair, restoration, or regeneration of living tissues using biomaterials, cells and factors alone or, in combination"[3] which accurately summarises the close interaction between these distinct academic subjects, as well as the multi-faceted relationship between the physical and biological fundamentals (Figure 1).

One of the key defining parameters for tissue engineering is the ability to control or direct the growth, differentiation, and behaviour of a specific cell population by modulating its culture substrate - a crucial factor that needs to be considered especially for stem cells due to their pluripotent nature. These scaffolds (also known as matrices, biological constructs, framework) are considered to be the most important element within tissue engineering strategies because they not only provide the mechano-architectural framework, at the micro- and macro-scale, required to generate the biological "mass" of a tissue or rudimentary organ, but they also allow the appropriate cell signalling and biological pathways, via extra-cellular matrix (ECM) and cell-to-cell interactions, to occur during culture and growth. In addition, they must also provide an appropriate environment whereby the cells are able to maintain the correct phenotype and synthesise or express the required proteins, growth factors, and molecules for that specific tissue function[4].

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function[1]. These biologically suitable substitutes for organs and tissues can be used for pharmaceutical, diagnostic, or research purposes. However, the eventual goal is to create autologous, engineered transplant material that can be used to replace tissues that have been damaged by disease or injury[2].

MATERIALS AS SCAFFOLDS

The selection of the most appropriate material for

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tissue engineering applications is frequently seen as the most challenging aspect of the field due to the intimate relationship between a cell and its culture substrate. Several parameters have been identified as being crucial for selecting a suitable scaffold including:

1. Biocompatibility: the material must not impact negatively on the cells that are to be cultured on it, i.e. it will allow the attachment, spreading, proliferation, differentiation, and/or migration of the cells (within the correct phenotype).
2. Appropriate characteristics: the material must possess the correct mechanical and physical characteristics in its native and three-dimensional (3D) form. It should also be easy to generate, manufacture, and manipulate. The cost of the material, either as a direct cost or indirect cost - via addition modification - is also often considered.
3. Toxicity: the material, including any breakdown products, must neither be harmful nor elicit an immune response.
4. Ethics: the material should ideally be derived from an ethically-derived source where appropriate, e.g. non-animal based, "green technology", non-human organ harvested.

As such, the scaffold-based materials used for tissue engineering applications[5] and associated cell-based screening models, are often identified as synthetic or (natural) biological materials. Synthetic materials have been obvious candidates for these applications due to their relative ease of procurement, as well as the natural ability for manipulation and control of their chemical and physical characteristics to suit a specific application. In contrast, biological materials - being naturally compatible - allow the appropriate cell-to-interface responses to occur which ultimately result in the correct cell or biological characteristics and behaviour to be expressed[6]. A large number of natural scaffolds currently in use employ biopolymers that can be found in existing ECMs. Examples include protein-based materials (e.g. fibrin, collagen, and gelatin) and polysaccharide-based materials (e.g. alginate, chitosan, glycosaminoglycans, hyaluronic acid, and methacrylate)[7-10]. Thus far, several cancer models that utilise synthetic scaffolds have been created[11], however only a small number of 3D models use natural materials[12, 13]. Curiously, a multitude of biopolymers have thermoresponsive solubility behaviour, opening up opportunities to establish systems that dissolve or gel at body temperature[14]. A summary of the range of current materials used for tissue engineering is shown in Table 1.

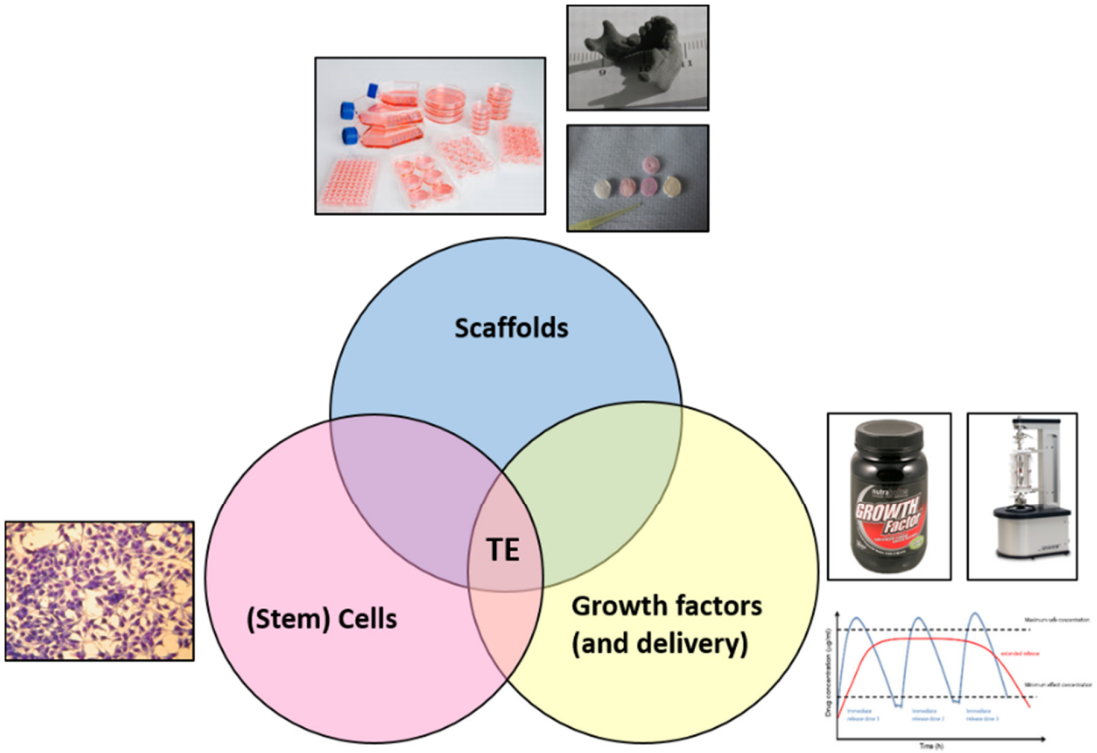


Figure 1. Schematic summarising the relationship and overlap between distinct subject areas within tissue engineering (TE).

2D vs 3D scaffolds

The advancement of novel biomaterials for *in vitro* cell culture in three-dimensional (3D) environments has become more prevalent in recent years[16-21]. The catalyst for this advancement is to overcome the limitations of current two-dimensional (2D) cell culture practices. 2D cell culture is ubiquitously employed to study a vast array of biological processes, despite clear structural and mechanical differences compared to the *in vivo* environment. *In vivo*, cells are situated within a complex extracellular matrix (ECM). The physical and biochemical properties of the ECM have a significant impact on a multitude of critical physiological and pathological processes[22].

In order to suitably recreate the *in vivo* environment in a controlled *in vitro* setting, the mechanical and chemical inputs have to be carefully modified, as they affect the ability of the cells to grow, proliferate, differentiate, and mature. Scaffolds help mimic the physical *in vivo* environment, allowing the cells to grow with appropriate morphologies. The scaffolds enable cell attachment and migration, retention, and presentation of biochemical factors. As well as providing mechanical support, and allowing the diffusion of nutrients, oxygen, and waste[2]. Significant differences of the biology and morphology between cells grown on 2D and 3D environments has been observed[23, 24]. For example, cancer cells cultured *in vitro* lose many of their *in vivo* features, due to a lack of environmental signals[16], caused by the deprivation of the tissue matrix that regulates tumour progression. This results in cell phenotype and gene expression changes. Ultimately, important aspects of tumour biology (most importantly angiogenesis and metastasis) cannot be properly assessed in monolayer culture[25-28].

In contrast, cancer cells cultured in a 3D scaffold exhibit decreased proliferation rates and an increased chemotherapy resistance, compared to cells grown in 2D monolayers, appearing to revert back to the original tumour phenotype[29].

The need for accurate drug screening models

Within the drug discovery process, traditional *in vitro*-based studies rely on the culture of cells as a monolayer on standard tissue culture plastic; often referred to as two-dimensional (2D) cell culture. However, this pseudo microenvironment does not accurately mimic the native conditions experienced by a cell *in vivo*, i.e. a 3D growth environment. Accordingly, using an inadequate and flawed experimental setup can lead to erroneous decisions during drug development - resulting in (expensive) false-positive therapeutic drugs being identified, progressed, and/or approved during the toxicity screening process. It has been reported that 3.5% of drugs, approved from 1980-2009 in the US, were withdrawn from the market due to safety concerns[30]. As such, the ability to accurately mimic the real environment experienced by specific organs, tumours, or localised tissue would be considered invaluable during the early stages within the drug discovery pipeline.

Drug testing: 2D versus 3D cell culture

Prior to any *in vivo* studies being performed, preliminary work is often carried out using simplified *in vitro* tests - the use of an appropriate cell line in tissue culture plastics such as culture-flasks, petri-dishes or cover slips; more than 70% of cancer and molecular biologists still rely on this technique before progressing to testing in animals[31]. However, it is now known that cells grown in a 2D environment lack the required 3D tissue architecture and cell-to-cell interactions experienced *in vivo*[32]. In addition, cells cultured on synthetic plastics and/or non-biological substrates have been reported to respond differently, as well as not being able to elicit/express the correct biological behaviour or phenotype appropriate to its characteristic[33, 34]. A summary of the differences experienced by cells when cultured in a 2D versus a 3D environment is shown in Table 2.

Therefore, a number of 3D methods have been accordingly developed over the years to take into consideration the spatial organisation of a cell within it microenvironment, in an attempt to address the missing link between monolayer cell culture and *in vivo* animal use (see reviews by Breslin and O'Driscoll, 2013[36]; Knight and Przyborski, 2015[37]; Baker and Chen, 2012[23]; Haycock, 2011[19]). A list of 3D models currently in use for drug toxicity screening is shown in Table 3.

