Advanced 3D Cell Culture Techniques in Micro-Bioreactors, Part I: A Systematic Analysis of the Literature Published between 2000 and 2020

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Keywords: in vitro models, HTS, tissue engineering, cellular microenvironment, material, microfluidics, scaffolds, 3D cell culture, micro-bioreactor

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Review

Advanced 3D Cell Culture Techniques in Micro-Bioreactors, Part I: A Systematic Analysis of the Literature Published between 2000 and 2020

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Abstract: Bioreactors have proven useful for a vast amount of applications. Besides classical large-scale bioreactors and fermenters for prokaryotic and eukaryotic organisms, micro-bioreactors, as specialized bioreactor systems, have become an invaluable tool for mammalian 3D cell cultures. In this systematic review we analyze the literature in the field of eukaryotic 3D cell culture in micro-bioreactors within the last 20 years. For this, we define complexity levels with regard to the cellular 3D microenvironment concerning cell–matrix-contact, cell–cell-contact and the number of different cell types present at the same time. Moreover, we examine the data with regard to the micro-bioreactor design including mode of cell stimulation/nutrient supply and materials used for the micro-bioreactors, the corresponding 3D cell culture techniques and the related cellular microenvironment, the cell types and in vitro models used. As a data source we used the National Library of Medicine and analyzed the studies published from 2000 to 2020.

Keywords: micro-bioreactor; 3D cell culture; scaffolds; microfluidics; material; cellular microenvironment; tissue engineering; HTS; in vitro models

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1. Introduction

Micro-bioreactors (MBRs) represent specialized bioreactor systems that were, unlike their big brothers, namely classical bioreactors for biotechnological applications or industrial production, developed for growing mammalian cells and/or tissues in vitro on a smaller scale. In fact, MBRs may be considered as an intermediate stage towards more complex organ-on-a-chip (OoC) systems as they incorporate design elements of classical bioreactors, e.g., stirred or perfused flasks/chambers instead of large-scale tanks, and novel technologies including microfluidic components, cell-scaffolds, and setups enabling microscopic analysis and cell-based assays which are also part of OoC systems. Despite the technological advances in the field of OoC systems, MBRs are still prevalent in cell-based biomedical research and have become invaluable tools in eukaryotic 3D cell culture research because they (i) provide controllable 3D cell culture conditions usually including an active fluid flow supply, (ii) enable, unlike large-scale bioreactors, the usage of small amounts of chemical entities and low cell numbers when primary cell and/or tissue availability is limited, and (iii) depending on the design, are frequently amenable to microscopic imaging, or other, even more sophisticated analysis techniques, which facilitates cell culture evaluation during bioreactor operation. This has led to an ever-increasing amount of applications which provide insights that have not been possible before. In this context, a multitude of in vitro studies using 3D cell culture techniques individually or in combination with MBRs identified the following parameters which are relevant for the modulation of cell behavior: (i) spatial and temporal gradients of signaling molecules (e.g., growth factors, cytokines, and hormones), (ii) spatial distribution of cell–extracellular matrix (ECM) interactions, which are also inevitably coupled with the molecular and mechanical ECM properties, (iii) the spatial distribution of homologous and heterologous cell–cell contacts, and (iv) biomechanical forces emerging from interstitial fluid flow and tissue deformation.

Against this background and within the scope of this special issue of *Processes* bearing the same title, we would like to give an overview of the work published in the last 20 years in the area of 3D cell culture in micro-bioreactors and to thereby address the following questions:

- What has been done in the last 20 years in the field of micro-bioreactor design and what are the applications of such systems?
- Which systems have prevailed up till now?
- What are currently the most common techniques of 3D cell culture-based MBRs?
- Where is the journey taking us?

To answer these questions, we reviewed the studies in the field of 3D cell culture in MBRs between the years 2000 and 2020 (9 July 2020) systematically with respect to MBR design, the corresponding 3D cell culture techniques and the related cellular microenvironment, the mode of cell stimulation and/or nutrient supply, the materials used for MBRs and scaffold fabrication, the applications of the systems and the used cell type or in vitro model.