General problems with scaffolds

The lack of vascularisation is the major limitation of most 3D cell culture models, causing restrictions in the diffusion of nutrients and the supply of oxygen. This limitation reduces the size of engineered tissues, as well as cell viability and function[39]. Artificial 3D matrix systems that can mimic the ECM have materialised as potential strategies towards creating cell culture systems that are more realistic.

The creation of optimised predictive cell model systems are needed in pre-clinical drug

discovery to improve the current 10% success rate in clinical drug testing[40].

Additionally, optimised 3D cell culture systems are required for tissue engineering[41], transplantation[41], and human stem cell biology (e.g. induced pluripotent stem cells)[42].

Biomaterials as scaffolds

Both synthetic and naturally derived materials are currently used to make scaffolds for tissue engineering applications[5]. However, the development and use of these 3D biocompatible scaffolds must overcome several challenges. The biomaterials used must be biodegradable, non-immunogenic, biocompatible, allow for surface modifications, and ultimately be cost effective[5]. Importantly, the procurement of biological scaffolds is often associated with human and/or animal derived sources which is disadvantageous due batch-to-batch variation, limited donor availability, and ethics. A large number of natural scaffolds currently in use employ biopolymers that can be found in existing ECMs. Examples include protein-based materials (e.g. fibrin, collagen, and gelatin) and polysaccharide-based materials (e.g. alginate, chitosan, glycosaminoglycans, hyaluronic acid, and methacrylate)[7-10]. Thus far, several cancer models that utilise synthetic scaffolds have been created[11], however only a small number of 3D models use natural materials[12, 13]. Curiously, a multitude of biopolymers have thermoresponsive solubility behaviour, opening up opportunities to establish systems that dissolve or gel at body temperature[14].

Artificial scaffolds vs Decellularised scaffolds

Two main methods of 3D cell scaffold production have been developed. The first method uses artificial scaffolds that have been synthesised from (bio)polymers. These artificially created scaffolds allow for phenomenal control over the material, enabling the fine-tuning of the various structural and biochemical properties of the scaffold[43-47]. The second method, decellularisation, has been used to produce natural 3D scaffolds from existing tissue[45-51]. The decellularisation process uses a range of reagents to lyse the cells and then remove them from the ECM of the tissue sample[52, 53]. Although this method lacks the precise control over the scaffold's structural and biochemical properties, the outcome is an easily obtained, naturally derived scaffold that has been used repeatedly in the creation of functional organs[49, 50, 54-56].

Cross-linking and growth factors

Cross-linking is the formation of chemical links between molecular chains in order to form a three-dimensional network of connected molecules[57].

Table 2. Cellular characteristics of cells cultured in a 2D environment compared to a 3D environment (adapted from Edmondson *et al.*, 2014[35])

Cell characteristic	2D culture environment	3D culture environment
Cell cycle	Majority of cells within same stage of cell cycle due to uniform exposure to stimuli	Spheroids containing a mixture of cells at different stages: proliferating, quiescent, hypoxic and necrotic populations
Exposure to medium and/or drugs	Cells experience uniform exposure to medium and/or drugs	Mass transfer and localised gradients limitations for nutrients, growth factors and/or drugs. Prone to penetration issues and occurrence of necrotic core
Gene/protein expression	Often display differential gene and protein expression levels compared to animal models	Often display similar gene and protein expression levels compared to animal models
Morphology	Attached and spread cells as a single monolayer: stretched sheet-like characteristics	Natural shape as aggregated or spheroidal structure
Proliferation	Often faster than *in vivo*	May proliferate differently compared to 2D culture system depending on cell line and/or 3D system
Sensitivity	Frequently succumb to treatment and drugs appear to be very effective	More resistant than that of 2D culture and therefore better predictor of *in vivo* drug responses

It is used in tissue engineering to improve the mechanical properties of biomaterial scaffolds[58]. Cross-linking agents, such as glutaraldehyde, have been used to reduce the rate of degradation[2].
Biological growth factors are commonly used as they encourage the infiltration of cells into the 3D scaffold, and also the differentiation into the specific cell and tissue type[59]. Commonly used growth factors include vascular endothelial growth factor (VEGF)[60], bone morphogenetic proteins (BMPs)[61], basic fibroblast growth factor (bFGF or FGF-2)[62], and transforming growth factor-β (TGF-β)[63].

Artificial scaffold design

The most commonly used fibre structures created for the biomedical industry are knitted, braided, woven, and non-woven[64].

- *Knitted structures* usually involve the largest number of individual fibres, resulting in greater intricacy and performance capabilities. They are often used when applications will undergo lots of stresses and stretching[64].
- *Braided structures* result from the intertwining of three or more fibre strands. This allows for the creation of flat or hollow structures, which have high tensile strength but without a large surface area[64].
- *Woven structures* can produce a wide range of different weaves. These structures are dense but light, do not stretch, and are capable of retaining their shape, making them ideal for supportive functions[64].
- *Non-woven structures* are assembled from fibres into complex 3D architectures. These structures largely comprise through-pores, rather than the blind or closed pores that can be found in other types of porous scaffold (Figure 2). Non-woven structures provide a much larger surface area than almost all biomedical textiles, and are commonly used as scaffolds. This is because they can be customised through layer thickness, specific spacing, and material integrity to encourage cell growth. Previous research has attempted to change the porosity in scaffolds produced by electrospinning[65-67], but this has been found to reduce the mechanical strength[68].

A major constraint of non-woven scaffold fabrication has been the problem of how to precisely control the pore size, as well as how to create distinct internal channels within said scaffold[69]. Formerly, the channelling of porous scaffolds with the aim of improving cell penetration and the exchange of nutrients and gases has been mainly limited to nonfibrous scaffold assemblies, such as foam, sponge, and hydrogel scaffolds[70-72].

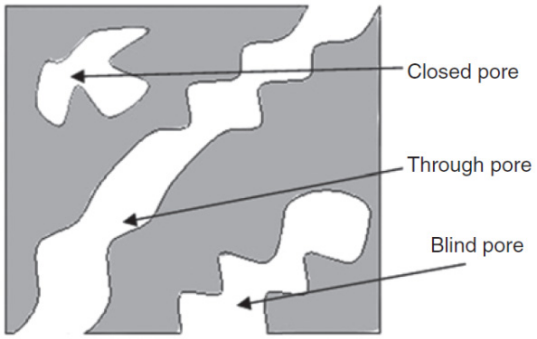


Figure 2: Different pore configurations[73]

Hydrogels

Hydrogels are water-swollen, cross-linked polymer networks[74]. This network of interconnected pores allows for the retention of high water content, as well as the efficient transport of nutrients, oxygen, and waste products[75]. These properties make hydrogels an auspicious class of materials for 3D cell culture[44, 75-80], and so far, both synthetic and natural source hydrogels have been used in cell culture[81].

Polysaccharides

A multitude of different types of polysaccharides have been used as scaffolds for tissue engineering, however they usually require a separate cross-linking step to actually form the hydrogel network[82].

Chondroitin Sulfate

Chondroitin sulfate (CS) (Figure 3) is a glycosaminoglycan (GAG) comprising alternating disaccharide units of N-acetyl-D-galactosamine and D-glucuronic acid[83]. CS boasts excellent biological characteristics[14], although because it is readily water-soluble, chemical cross-linking is required for *in vitro* or *in vivo* use. Most commonly used is a mixture of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS)[84]. Unfortunately, cross-linking using EDC frequently resulted in some collapse of the matrix in aqueous media, although this could be prevented to a certain extent by cross-linking in the presence of ethanol[85].

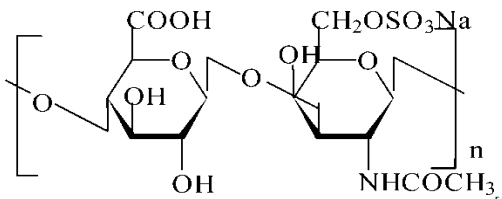


Figure 3. Chemical structure of chondroitin sulfate[14]

CS-based hydrogels have found widespread use in tissue engineering. Gelatin and CS comprised hydrogels have been used to create controlled release systems for antibacterial proteins[86]. Change *et al.* used gelatin-CS-hyaluronan tricopolymer scaffolds to mimic natural cartilage[87, 88]. Bilayer gelatin-CS-hyaluronan biomatrices have been investigated for use in treating wounds, with the results demonstrating that the skin substitute promoted the wound healing process and assisted in the regeneration of full-thickness skin defects[89, 90]. An overview of the biomedical applications of chondroitin sulfate is given in Table 4.

Hyaluronic Acid

Hyaluronic acid (also called hyaluronan, hyaluronate, HA) (Figure 4) is the only non-sulfated GAG, comprising alternating disaccharide units of D-glucuronic acid and D-N-acetylglucosamine, that are linked together by alternating β-1,4 and β-1,3 glycosidic bonds[91, 92]. HA is one of the chief components of the extracellular matrix in the skin, cartilage, and the vitreous humour[93, 94].

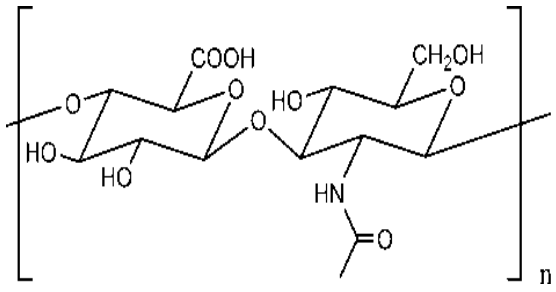


Figure 4. Chemical structure of hyaluronic acid[14]

HA has been combined with alginate[95], poly-L-lysine[96-98], and acrylate-functionalised PEG to create different scaffolds for a range of tissue engineering applications, including nerve regeneration[99] and spinal cord repair[100]. Unfortunately, the mechanical properties of the previously mentioned HA scaffolds were insufficient for use in hard tissue engineering (such as cartilage repair). So a number of research groups have looked at developing HA-based composites that incorporate synthetic polymers such as poly lactic-glycolic acid (PLGA)[101] and poly(propylene fumarate)[102]. Aside from porous HA scaffolds, GAG-based microbeads and nanofibres have been developed using phase separation and electrospinning, respectively[14]. Additionally, HA and stem cells have been combined to serve as injectable material for tissue augmentation[103]. An overview of the biomedical applications of hyaluronic acid is given in Table 5.