In this first part of the review, we analyze the published papers in terms of MBR design; this includes the used 3D cell culture technique, the mode of fluid flow and MBR materials, the applications of such systems and the origin and type of the cells in the last 20 years. On the basis of the literature research results we have structured the different approaches for better clarity by means of the complexity of the corresponding 3D cell culture techniques used in the systems, hereafter referred to as "complexity level", defined the main fluid flow mode and classified the micro-bioreactor types according to their principal design. Furthermore, as organ-on-a-chip (OoC) systems are sometimes very hard to distinguish from MBRs with incorporated microfluidics we try to elucidate what distinguishes MBRs from OoC systems, although barriers are fluent.

In review part two, we provide a more detailed description of the results of our literature research structured according to the aforementioned complexity levels and with focus on the existing MBR types and their applications, as well as on common cell/tissue types cultured in such systems. We then disseminate the different types of MBR applications including simulation studies and finally give an outlook to future developments in the field of 3D cell culture in MBR. With this review, we hope to be able to adequately introduce the reader into the great contributions of this special issue of *Processes* on Advanced 3D-Cell Culture Techniques in Micro-Bioreactors.

2. Methods and Definitions

For the detection and analysis of the relevant literature, the PubMed® database of the National Library of Medicine of the National Center for Biotechnology Information (NCBI) at the U.S. National Library of Medicine (https://pubmed.ncbi.nlm.nih.gov/) was used. The following search terms were defined to include different spellings of the keywords as well as their synonyms:

1. "3D cell culture" AND "microbioreactor";
2. (bioreactor OR microbioreactor OR micro-bioreactor) AND ("three-dimensional cell culture" OR "3D cell culture" OR "3-D cell culture");
3. (microbioreactor OR micro-bioreactor) AND "tissue engineering".

Moreover, secondary literature meeting the same search term results were included (Figure 1). Based on the results of our literature research we could extract the following parameters which are characteristic for MBRs and were used to define MBRs in the present review:

- culture volumes of less than 500 mL;
- provision of an active fluid flow, being realized by a pump, a stirring mechanism, piston movements or mechanical movement of the cell-based constructs in culture medium;
- 3D cell culture approach with or without scaffold.

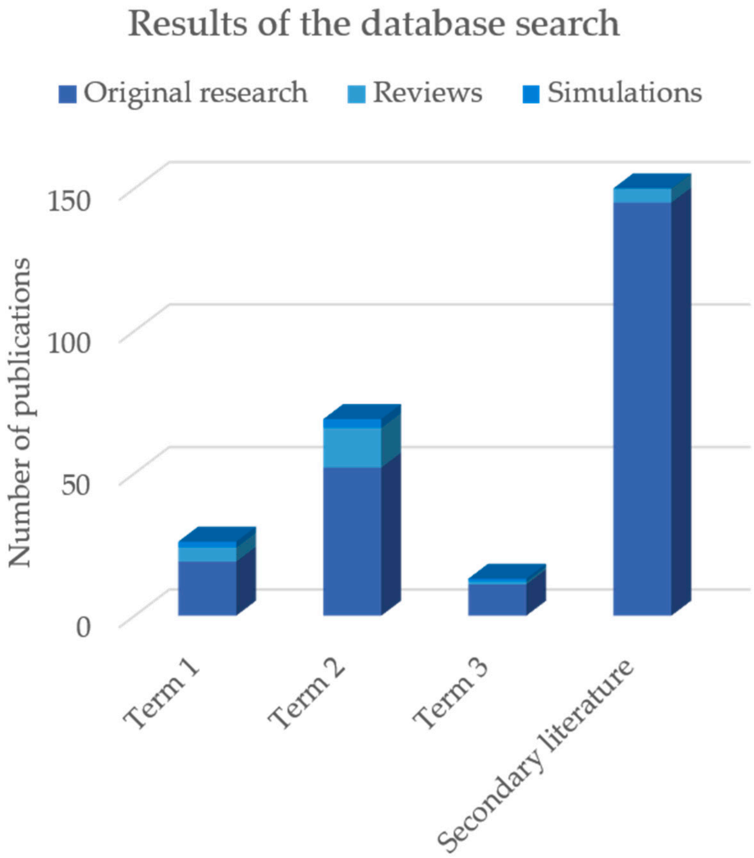

Figure 1. Number of publications yielded by the search terms in the period from 2000 to 2020. Term 1: "3D cell culture" AND "microbioreactor"; Term 2: (bioreactor OR microbioreactor OR micro-bioreactor) AND ("three-dimensional cell culture" OR "3D cell culture" OR "3-D cell culture"); Term 3: (microbioreactor OR micro-bioreactor) AND "tissue engineering".