Chitosan

Chitosan (Figure 5) is the partially deacetylated derivative of chitin. This linear polysaccharide comprises randomly distributed β-(1-4)-linked N-acetyl-β-d-glucosamine and D-glucosamine[104], and is primarily obtained from the shells of crustaceans.

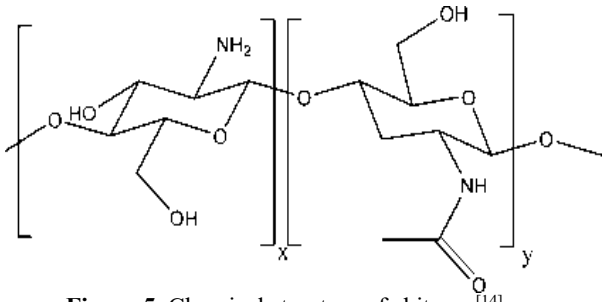


Figure 5. Chemical structure of chitosan[14]

Chitosan microspheres have been created by the addition of chitosan solution droplets into a solution of sodium tripolyphosphate[105], with the goal of developing a chitosan matrix that can be used for bone tissue engineering. Chitosan scaffolds have been successfully used in tendon tissue engineering[106], additionally, scaffolds containing elongated channels have demonstrated the potential for use in nerve regeneration[107]. Different techniques that are used to create porous scaffolds include supercritical fluid technology[108, 109] and stereolithography[110, 111]. Chitosan-based scaffolds and nanofibres have been used in bone regeneration, either utilising plain chitosan[112, 113] or by combining it with synthetic polymers such as poly(L-lactic acid)[114], poly(butylene-succinate)[115], or with ceramics such as hydroxyapatite[116-127]. ECM constituents, such as collagen, are frequently incorporated into chitosan-based scaffolds when being used for tissue regeneration[128, 129]. As well as combining collagen[130, 131] and its derivatives[120, 132-134] with chitosan, synthetic polymers such as poly(ethylene glycol)[135, 136] and Pluronics®[137, 138] have also been used. Additionally, injectable chitosan-based hydrogels have been investigated for their use in tissue engineering[139, 140]. An overview of the biomedical applications of chitosan is given in Table 6.

Table 4. Overview of Biomedical Applications of Chondroitin Sulfate[14]

Type of chondroitin sulfate	Application
gelatin/chondroitin-6-sulfate/hyaluronan, methacrylate- and aldehyde-modified chondroitin sulfate, chondroitin sulfate/chitosan/dermatan sulfate, poly(L-lactide)-*g*-chondroitin sulfate, poly(ethylene glycol)/chondroitin sulfate	Cartilage
EDC cross-linked chondroitin sulfate/collagen/elastin, EDC cross-linked chondroitin sulfate/collagen, thiolated chondroitin sulfate/hyaluronan/gelatin	General tissue engineering application
chondroitin sulfate/collagen	Heart
gelatin/chondroitin-6-sulfate/hyaluronan, glutaraldehyde cross-linked gelatin/chondroitin-6-sulfate	Intervertebral disc
chondroitin sulfate/heparin/collagen	Liver
chondroitin sulfate/collagen	Lung
EDC cross-linked chondroitin-6-sulfate/gelatin/hyaluronan	Skin
chitosan/chondroitin sulfate, chondroitin sulfate/Pluronic F127 nanogel, chondroitin sulfate spheres	Drug release

Table 5. Overview of Biomedical Applications of Hyaluronic Acid[14]

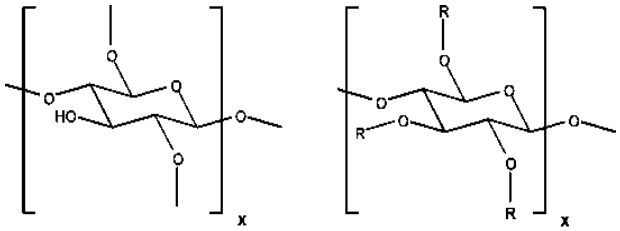
Type of hyaluronic acid	Application
ester-containing hyaluronic acid	Adipose tissue
amine/aldehyde-containing hyaluronic acid, hyaluronic acid/poly(vinyl alcohol), MMP-sensitive hyaluronic acid	Bone
hyaluronic acid/collagen I, hyaluronan/gelatin/chondroitin-6-sulfate, adipic dihydrazide-modified collagen/hyaluronic acid, fibrin/hyaluronic acid, chitosan/hyaluronic acid, carrageenan/fibrin/hyaluronic acid	Cartilage
thiolated hyaluronan/poly(ethylene glycol) diacrylate, hyaluronic acid/gelatin gradient, poly(N-isopropylacrylamide)/hyaluronic acid, hyaluronic acid/pendant L-benzoyl-cysteine, methacrylated hyaluronic acid, collagen/hyaluronan/chitosan, collagen/hyaluronic acid, silk fibroin/hyaluronan	General
acryl-modified hyaluronic acid/poly(ethylene glycol) acryl	Gene therapy
ester-containing hyaluronan/butyric and retinoic acid, methacrylated hyaluronan, divinyl sulfone cross-linked hyaluronan	Heart
benzyl esters of hyaluronic acid, hyaluronan/gelatin/chondroitin-6-sulfate	Intervertebral disc
benzyl esters of hyaluronic acid hyaluronic acid	Liver Muscle
photo-cross-linked hyaluronic acid, collagen/hyaluronic acid, fibroin/hyaluronic acid, antibody-modified hyaluronic acid, hyaluronic acid/polylysine	Nerve
hyaluronic acid derivatives, carbodiimide-cross-linked hyaluronic acid	Ophthalmology
benzyl esters of hyaluronic acid, hyaluronan-gelatin, EDC cross-linked hyaluronan/chondroitin-6-sulfate/gelatin, adipic dihydrazide derivatives of hyaluronic acid/PEG-propiondialdehyde, hyaluronic acid/chitosan/gelatin	Skin
thiol-modified hyaluronic acid	Spinal cord
methacrylated hyaluronic acid	Vascular tissue

Table 6. Overview of Biomedical Applications of Chitosan[14]

Type of chitosan	Application
glutaraldehyde-cross-linked collagen/chitosan	Adipose tissue
freeze-dried chitosan/gelatin, electrospun collagen/chitosan nanofibre	Blood vessel
sintered chitosan microspheres, poly(ε-caprolactone)/poly(vinyl alcohol)/chitosan, chitosan/fibroin/hydroxyapatite, β-TCP/chitosan, β-FGF-loaded hydroxyapatite/chitosan, polycaprolactone/chitosan, chitosan/alginate multilayer scaffold, chitosan/gelatin, titania/chitosan composite, photo-cross-linkable chitosan, chitosan/collagen, ceramic nanoparticles/chitosan, chitosan/polyethylene glycol dimethacrylate/*N,N*-dimethylacrylamide, silk/chitosan, nanohydroxyapatite/chitosan/carboxymethyl cellulose	Bone
chitosan beads, EDC-cross-linked collagen/chitosan/GAG, chitosan/poly(butylene succinate), CS/dermatan sulfate/chitosan, chitosan/hyaluronic acid, chitosan/polyester-based, insulin-loaded chitosan, chitosan/gelatin, alginate/chitosan, chitosan/gelatin/hyaluronan, chitosan/Pluronic, polyethylene oxide/chitosan, glutaraldehyde/oxidised dextran/chitosan	Cartilage
hydroxypropyl chitosan/gelatin	Corneal stroma
chitosan/starch, hydroxyapatite/chitosan, chitosan/soy protein/TEOS, collagen/hyaluronan/chitosan, genipin-cross-linked chitosan, thiolated chitosan, electrosprayed chitosan microbeads, chitosan/poly(vinyl alcohol), poly(caprolactone)/chitosan, chitosan/collagen, nanofibrous PLLA/chitosan fibres, disulfide cross-linked chitosan, chitosan/poly-L-lysine, chitosan/gelatin, chitosan-*graft*-β-cyclodextrin, calcium phosphate/chitosan, carboxymethyl chitosan-*graft*-D-glucuronic acid, chitosan/PEG/gelatin, chitosan-*g*-lactic acid, chitosan/phospholipid	General tissue engineering applications
chitosan/glycerophosphate, chitosan/glycerophosphate/hydroxyethyl cellulose	Intervertebral disc
collagen/chitosan, silk fibroin/chitosan/heparin, chitosan/gelatin	Liver
alginate dialdehyde cross-linked chitosan/calcium polyphosphate	Meniscus
poly(lysine)-functionalised chitosan, polypyrrole/chitosan, PLGA/chitosan/HA, chitosan/polyglycolic acid	Nerve
chitosan/gelatin/glycerol phosphate	Nucleus pulposus
DTBP-cross-linked chitosan, gold colloid/chitosan, collagen/chitosan, bFGF/chitosan, β-glycerol phosphate/collagen/chitosan	Skin
chitosan-based hyaluronan, chitosan microchannel	Tendon

Cellulose Derivatives

Cellulose (Figure 6) is an organic polysaccharide comprising D-glucose subunits linked together by β(1–4) glycosidic bonds[141]. It is also the major structural component of plant cell walls. Unlike starch and glycogen, minimal nutritional benefit can be obtained from cellulose because the glycosidic bonds can only be digested by the enzyme cellulase[5].