By using this search strategy, 326 papers were retrieved of which 92 were excluded because they did not fulfil above mentioned MBR-definitions or because information on the used bioreactor type was insufficient. In addition to 192 relevant publications, we found 26 reviews and 10 publications which were dedicated to the simulation of flow rates, shear stress, or also oxygen distribution (Figure 1). These studies are discussed in the second part of the review. A total of 192 publications was the basis of the analysis with the corresponding figures and all papers were analyzed with regard to the following parameters: complexity level, bioreactor type, materials used for fabrication, scaffold- or non-scaffold-based culture technique, species origin of the cells used, type of organ, type of cells, and application.

Since 3D cell culture comprises many different techniques, we defined as mentioned before, complexity levels with regard to the culture configuration to structure the different strategies. Cellular complexity levels of the 3D cell culture configurations were defined as follows (Figure 2A):

1. Complexity level 1: cells immobilized in (hydro-)gels as monoculture (one cell type) or coculture (at least two cell types);
2. Complexity level 2: multicellular aggregates consisting of one cell type in 3D scaffolds or in scaffold-free cultures;
3. Complexity level 3: multicellular aggregates consisting of at least two cell types in 3D scaffold-based or in scaffold-free cultures.

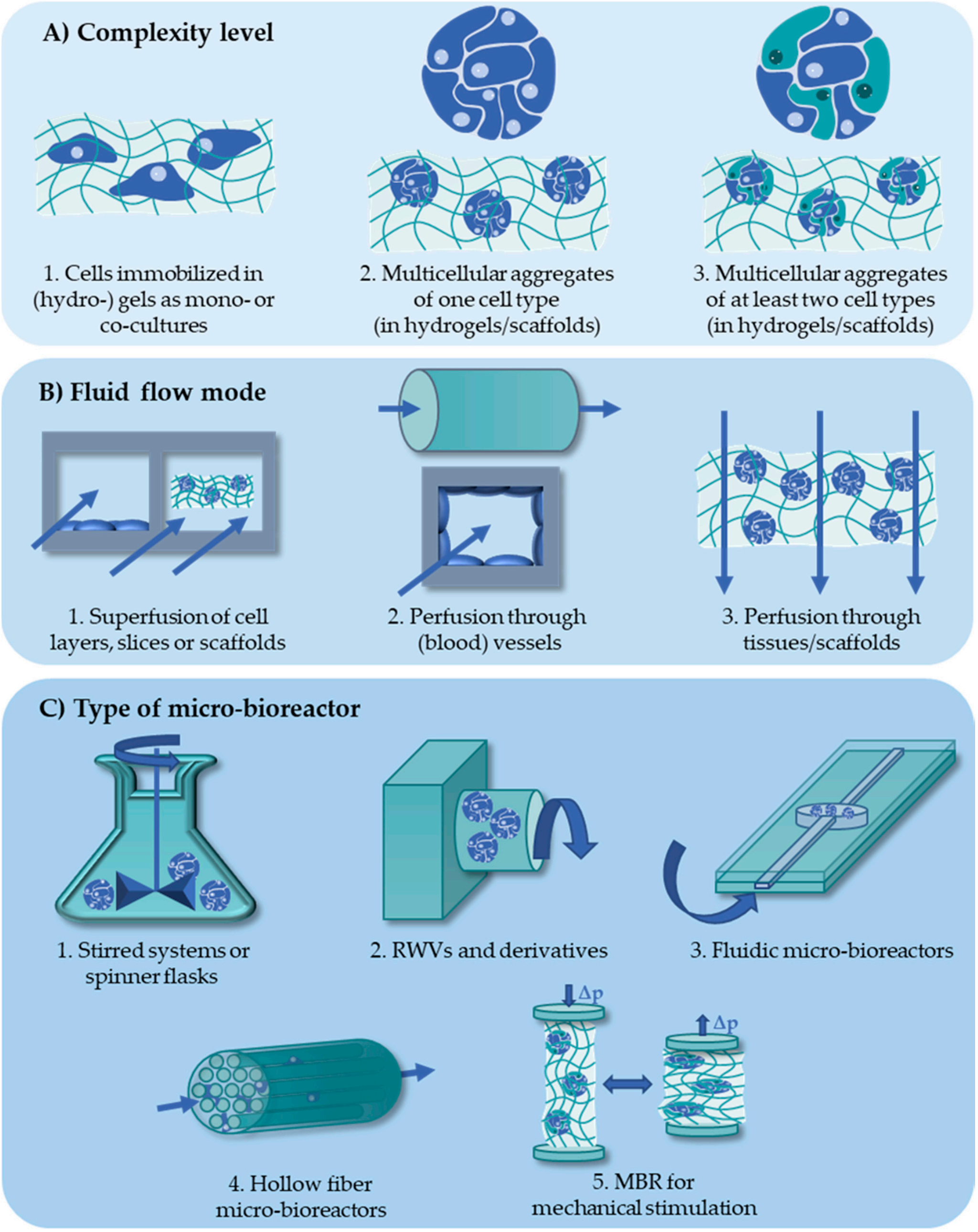

Figure 2. (**A**) Complexity levels defined by means of the applied 3D cell culture technique and with respect to the existing 3D microenvironment. (**B**) Differentiation between superfusion and perfusion of different 3D cell cultures. (**C**) Frequently used micro-bioreactor (MBR) types: 1. based on spinner flasks or stirred tank systems, 2. rotating wall vessels (RWVs), 3. fluidic MBR, 4. hollow fiber bioreactors, and 5. MBR for mechanical stimulation. The sketches show examples of the structure of the respective MBRs. Details are discussed in review part II.