R= -CH_3 of -$CH_2CH(CH_3)OH$ of -H

Figure 6. Chemical structure of methyl cellulose (left) and (hydroxypropyl)methyl cellulose (right)[14]

Native plant derived nanofibrillar cellulose has been used in hepatocyte 3D cell culture[81], but it is not commonly used in the field of tissue engineering. This is possibly due to the difficulty of isolating the nanofibres from the plant cell walls[142-144]. Alternatively, synthetically produced cellulose scaffolds have seen use in a wide variety of 3D mammalian cell culture[17, 145-148], including being combined with hydroxyapatite for use in bone tissue engineering[122, 149-152]. Cellulose is also frequently combined with proteins[153], polysaccharides[154], or both[122, 155].

Bacterial cellulose has been used for a variety of tissue engineering purposes[149, 156-158], including hard tissue such as bone and cartilage[78, 159], because it has been reported that bacterial cellulose supports the proliferation of mammalian cells[160-162]. Unfortunately, bacterial cellulose cannot be enzymatically degraded *in vivo*, resulting in the necessary production of modified bacterial cellulose[163, 164]. At the present time it is premature to speculate on the suitability of bacterial cellulose as implantable scaffolds, because there are unknowns about their *in vivo* biocompatibility. The immunogenicity and abiding stability of implanted cellulose-based biomaterials is still under investigation[156, 160]. An overview of the biomedical applications of cellulose derivatives is given in Table 7.

Alginate

Alginate is a polysaccharide, comprising units of *β*-D-mannuronic acid and α-L-guluronic acid[165], that is found in the cell walls of brown algae. Alginate has shown its usefulness for tissue engineering as it is mucoadhesive, biocompatible, and non-immunogenic[166]. Even though it does not naturally possess cell interactive properties, numerous compounds including proteins[167, 168] and cell-interactive peptides or growth factors[169] have been coupled to the alginate backbone to overcome this problem[170]. Alginate is also frequently combined with calcium phosphates for use in bone tissue engineering[167, 171, 172]. Alginates are showing promise in the field of pharmaceutics because of their propensity to form an ionotropic gel after the addition of multivalent cations[170, 173]. An overview of the biomedical applications of alginates is given in Table 8.

Proteins

Collagen

Collagen is the main structural protein of the ECM, and is the most abundant protein in vertebrates[174], with more than 12 types of collagen found across a variety of tissues[175-183]. The majority of porous collagen-based scaffolds are made using stereolithography or freeze-drying, although a novel technique for the cryogenic plotting of 3D scaffolds has been developed[184]. Specific scaffold designs and methods have been used for specific tissue regeneration purposes. Examples include cylindrical tubes for use in blood vessel regeneration made using a rotating cylinder[185], and nanofibres created using electrospinning[186-192].

For use in bone tissue regeneration, calcium phosphates are often combined with the porous collagen scaffold[193-199]. Additionally, multiple researchers have developed composite scaffolds with synthetic polymers[200-205], or modified GAGs[197, 206-211], to form semi-interpenetrating polymer networks (SIPN)[212, 213]. As with alginate, the cell-interactive properties of the collagen-based matrices have also been improved, with specific peptides[214], growth factors [215-218], or both having been incorporated into the matrix. Recombinant human-like collagen has been developed with an eye towards safety issues, such as the risk of pathogen transmission from animals to humans[131, 219, 220]. An overview of the biomedical applications of collagen is given in Table 9.

Table 7. Overview of Biomedical Applications of Cellulose Derivatives[14]

Type of cellulose	Application
Ca^{2+}-activated cellulose, cellulose/lactide, bacterial cellulose, nanohydroxyapatite/bacterial cellulose	Bone
cellulose/collagen, injectable cellulose	Cartilage
bacterial cellulose	Cornea
various cellulose-based hydrogels	General
cellulose acetate and regenerated cellulose	Heart
bacterial cellulose	Muscle
cellulosic hydrogels	Nerve
carboxymethyl cellulose	Nucleus pulposus
bacterial cellulose	Vascular

Table 8. Overview of Biomedical Applications of Alginate[14]

Type of polymer	Application
alginate/elastin/PEG, angiogenic factors/alginate	Blood vessel
alginate microbeads, alginate/gelatin/hydroxyapatite, oxidised alginate/gelatin/tricalcium phosphate, chitosan/alginate, alginate/poly (lactic-*co*-glycolic acid)/calcium phosphate, collagen/alginate/nanohydroxyapatite	Bone
sodium alginate, chitosan/alginate, gelatin/alginate	Bone marrow
alginate/fibrin, agarose/alginate/gelatin, chitosan/alginate/hyaluronate, PLGA/alginate, transforming growth factor-β(1) loaded alginate	Cartilage
alginate, alginate-*cis*-aconityl-daunomycin, calcium alginate/silk fibroin, hyaluronic acid/alginate, PLGA/Ca-alginate	Drug delivery
alginate, alginate/poly(vinyl alcohol), laminated alginate, carbon nanotube/alginate, iron-cross-linked alginate, alginate/poly(L-lysine)-hyaluronic acid, alginate/chitosan, copper-capillary alginate	General
injectable alginate, gelatin/alginate	Heart
alginate/chitosan	Ligament
macroporous alginate, alginate/galactosylated chitosan, sodium alginate	Liver
chitosan/calcium polyphosphate	Meniscus
photo-cross-linked alginate	Nucleus pulposus
gelatin/alginate	Skin
alginate	Spinal cord
alginate/chitosan	Tendon

Table 9. Overview of Biomedical Applications of Collagen[14]

Type of polymer	Application
glutaraldehyde-cross-linked collagen/chitosan, bFGF/collagen, collagen microbeads	Adipose tissue
compressed collagen	Bladder
collagen/cell assembly, p(DLLA-*co*-TMC)/collagen, collagen-chitosan nanofibre, PLGA microsphere/collagen, fibroin/collagen, TMC/DNA-containing collagen, collagen/citric acid derivative, polylactide/silk fibroin/gelatin	Blood vessel
collagen/nanohydroxyapatite, dense collagen, polyvinyl alcohol/collagen/hydroxyapatite, collagen microspheres, collagen/nanotube, collagen I/PLGA-β-TCP, collagen fibre/PLA, collagen/glycosaminoglycan, nano-HA/collagen/PLLA, collagen/OP-1, PCL/collagen, RhBMP-2 microspheres/chitosan/collagen, adenovirus vectors/collagen/chitosan	Bone
collagen/chitosan/GAG, adipic dihydrazide-modified collagen/hyaluronic acid, PLGA/collagen, micronised collagen sponges, type II collagen, collagen propeptides, type II collagen/chondroitin sulfate/hyaluronan, collagen/HA/chondroitin sulfate	Cartilage
dendrimer-cross-linked collagen, hydroxypropyl chitosan/gelatin	Cornea
CO(3)Ap-collagen	Dental
photo-cross-linked collagen, EDC-cross-linked electrospun collagen, poly(lactic-*co*-glycolic acid)/collagen, PHBV/collagen, collagen/hyaluronan/chitosan, collagen/hyaluronic acid, TPU/collagen, collagen/glycosaminoglycan, poly(lactic acid-*co*-caprolactone)/collagen, stromal cell-derived factor 1α-loaded heparinised collagen, collagen/hyaluronan/chitosan, gelatin/alginate	General
type I collagen, collagen/GAG	Heart

Table 9. Continued…..

type I and II collagen/GAG	Intervertebral disc
collagen/silk	Ligament
poly(lactic-*co*-glycolic acid)/collagen, collagen/chitosan/heparin	Liver
cross-linked atelocollagen	Muscle
collagen/microchannels, collagen/hyaluronic acid, collagen/heparan sulfate	Nerve
collagen II/hyaluronan/chondroitin-6-sulfate, collagen	Nucleus pulposus
UV-cross-linked collagen	Ophthalmology
PLGA/collagen	Pancreas
compressed collagen, cross-linked collagen/chondroitin sulfate/hyaluronic acid, β-glycerol phosphate/collagen/chitosan, collagen/elastin, electrospun collagen/PCL, poly[(D,L-lactide)-*co*-glycolide]/collagen	Skin
collagen	Urological

Gelatin

Gelatin is a soluble protein produced by the partial hydrolytic degradation of collagen. The primary sources of gelatin are pig skin (46%), cow hide (29.4%), and pork and cattle bones (23.1%)[221]. Gelatin is frequently used for biomedical applications because of its wide variety of chemical properties, such as gel formation, shear thickening, protective colloid function, and film-forming capacity[222]. As gelatin has a sol-gel transition temperature of approximately 30°C, it must be chemically cross-linked in order to prevent dissolution at body temperature. As a result of the number of side chains present in gelatin, a wide array of modification methods could be used[223, 224]. However, the chosen reagents must be water-stable as gelatin will only dissolve in water and some alcohols. Cross-linking reagents that have been used include glutaraldehyde[225], carbodiimides[226], diisocyanates[227, 228], polyepoxy compounds (PCs)[229], genipin[230-232], and acyl azides[233].

Created using cryogenic treatments and lyophilisation[234-236], gelatin combined with methacrylamide has been used to produce porous scaffolds that support the adhesion, spreading, and proliferation of human cells (endothelial cells, fibroblasts, epithelial cells, glial cells, and osteoblasts)[237]. More common techniques used to produce porous gelatin-based scaffolds for tissue engineering are freeze-drying and phase separation[236, 238, 239]. Since gelatin is derived from collagen, it is commonly combined with calcium phosphates[240-244] and/or GAGs[89, 90, 245-247] when targeting specific tissues. An overview of the biomedical applications of gelatin is given in Table 10.

Elastin

Elastin is a major component of mechanically active tissues that require elasticity, such as lungs, arteries, skin, and elastic ligament and cartilage[248]. Commercially available dermal skin substitutes comprising elastin and collagen (such as MatriDerm® and AlloDerm®) have frequently been evaluated for their uses in wound healing[249-251]. Because of the vast amount of covalent cross-linking present in native elastin, it is not commonly used as cell carriers for tissue engineering[252]. However, modified elastin has been used to create porous scaffolds using CO_2[253], as well as nanofibres made by electrospinning[254-258]. Additionally, elastin-like polymers demonstrate excellent biocompatibility as they are similar to natural elastin and their degradation products are native amino acids[259]. An overview of the biomedical applications of elastin is given in Table 11.

Fibroin

Fibroin is a naturally-produced hydrophobic glycoprotein synthesised by spiders, as well as numerous insects, including the silkworm *Bombyx mori*[260]. The primary structure of fibroin almost entirely consists of the recurrent amino acid combination $(Gly\text{-}Ala\text{-}Gly\text{-}Ala\text{-}Gly\text{-}Ser)_n$[166]. Silk fibroin shows remarkable promise as a material for implantation as it is tissue compatible, minimally immunogenic, and non-toxic[261]. Additionally,

silk-based biomaterials are biocompatible with numerous cell types, able to promote successful cell growth and proliferation[262-264]. Silk fibroin can be processed into a wide array of different forms including films[265-267], gels[268, 269], nanofibres[270-274], scaffolds[275], membranes[276], hydrogels[277, 278], nanoparticles[277, 279, 280], and powders[281, 282]. This versatility makes it extremely useful to the field of biomaterials and drug delivery[166]. To produce different scaffolds, fibroin has been combined with gelatin for ligament tissue engineering[239], hydroxyapatite for bone tissue engineering[283, 284], as well as other proteins[189, 219, 285, 286] and glycosaminoglycans[287, 288]. An overview of the biomedical applications of fibroin is given in Table 12.

Summary of desired scaffold properties

Scaffolds for tissue engineering need to support cell growth in three dimensions in order to be successful. The most effective scaffolds have a surface area large enough for cell attachment, and are highly porous in order to facilitate continuous nutrient diffusion[289]. Scaffolds can be created using a range of different methods and techniques, including dissolvable porogen-fused scaffolds, 3D printing, laser-sintering, as well as electrospun fibres, hydrogels and nonwovens[290]. The materials that are used to create the scaffold must be biocompatible and, if required, degradable. Especially important in bone tissue and ligament formation, until the new tissue becomes load bearing, the porosity of the scaffold must not compromise its mechanical performance[291].

Table 10. Overview of Biomedical Applications of Gelatin[14]

Type of polymer	Application
gelatin sponge	Adipose tissue
gelatin/poly(ε-caprolactone) nanofibres, VEGF immobilised gelatin, polyethylene-glycol diacrylate/gelatin, chitosan/gelatin, gelatin/PET nanofibres, gelatin/PES fibres, gelatin/PTFE	Blood vessel
hydroxyapatite chitosan/gelatin, gelatin/poly(α-hydroxy acids), glutaraldehyde cross-linked gelatin, hydroxyapatite/gelatin, *β*-tricalcium phosphate/gelatin, gelatin/poly(*ε*-caprolactone) nanofibres, gelatin microcarriers/polyester, micro- and nano-hydroxyapatite/chitosan/gelatin, rhBMP-2-loaded gelatin/nano-hydroxyapatite/fibrin, poly[(L-lactide)-*co*-(epsilon-caprolactone)]/gelatin, gelatin-based photopolymers	Bone
gelatin/chondroitin-6-sulfate/hyaluronan, plasmid DNA/chitosan/gelatin, gelatin microparticle/OPF, gelatin microparticle/poly(D,L-lactide-*ε*-caprolactone), TGF-*β*1-loaded gelatin, ceramic/gelatin, esterified hyaluronan/gelatin, gelatin/chitosan/hyaluronan	Cartilage
transglutaminase cross-linked gelatin, proanthocyanidin cross-linked chitosan/gelatin, gelatin/poly(D,L-lactide), gelatin fibres, PHBHHx/gelatin, PVA/gelatin, PNIPAM/gelatin, gelatin- and fibronectin-coated PE multilayer nanofilms, gelatin/montmorillonite/cellulose, chitosan/PEG/gelatin, gelatin/(hydroxyphenyl)propionic acid, gelatin microparticles, gelatin/chitosan cryogels, genipin-cross-linked PCL/gelatin nanofibres, silk sericin/gelatin, α-chitin/gelatin, agarose/gelatin cryogel, hyaluronan/gelatin	General
gelatin/polyurethane, photo-cross-linked gelatin, alginate/gelatin	Heart
gelatin/chondroitin-6-sulfate/hyaluronan, gelatin, glutaraldehyde cross-linked gelatin/chondroitin-6-sulfate	Intervertebral disc
gelatin/silk fibroin	Ligament
cross-linked sodium alginate/gelatin, chitosan/gelatin	Liver
gelatin/PCL nanofibres	Muscle
photo-cross-linkable gelatin, gelatin/(hydroxyphenyl)propionic acid	Nerve
chitosan/gelatin/glycerol phosphate	Nucleus pulposus
gelatin/agarose	Pancreas
glutaraldehyde cross-linked gelatin	Skin

Table 11. Overview of Biomedical Applications of Elastin[14]

Type of polymer	Application
collagen/elastin, alginate/elastin/PEG, collagen/elastin/PCL, copper non-parasitic/elastin, bFGF/elastin, polydioxanone/elastin/collagen, poliglecaprone/PCL/elastin/gelatin, polyglyconate/elastin	Blood vessel
BMP-containing elastin	Bone
hexamethylene diisocyanate-cross-linked α-elastin, recombinant elastin, tropo-elastin, collagen/elastin, collagen/elastin/chitosan/poly(lactic acid), poly(lactide-*co*-glycolide)/gelatin/elastin	General
elastin-like proteins	Nerve
recombinant elastin	Ocular
collagen/elastin	Skin

Table 12. Overview of Biomedical Applications of Fibroin[14]

Type of polymer	Application
non-mulberry and mulberry silk gland fibroin	Adipose tissue
fibroin, collagen/fibroin, polylactide/silk fibroin-gelatin, fibroin modified-polyhydroxyalkanoate	Blood vessel
silk fibroin/chitosan/PLLA, chitosan/fibroin-hydroxyapatite, non-mulberry silk gland fibroin, non-mulberry and mulberry silk gland fibroin	Bone
silk fibroin modified porous poly(e-caprolactone), plasma-treated fibroin	Cartilage
alginate/fibroin, silk fibroin/gelatin	Drug delivery
gelatin/silk fibroin, hyaluronan/silk fibroin, chitosan/silk fibroin, fibroin/recombinant human-like collagen, *Antheraea assama* silk fibroin, nano-hydroxyapatite/fibroin, silk fibroin-modified PHBHHx, polylactide/silk fibroin-gelatin	General
gelatin/silk fibroin	Ligament
fibroin/recombinant human-like collagen, PLLA/fibroin, chitosan/silk fibroin, chitosan/silk fibroin/heparin	Liver
Antheraea pernyi silk fibroin	Tendon

Why animals are used?

History of animal testing

Experiments on animals have been performed since the inception of biomedical research. Starting with Greek physician-scientists, such as Aristotle (384–322 BC) and Erasistratus (304–258 BC), experiments have been carried out on living animals to advance our knowledge and understanding of anatomy, physiology, pathology, and pharmacology[292]. Today, animal testing is used for a variety of purposes, including tests on drug activity and affinity[293], toxicological screenings, vaccines[294, 295], and as tools to understand the effects of medical procedures and surgical experiments[296].

The importance of drug testing using animals became apparent in the 20th century with a variety of famous, and tragic, cases of drug toxicity. In 1937, an American pharmaceutical company made a preparation of the antibiotic sulfanilamide, and used diethylene glycol as the solvent. Unfortunately, diethylene glycol is toxic to humans, and the subsequent mass poisoning caused the deaths of one hundred and five patients. This, and similar, incidents resulted in the creation of the 1938 Federal Food, Drug, and Cosmetic Act, which required drug toxicity testing on animals before they could be marketed[297].

Another drug-related tragedy occurred in the 1950s-1960s with thalidomide. Originally marketed as a tranquilliser and painkiller,

thalidomide was discovered to prevent morning sickness, consequently thousands of pregnant women took thalidomide to relieve their symptoms. As a result of thalidomide's teratogenicity, more than 10,000 children across 46 countries were born with malformations or missing limbs (phocomelia)[292]. Confusingly, rodents used in the original thalidomide toxicity studies did not display signs of teratogenicity. However, subsequent research has demonstrated that rats are thalidomide-resistant, compared to rabbits which are thalidomide-sensitive[298]. After thalidomide it was recognised that inter-species differences required consideration. This produced the requirement that developmental toxicity testing for pharmaceuticals is performed using two different animal species, one of which is not a rodent[296].

Numbers of animal testing

Each year, millions of animals are used across the world in toxicity testing and biomedical research, with the focus on developing cures for human diseases. It is estimated that 17.3 million animals were used in 2005, making the United States the leading global user with 1.2 million. Japan was second with 11.2 million, and China third with 3.0 million animals used[299]. In 2013, 4.12 million scientific procedures were carried out in Great Britain, an increase of 11,600 compared to 2012[300], with mice, fish, and rats the most commonly used species (Figure 7).