With respect to the fluid flow mode in the context of microfluidic bioreactors, the term perfusion is now used almost inflationary for all fluidic models. In the medical context, perfusion is defined as the flushing of a blood vessel or the lymphatic system with a liquid. In a broader meaning, perfusion also refers to the fluid flow through three-dimensional cell constructs or scaffolds. Systems in which only cell layers are flushed, or tissues or scaffolds are flushed around them, do not fulfil these definitions. This is generally called superfusion or perifusion [1]. The perfusion of tissues/scaffolds can be clearly differentiated from superfusion. However, the distinction between superfusion and perfusion of (blood) vessels is difficult to determine in the current in vitro models. Therefore, in the context of this review, systems in which the medium flows through a channel surrounded by cells are considered to be part of a perfusion system (Figure 2B).

In order to realize a medium flow around or through the 3D cell-constructs/scaffolds or tissues in the MBRs, several bioreactor types have been established in the last years including (1) stirred systems or spinner flasks, (2) rotating wall vessels (RWVs), (3) microfluidic MBRs, and (4) hollow fiber bioreactors. Especially in the case of microfluidic MBR there are various designs enabling perfusion of the cell-based constructs/scaffolds in addition to superfusion (Figure 2C). Furthermore, in MBRs designed for mechanical stimulation ((5) in Figure 2C) the medium flow or mass transport through the 3D cell culture emerges by stretching or compressing the cell-based construct/scaffold or tissue. These are mostly in-house developments of the labs and details are presented in the second part of this review.

3. Results

In order to give the reader an overview of the MBR configurations, the materials used for fabrication, and 3D cell culture techniques employed in the available literature, we analyzed the following parameters in 5-year periods: (i) the complexity level of the underlying 3D cell culture technique (Figure 3), (ii) the frequencies of the MBR types (Figure 4), (iii) the materials used for MBR fabrication (Figure 5), (iv) the complexity level for scaffold-based and scaffold-free applications in relation to the MBR type (Figure 6), (v) the application field of the MBR types (Figure 7), and (vi) the type and origin of cells that are used for the different approaches (Figure 8).

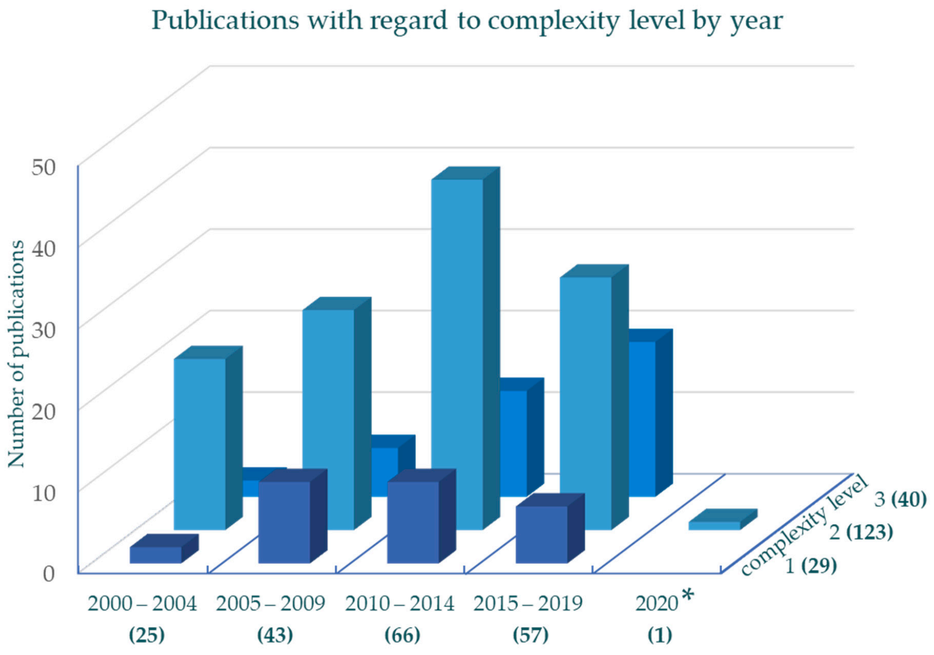

Figure 3. Distribution of studies in the period from 2000 to 2020 with regard to complexity level (for definitions, see "Section 2"). The number of the respective publications is shown in brackets. * status as per 9 July 2020.

Regarding the distribution of the studies over time with regard to publication date and complexity level, it can be seen from Figure 3 that applications of complexity level 2 contribute a considerable portion to the total number of publications (123 out of 192). Complexity levels 1 and 3 are less represented in this area and share a similar proportion with 29 and 40 publications, respectively. This distribution reflects the notion that 3D aggregates of the same cell type are the most popular model systems in the field of MBR culture.

Of the 192 publications falling in the time frame between 2000 and 2020 (9 July 2020), an increase in the publication number from 25 in the 5-year period of 2000–2004 to 43 in the period of 2005–2009, to 66 in the period of 2010–2014 can be observed. In the period of 2015–2019 a slight decrease to 57 publications in total can be detected. With respect to the temporal distribution of the study numbers within the complexity level groups, our analysis further demonstrates that the number of studies using cell culture configurations with complexity levels 1 and 2 in MBRs peaked from 2005 to 2014 and from 2010 to 2014, respectively. In contrast, the number of published studies on complexity level 3 approaches in MBRs increased continuously during the evaluation period. This trend indicates that cell culture models made of different cell types gain in importance within the area of 3D cell culture in MBRs.