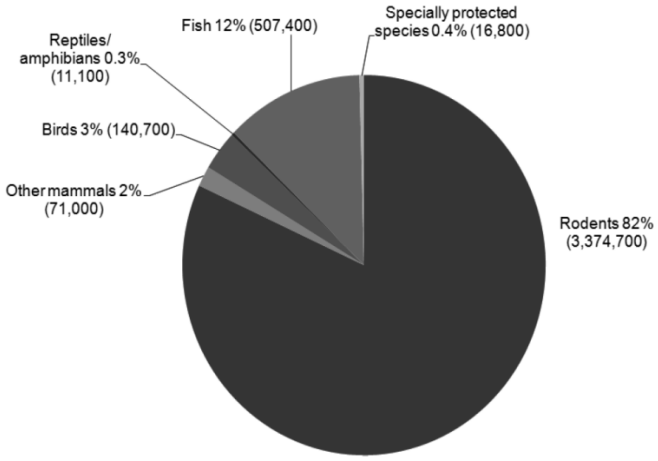


Figure 7. Procedures by species of animal, 2013[300]

In Great Britain, in 2013, the number of animal procedures used for toxicity testing decreased by 0.5% to 375,000. In contrast, the number of non-toxicity procedures increased by 0.4% to 3.75 million. These non-toxicity-based procedures cover the breeding of genetically modified animals (GM), and animals with harmful genetic mutations (HM), for research. The main fields of research were immunology, cancer research, physiology, anatomy, and genetics[300].

Procedures within animal testing

The term 'regulated procedure' refers to any act, carried out for a scientific purpose, that may cause an animal a greater level of pain, suffering or distress than would be caused by the insertion of a hypodermic needle[301]. A procedure can be as mild as an injection, or as severe as an organ transplant[302]. Procedures are classified as 'non-recovery', 'mild', 'moderate', or 'severe' on a case-by-case basis, based upon the degree of pain, suffering, distress, or lasting harm that will be experienced by the animal[303].

Non-recovery procedures are performed under permanent general anaesthetic, with the animal being euthanised when the procedure is finished[303], an example being to make a hole in the intestines of an animal to cause sepsis in order to monitor the effects[304]. Mild procedures result in an animal being likely to experience short-term mild pain/suffering/distress[303], such as from short-term social isolation or superficial surgical procedures[305]. Moderate procedures result in an animal being likely to experience short-term moderate pain/suffering/distress, or long-lasting mild pain/suffering/distress. They may also undergo procedures that are likely to cause moderate impairment of their well-being or general condition[303]. This can include organ transplantation or exposing the animal to a fearful stimulus whilst restraining[306]. Severe procedures result in an animal being likely to experience severe pain/suffering/distress, or long-lasting moderate pain/suffering/distress. They may also undergo procedures that are likely to cause severe impairment of their well-being or general condition[303]. This can include inescapable electric shocks, or any tests that deliberately result in death[307].

The Draize test

One of the most famous, and arguably most controversial, testing methods is the "Draize test". Devised in 1944 by US Food and Drug Administration (FDA) toxicologist John Draize, the procedure was initially used for assessing the damage caused by acute, intermediate, and chronic exposure of cosmetic-based compounds to the skin, penis, and eyes of rabbits[308]. Subsequent to Draize's initial publication, the FDA used the aforementioned techniques to assess the safety of several substances, such as insecticides, sunscreens, and antiseptics[309]. The method used

by Draize was widely adopted, with scientists referring to this method simply as the 'Draize technique' or the 'Draize test'[310]. However, despite its widespread adoption and "gold standard" status, Draize testing was never formally validated[311]. Somewhat ironically, the original reason for devising methods to test for ophthalmic toxicity was not to screen medications for public safety. Instead it was with the aim of identifying chemicals that could be weaponised to harass, harm, and blind enemy soldiers[312].

Animal testing controversy

The continual increase in the use of GM and HM animals, as well as a number of large-scale chemical testing programs operating within the US and Europe, are causing an increase in the number of animals used in laboratory experiments[313]. Yet biomedical research using animals remains a highly controversial and emotive subject.

Proponents often claim that research involving animal testing is vital for preventing, relieving, or curing human diseases[314, 315], that the most notable medical achievements have only been possible because of animal testing[316], and that the physiological and biological complexity of humans requires the use of animal models in order to successfully interpret the results of biomedical investigations[317].

However, such claims are hotly contested[318-320]. Whilst segments of the animal rights community have long challenged the merit of preclinical studies that use animals as barometers for human response, recently the worth of animal studies has been questioned by the scientific community itself[321-323]. An increasing catalogue of evidence questions the validity of animal studies as experimental human models[324-327], with the lack of consistency of animal efficacy data with the outcomes of human trials resulting in questions over the fundamental role that animal-based studies have in preclinical studies[328-330], and the methods used within[331, 332].

Animal experiments commonly used as part of pharmaceutical research suffer from varying degrees of success, as the translation from animal to human depends upon the parameter being tested. For example, converting oral drug bioavailability from animal data to humans is a classic failure as no acceptable correlation between species has ever been identified[333]. Another example is that the volume of drug distribution between humans and animals is broadly similar, whereas predicting the drug distribution to individual cell types/tissues is more difficult[334]. Olson *et al.* reported that approximately 50% of the drugs that are acknowledged to cause human liver injury were not recognised as hepatotoxic by the animal testing[335]. This lack of concordance is probably caused by the significant differences in liver pathways between animals and humans[336-338].

Aside from the ethical concerns, drawbacks of animal testing include the required skilled/trained manpower, time consuming protocols, and the high cost of breeding and housing[339].

Alternatives to animal testing

The experimental protocols involved in animal testing have remained comparatively unchanged for over 40 years[340], yet consumers constantly expect greater safety and information about their products. Across the world, approximately £10 billion a year is spent on animal experiments, of which about £2 billion is spent on toxicology studies[340]. The costs of using, housing, and maintaining the vast numbers of animals required for use in the toxicity testing of a single compound can exceed millions of pounds[339, 341]. A number of ethical, business, and legal concerns, as well as continual scientific advances, have driven the demand for alternative, animal-free testing that is more accurate and relevant to humans[342].

Across Europe, there has been a political shift in the attitude about animal testing, moving towards testing methods that use animals minimally, if at all. Since implementing the Animal Welfare Guideline 86/609/EC in 1986, it is the policy of all European Union institutions to support the development and use of alternative testing methods[343]. Animal testing on cosmetic ingredients has been completely banned in Europe since March 2009[344]. Additionally, current European Union chemicals legislation (Registration, Evaluation, and Authorisation of Chemicals - REACH) recommends animal testing only as a last resort, when there is an absence of *in vitro* or *in silico* alternatives[345-347].

More effort has been spent on producing viable alternative testing techniques to the Draize test than all other *in vivo* toxicity tests combined[348]. Opponents of the Draize eye and skin irritation tests often cite its subjective and time-consuming nature[349], variable estimates[311, 350], insufficient relevance of test chemical application[341], high doses[351], non-standardised test methods[352], and over-prediction of human responses[353]. Interspecies differences are the main source of over-predicting human response from the Draize test[354], such as rabbit skin being much more reactive than human skin[355], and the anatomy of rabbit eyes differing quite substantially from human eyes. Compared to humans, rabbit corneas

are thinner, produce fewer tears, blink less often, their ocular surface is less sensitive[348], they have larger conjunctival sacs, and they have a third eyelid[356]. A suggested "gold standard" alternative to the Draize test for eye irritation would be the human response[357], using a testing strategy that uses a very large number of humans in order to fully represent human diversity. The test subjects would be unknowingly ("blinded", if you will) exposed to the substance and the effects assessed[340]. Many research articles that engage with members of the public produce the suggestion of using convicts, usually limited to murderers and paedophiles, as an alternative to animal testing[358]. However, because such experimentation is unrealistic and unethical, human data can only be obtained from accidental exposure or clinical studies.

To overcome some of the problems associated with animal testing, and to avoid unethical procedures, a strategy of 3R's (reduction, refinement, and replacement) is applied to find more palatable alternatives[359]. This strategy encourages the use of minimal animals in an experiment, with their use carefully planned and streamlined to minimise pain and distress, and conscious living vertebrates should be substituted with alternative methods and lower organisms[339, 359, 360].

A variety of different models have been suggested as alternatives to animal testing, these models include;

- **Computer models**

Computational models and simulations are used for the toxicity testing of chemicals and potential drug candidates, without the need for animal testing. Only the most auspicious molecules are carried forward for use in *in vivo* experiments[361]. Computer Aided Drug Design (CADD) can predict the likely binding sites for potential drug molecules, preventing the testing of compounds with no biological activity. Drug molecules can also be designed for a specific binding site, with animal testing performed to corroborate the results[362]. Structure Activity Relationships (SARs) predict the biological activity, such as carcinogenicity and mutagenicity, of a drug candidate based upon its molecular structure[363]. The benefits of computer models are their greater speed over conventional animal models, with the procedures used being relatively inexpensive[364].

The sheer complexity of producing QSAR software can give rise to multiple factors that contribute to the relatively poor performance of QSAR software in predicting the carcinogenic potential of pharmaceuticals. These include the inadequate representation of the molecular diversity of drugs, and of the biological and toxicological complexity of chemically-induced carcinogenicity, as well as the SAR evaluation criteria itself. Yet despite these hurdles, more-recent QSAR databases have demonstrated their effectiveness in predicting the carcinogenicity of test compounds, with one study describing a QSAR system that demonstrated 97% sensitivity for rodent carcinogens and 98% specificity for non-carcinogens[364].

- **Cells and tissue cultures**

The use of *in vitro* cell and tissue culture involves the removal of cells and tissues from an animal source, and their subsequent growth as a monolayer or in suspension. Cell and tissue culture methods are commonly used for the initial screening of potential drug molecules or chemicals in order to investigate toxicity, efficacy, drug disposition, drug-drug interactions (DDIs), drug clearance, and major metabolites[365-368]. The benefits associated with cell and tissue culture testing are that the protocols are simpler, quicker, and cheaper than directly testing on animals[369-371], with toxicity able to be understood at the cellular or molecular level[341]. However, *in vitro* cell and tissue culture experiments cannot always reliably predict the *in vivo* properties of compounds, as a multitude of important biological factors cannot be adequately replicated *in vitro*[372].

An alternative to the Draize test for ocular chemical irritancy has been proposed, one which uses bovine corneal organ culture *in vitro*. However, there are some inherent differences between the two models, such as layers of mucin and epithelium in the cornea, which form a highly impermeable line of defense against biological and chemical insults[373].