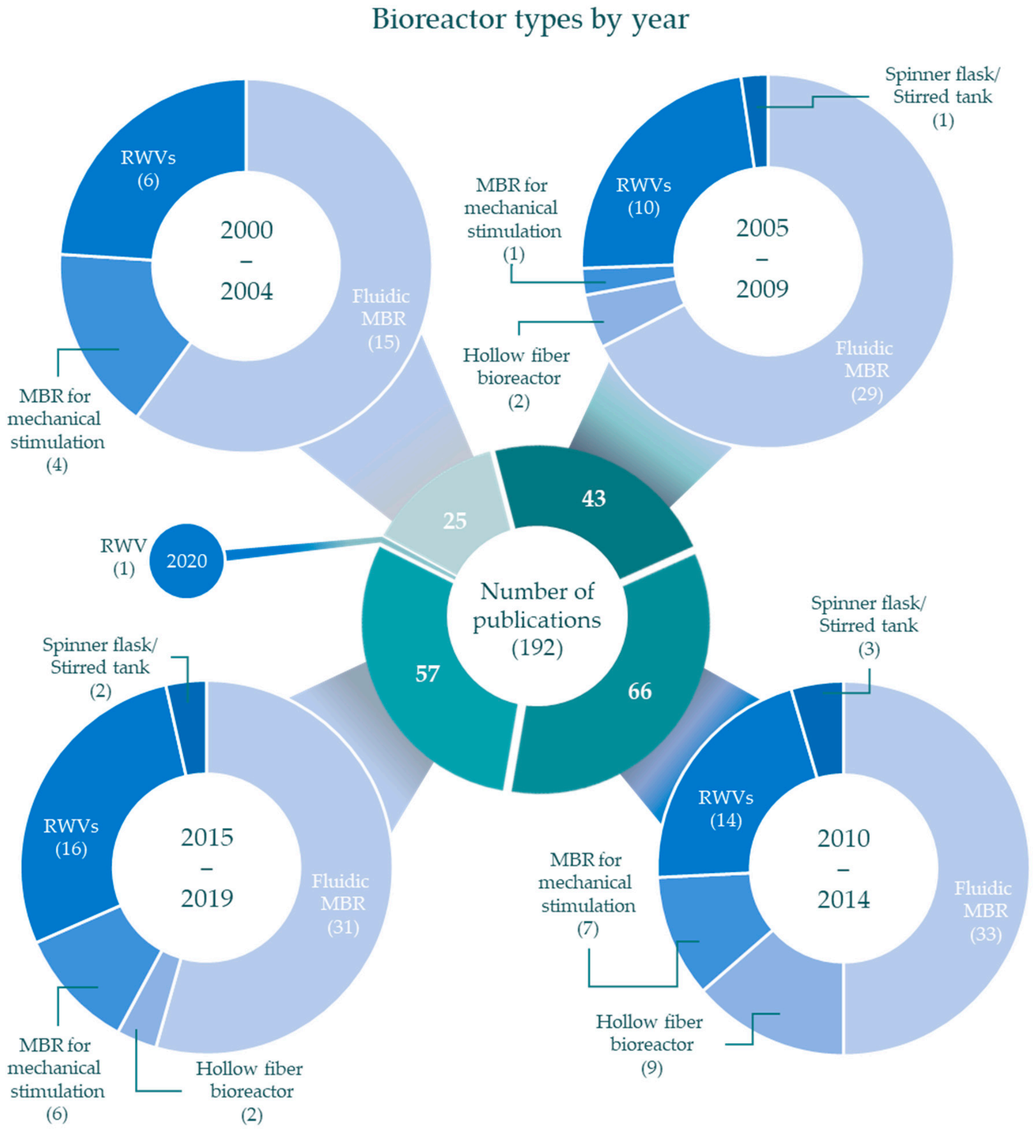

Figure 4. Distribution of micro-bioreactor types from 2000 to 2020 in the literature under study.

If the analysis is done with regard to the used bioreactor type in the same 5-year periods, it is clearly demonstrated that MBR systems with integrated microfluidics are the most prominent ones, followed by RWV type bioreactors (Figure 4). The use of MBRs for mechanical stimulation and hollow fiber MBRs take an intermediate position and vary between the specified periods. Furthermore, it is interesting to note that MBR types, developed at a very early stage of 3D-bioreactor culture, such as spinner flasks and hollow fiber systems, are still used nowadays.