The haemoglobin denaturation (HD) test, where the denaturation of haemoglobin by surfactants is evaluated, has been developed to predict the eye irritation potential of chemicals[374]. The *in vitro* test results are reported to be in good agreement with the Draize test. But the results revealed multiple limitations associated with the HD test, namely; it cannot be applied to coloured test substances with a strong absorption, it cannot

evaluate water-insoluble test substances, it cannot be applied to strong acids that exceed the buffering capacity of a phosphate buffer solution, and it cannot determine the potential for eye irritation caused by factors other than protein denaturation. Because of these limitations, the HD test alone is inappropriate for predicting eye irritation potential[375].

Wang *et al.*[365], evaluated a novel micro-patterned hepatocyte co-culture system for its ability to generate human *in vivo* metabolites. The co-culture system produced 82% of the excretory metabolites, exceeding the performance of hepatocyte suspension incubations and other *in vitro* systems. Unfortunately, *in vitro* systems possess some shortcomings when used to predict the total *in vivo* metabolism profiles in humans. Some systems are limited by the number/variety of drug-metabolising enzymes present, whilst others cannot perform the multiple sequential reactions required before the drug-related material is excreted.

A more recent study by Chan *et al.*[368], looked at overcoming the short incubation times of primary hepatocytes in suspension used to predict *in vivo* clearance, as 4-6 hours is not long enough to accurately evaluate the metabolic stability of slowly metabolised compounds. Using a micro-patterned hepatocyte-fibroblast co-culture system (HepatoPac®), continuous incubations were performed for up to 7 days. Hepatic clearance was accurately predicted for 13 of 17 compounds (76%; predicted clearance within 3-fold of observed human *in vivo* clearance values).

Testing with incubated hen's eggs is arguably a grey area between *in vivo* and *in vitro* systems, but is regarded as a preferable alternative to the Draize irritation test[376]. The Hen's Egg (or Hühner-Embryonen) Test-Chorioallantoic Membrane (HET-CAM) is a rapid, sensitive, and inexpensive toxicity test. HET-CAM has a number of advantages over other alternative tests, particularly cytotoxicity tests, because the technique is applicable to all types of chemicals, regardless of their physical properties or solubility[377]. A study by Debbasch *et al.*[378] reported that the results of HET-CAM testing with strong irritants correlated well with Draize testing, but appeared less suited to identify mild irritants.

The Syrian hamster embryo (SHE) cell transformation assay has been described as the most predictive short-term assay for rodent carcinogens, as it detects morphological cell transformation, the earliest identifiable stage in carcinogenesis[379]. Pienta *et al.*[380] demonstrated a 91% correlation between the morphological transformations of SHE cells and the reported carcinogenic activity of a multitude of carcinogenic and non-carcinogenic chemicals. Although the SHE assay is still undergoing improvement, most of the difficulties encountered in earlier versions have been overcome by culturing SHE cells at pH 6.7[379, 381]. An overview of the *in vitro* alternative methods to evaluate irritation is given in Table 13.

- **Alternative organisms**

Various ethical arguments have resulted in a number of restrictions on the use of higher vertebrates, such as rats and monkeys, in animal testing. To overcome these restrictions and ethical arguments, the use of alternative organisms, such as lower vertebrates and invertebrates, has been suggested.

Lower vertebrates, including the zebrafish (*Danio rerio*), are appealing alternatives to animal testing because of their genetic relatedness to higher vertebrates (including mammals), as well as the decrease in ethical issues, working space, cost of laboratory solutions, test chemicals, and manpower involved compared to animal testing[361, 382].

During early development, the body of the zebrafish is almost transparent, which helps easy visual access to the internal anatomy. This allows direct observation of developmental stages, the identification of phenotypic traits during mutagenesis, easy screening, assessment of endpoint of toxicity testing, and direct observation of gene expression through light microscopy[361].

As zebrafish embryos can survive for several days in a single well of a 384-well plate through the absorption of yolk, and can be visually assessed for malformation[383], it is possible to rapidly treat and screen large libraries of molecules for toxicity or therapeutic value. Using fluorescent transgenics, Peterson *et al.*[384], screened 1100 small molecules and identified several that altered organ development. Likewise, Milan *et al.*[385], assessed 100 molecules that cause cardiac QT prolongation in humans, but manifested as bradycardia and AV block in zebrafish.

Currently, acute toxicity studies using zebrafish are very limited. Examples of toxic

substances investigated include lead and uranium[386], malathion[387], metronidazole[388], anilines[389], and colchicine[390]. The main reason few studies have utilised juvenile and adult zebrafish is because the value of zebrafish lies in its genetics and developmental biology[382].

Invertebrates, including the common fruit fly (*Drosophila melanogaster*) have been extensively used as alternatives for animal testing to investigate a variety of diseases[361] and biomedical applications[391]. The results of tests on the common fruit fly is widely applicable to humans because of similarities in genetics[392, 393], anatomy[394], and CNS responses to drugs[394-396]. Although invertebrates have an undeveloped organ system and are not recognised to have an adaptive immune systems, which poses some limitations for their use in human diseases[361], some genes in organisms such as *C. intestinalis* are related to those in vertebrates and give rise to adaptive immunity[397].

The fruit fly is considered a multiple model organism, used to study a number of different concepts[395]. For example, the embryo is used to study neuronal development, axon path finding, and organogenesis, whereas the larva is used to study physiological and developmental processes and behaviours[361].

Fruit flies are also used to express the protein products found in human diseases and to compare the resulting pathologic conditions, serving as important tools to investigate neurodegenerative diseases like Alzheimer's, Parkinson's disease, and Huntington's disease[398-400].

A number of *Drosophila* phenotypes have been created as models for human disease[401]. Insulin signalling is very similar in flies and humans, making them ideal models to study the ways in which insulin regulates metabolism. However, *Drosophila* is not suitable to study all aspects of human metabolic control, as for example, the leptin signalling pathway is not present in the fly[402].

Drug delivery is a major challenge when using *Drosophila*, because of the extreme difficulty in standardising the amount of drug consumed in a fly's diet[403]. Alternative administration options, such as microinjection into the abdomen, have been suggested[404]. In spite of this challenge, multiple studies have used *Drosophila* for toxicity testing[403, 405-407]. Researchers have observed negative reproductive effects in *Drosophila* adults and cell lines after exposure to variety of different insecticides[406, 407]. Similarly, Avanesian and colleagues[403] studied methotrexate toxicity in flies and found ovarian impairment comparable to that observed in mammalian models.

Caenorhabditis elegans is a eukaryotic nematode, a transparent multicellular organism that has been used to study various diseases, such as Huntington's disease, Parkinson's disease, Alzheimer's disease, various immune disorder, cancer, and diabetes[408-412]. It has also been used in both LD_{50} and behavioural pattern studies[413], showing results comparable to those of mouse systems[414].

The extrapolation of responses to chemicals from invertebrates to vertebrates presents a number of different problems. For example, asexual or parthenogenetic invertebrates are not suitable for the evaluation of effects on gametogenesis[391]. Additionally, some routes of entry are typical of vertebrates (e.g., lung and skin), but their importance in the penetration of chemicals cannot be assessed using invertebrates. For example, the cuticle of arthropods and the skin of vertebrates are very different in structure and relative permeability to substances[415-418].

- **Microorganisms**

Brewer's yeast (*Saccharomyces cerevisiae*) is one of the main microorganism used in experiments because of its rapid growth, ease of replication and mutant isolation, dispersed cells, well defined genetic system, highly versatile DNA transformation system, and the fact that its membrane-bound and secretory organelles mimic the functions of mammalian cells[419]. *S. cerevisiae* is used to comprehend apoptosis and cell death regulators for cancer research[420], as well as the cellular biology involved in neurodegenerative diseases[421, 422].

The most commonly used test for mutagenicity is the Ames test for "reverse mutation" in *Salmonella typhimurium*[423]. Mutagenicity is detected by exposing an already mutated strain to potential mutagens. If the mutation is reversed, the bacteria regain their ability to produce the amino acid histidine, allowing them to multiply in a histidine-deficient culture medium. The Ames *S. typhimurium* reverse mutation and chromosomal aberration genotoxicity assays

have been accepted by regulatory agencies for many years[424].

Tennant *et al.*[425] reported successfully predicting the outcomes of 86% of 44 chemicals undergoing carcinogenicity testing, by using the *Salmonella* mutagenicity and sub-acute (90-day) rodent toxicity tests, combined with chemical structural information.

Justification for toxicology testing

Main aim

The primary aim of toxicity testing is to protect humans against the potential adverse effects of exposure to a wide variety of chemicals and substances, such as pharmaceuticals, cosmetics, household products, industrial chemicals, and agrochemicals[426]. The safety studies performed are used to identify the various toxicological endpoints; everything from skin irritation and corrosion, to acute systemic toxicity and carcinogenicity[347].

Toxicity studies in people

The methods available to investigate the toxicity of chemicals in humans is limited for obvious reasons. Therefore, the information on the effects of chemicals in humans normally comes from either accidental exposure or the suicidal uptake of high doses[347]. Studies performed on the ADME of chemicals in humans can be used to characterise their toxicity, and are recognised as important contributions for future risk assessment[505-513].

Why toxicity testing is required for drug development

Toxicity testing is very important in the arena of drug development. Currently it costs approximately £2-3 billion and 12-15 years to launch a single drug into the market[514, 515]. Lead compounds usually go through ADMET (absorption, distribution, metabolism, excretion, and toxicity) analysis *in vitro* and *in vivo* before being put forward for clinical trials in humans. However, of the compounds that pass through pre-clinical screening, almost 90% of them eventually fail during clinical trials, with one-third of failures ascribed to toxicity[516]. Additionally, about 90% of drug withdrawals are because of toxicity concerns, with drug-induced liver injury a chief source[517, 518].

CONCLUSION

Whilst progress has been made to develop a number of alternative techniques to *in vivo* testing, further progress is required to reduce the dependency of toxicity testing on live animals. Unfortunately, at the moment, *in vitro* methods cannot currently predict complex toxicological endpoints; however alternative testing methods could potentially reduce the number of animals used.

The information presented in this review clearly demonstrates the versatility of biopolymers. It is still too early to speculate about the *in vivo* applicability of biopolymer-based scaffolds, due to the many unknowns regarding the biocompatibility of these scaffolds *in vivo*, as the immunological response and long-term stability of implanted biomaterials is still being studied.

Multiple approaches to produce a 3D matrix that supports mammalian cell cultures are available, however a large number of these products are proprietary, costly, or require chemical synthesis. Furthermore, due to the interdisciplinary nature of tissue engineering, close collaboration between various research disciplines will be essential to develop the ideal biopolymer-based organ structures.

Successful production of biocompatible, biopolymer-based 3D organ structures that can be used for drug toxicity testing should eventually usher in the end of testing on animals. However, this will be dependent on artificial organ structures no-longer being cost prohibitive to produce, as well as the results produced using them being recognised by the relevant regulatory authorities.

CONFLICT OF INTEREST STATEMENT

The authors declare that no competing interests exist.

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Table 1. Materials used for Regenerative Medical Applications (adapted from Park and Bronzino, 2002[15])

Materials	Examples	Advantages	Disadvantages	Applications
Biologically-derived materials, proteins and polysaccharides	Agar, agarose, cellulose, chitosan, collagen, fibrin, hyaluronic acid, silk	• Biologically active and compatible	• Weak • Limited sources • Ethics • Immunoresponsive	Soft and hard tissue repair, replacement, topical wound dressing, drug delivery, cell scaffold/matrix for culture applications
Ceramics	Aluminium oxide, carbon derivatives, calcium phosphates, hydroxyapatite	• Very biocompatible • Inert • Strong in compression	• Brittle and not resilient • Difficult to manufacture and manipulate	Material coating, joint implants, scaffold material for cells
Composites	Carbon-carbon, wire or fibre reinforced cement	• Strong • Customisable	• Deforms with time • Degradable • Difficult to manufacture and manipulate	Joint implants, material coating
Metals	Gold, silver, platinum, stainless steel, alloys	• Strong • Tough • Ductile • Inert	• Dense • Difficult to manufacture and manipulate • Costs • May suffer from corrosion	Joint replacements, screw, wires, plates, material coating
Synthetic polymers	Nylon, silicone, polyester, polytetrafluoroethylene, rubber	• Easy to fabricate • Tough • Ease of manipulation	• Weak • Brittle • Deforms with time • Degradable	Sutures, soft tissue replacement, cell culture matrix, drug delivery, scaffold support, wound dressing

Table 3: Overview of Different 3D Culture Models used for Toxicity Studies (adapted from Fitzgerald *et al.*, 2015[38])

Sample model system	Cell lines	3D constructs or techniques exploited	Key findings
Cancer (breast)	MDA-MB-231, MCF-7, MDR-MCF-7, BT474, SKBR3, HS 578T	Non-mulberry silk fibroin protein scaffolds, multicellular spheroids, decellularised tissue	Higher drug concentrations required to achieve comparable reduction in cell viability and invasive potential compared to 2D cultures, 3D culture induces increase doxorubicin resistance in parental cells but not in MDR cells, 3D cells displayed reduced sensitivity relative to 2D cells, BT474 and SKBR3 cells more sensitive to treatment than 2D cultured cells

Cancer (lung)	Primary cells, SPCA-1 co-cultured with HFL1, H460M, NCI-H460, SA87	Microfluidic device, hyaluronan hydrogel	Discrepancies noted between 3D and 2D culture systems in addition to primary versus established cell lines, IC50 values for certain drugs were higher for cells in 3D culture compared to 2D culture, paclitaxel was significantly less toxic to tumour cells grown as multicellular aggregates
Cancer (prostate)	PC3, LNCaP, C4-2B	Collagen-based scaffold, hyaluronic acid-based hydrogels	Cell lines in 3D culture demonstrated higher drug resistance, higher and faster apoptotic resistance observed in cells cultured in 3D
Cancer (others)	Panc-1 (pancreatic), PA-1 (ovarian), COLO 205 (colorectal), U87 (glioblastoma), BGC-823 (gastric)	Fibroblast-derived 3D matrix, pre-conditioned ECM, multicellular spheroids, poly(lactide-co-glycolide) and derivatives, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and collagen peptide based nanofibers, Matrigel™, collagen-based gels	Increased drug resistance noted in 3D cultures compared to 2D system, higher drug exposure and duration required to exert comparable effect on 3D culture compared to cells as monolayers
Heart	Isolated rat myocytes, neonatal mouse, Wistar rat myocytes	Co-culture, collagen gel, Matrigel™	3D culture demonstrated acceleration of contraction kinetics, specific for testing of atorvastatin on contraction after establishing in 2D culture that it decreases isoprenylation of Gɣ
Kidney	NK-i2, HEK293, murine proximal tubule epithelial, Vero, Madin-Carby canine kidney, LLC-PK1, CaKi-1, rat MSC, LZ100, podocytes, primary human	Collagen, Matrigel™, hyaluronic acid hydrogel, fibrin microbeads, microfluidic chip, culture chamber, 3D organoid culture	3D models have different time of toxicity induction and lower LD50/sensitivity value, comparable data observed in 3D compared to animal kidney injury models, microbead system demonstrated multilayer growth and relative resistance to cell killing and lysis by oncolytic viruses, metabolism closely represented using microfluidic device, comparable biomarker expression of cells to animal and clinical studies when grown in 3D culture
Liver	Human hepatocytes, HepaRG, primary rat cells, HepaG2/G3a,	Hurel microfluidics, organoids, biochips, co-culture	Predictive hepatic clearance correlation superior in 3D culture, higher sensitivity for toxicity observed in 3D system

Table 13: *In vitro* Alternative Methods to Evaluate Irritation[426]

***In vitro* Alternatives**	**Model Systems**	**Endpoints**	**References**

In vitro eye irritation tests			
Red blood cell test	RBCs of bovine, sheep, rabbit, and calf	Haemolysis and haemoglobin denaturation	[427-432]
Haemoglobin denaturation	Bovine haemoglobin	Spectrophotometric changes in haemoglobin	[374, 375, 433-435]
HET-CAM	Chorioallantoic membrane of White Leghorn eggs	Haemorrhage, vasoconstriction, coagulation, trypan blue adsorption	[376-378, 436-447]
Isolated cornea	Isolated bovine cornea	Corneal opacity and permeability	[448, 449]
Isolated eyes	Isolated eyes of rabbits, cows, pigs, chickens	Corneal swelling, corneal opacity, fluorescein retention	[450, 451]
Cell culture	• Rabbit corneal epithelial cells • Human corneal endothelial cells • Bovine corneal stroma • Bovine epithelium • Madin-Darby canine kidney cells	Fluorescein leakage, neutral red release	[452-460]
Commercial kits			
• EYTEX®	Medium containing proteins, glycoproteins, and mucopolysaccharides	Turbidity	[461-463]
• Reconstituted corneal epithelium	3D model of human corneal epithelium, composed of normal human-derived epidermal keratinocytes	Tissue viability (MTT assay)	[464-466]
In vitro skin irritation			
Cell culture	• Human epidermal keratinocytes • Murine epidermal keratinocytes • Mouse immortalised fibroblasts (3T3)	Cell Viability (NRU or MTT assay), cytotoxicity (LDH and NAG release), metabolism (glucose utilisation), inflammatory mediator release (PGE-2, IL-6, IL-1α)	[467-478]
Commercial kits			
• Skintex™	Modified keratin/collagen membrane, containing an indicator compound and a globulin/protein macromolecular reagent solution	Turbidity	[479, 480]
• Human epidermal models	Human skin cultures	Cell death, release of IL-1α, cell viability	[479, 481]

In vitro skin corrosivity			
Commercial kits			
• Corrositex®	Biobarrier comprising a hydrated collagen matrix	Colour change	[482, 483]
• EpiDerm®	Normal human epidermal keratinocytes (NHEK)	Cell viability (MTT assay), membrane damage (extracellular release of IL-1α, LDH, and GOT)	[481, 484-487]
• EPISKIN™	Reconstructed organotypic culture composed of human adult keratinocytes	Cell viability (MTT assay), membrane damage (extracellular release of adenylate kinase, IL-1α, IL-8, LDH, and GOT)	[481, 485, 486, 488-492]
Transcutaneous electrical resistance	Isolated rat skin	Reduction in TER, degree of colour staining	[493-496]
In vitro phototoxicity			
Red blood cell phototoxicity	Mouse immortalised fibroblasts (3T3)	Photohaemolysis	[497]
3T3 NRU phototoxicity	• Mouse immortalised fibroblasts (3T3) • Human immortalised keratinocytes (HaCaT)	Cell viability (as determined by NRU)	[498-500]
Keratinocyte cell culture	• Human epithelial carcinoma cells (A431) • Mouse immortalised fibroblasts (3T3) • Primary human keratinocytes, isolated from human foreskin	Cell viability (NRU or MTT assay)	[501-503]
Skin equivalent model	Normal human keratinocytes, seeded onto a collagen-glycosaminoglycans-chitosan porous matrix populated by normal human fibroblasts.	Cell viability (determined by MTT assay, or extracellular release of IL-1α)	[504]

Abbreviations:
GOT, glutamicoxaloacetic transaminase; IL, Interleukin; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NAG, N-Acetylglucosamine; NRU, neutral red uptake; PG, prostaglandin; RBC, red blood cell; TER, transcutaneous electrical resistance.