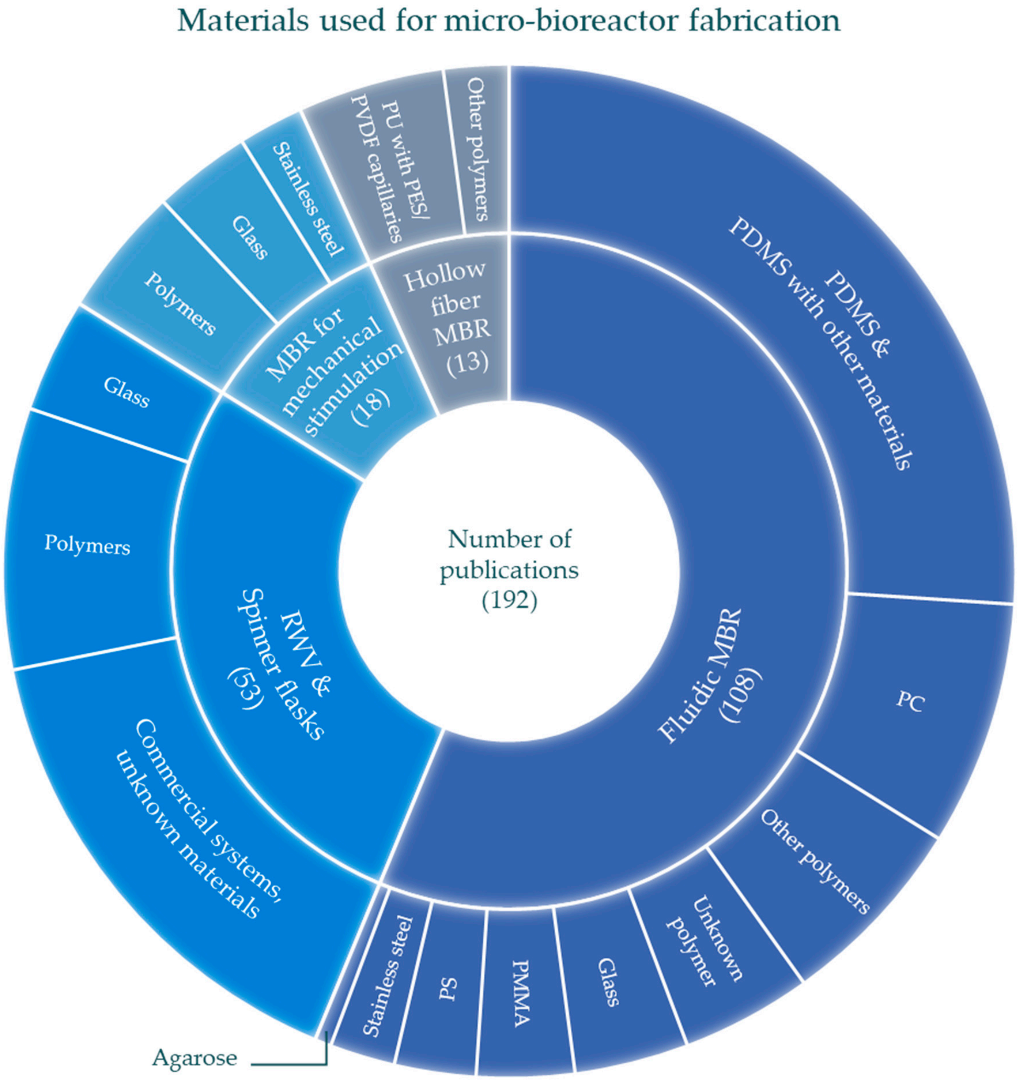

Figure 5. Distribution of studies in the period from 2000 to 2020 with regard to the materials used for MBR fabrication. The materials used for the most common types of MBR, namely fluidic MBR, RWVs and spinner flasks, MBR for mechanical stimulation and hollow fiber MBR. Abbreviations: PDMS (polydimethylsiloxane), PC (polycarbonate), PMMA (Poly(methylmethacrylate)), PS (Polystyrene), PU (polyurethane), PES (polyethersulfone), PVDF (polyvinylidene fluoride); "other polymers" include the publications with the less frequently applied materials stereolithography resin, polyethylene terephthalate, polylactic acid, polytetrafluoroethylene, PU (individually), polyethylene, polyoxymethylene, polypropylene, polyether ether ketone; the category "unknown materials" includes publications in which the applied MBR materials were not further specified.

When looking closer to the materials used for bioreactor manufacturing, it can be seen that polymer-based materials represent the major part. Polydimethylsiloxane (PDMS), individually or in combination with other polymers or glass, is thereby the most commonly used material and mainly applied when microfluidics is asked to be incorporated (Figure 5). This may be due to numerous in-house developments and optimizations in the field of fluidic MBR as PDMS is comparatively easy to process and to shape. Occasionally, 3D printed systems are also used [2,3]. Another large group of widely used MBRs, the RWV bioreactor type, is usually based on commercially available systems, e.g., from Synthecon. These are available as plastic or glass versions. However, the RWV version used in the corresponding publications were not always specified and therefore summarized under unknown materials in Figure 5. In addition to the polymer-based materials, stainless steel, besides glass, is often used in microfluidic systems and MBRs for mechanical stimulation. As the latter MBR type is primarily used in bone or cartilage tissue reconstruction to simulate the in vivo situation by applying mechanical forces, tougher materials like stainless steel are used for construction to meet the biomechanical requirements. In the group of hollow fiber MBRs, polyethersulfone (PU) and polyvinylidenedifluoride (PVDF) predominate as these materials are mainly used for the capillaries or hollow fibers of the MBR.

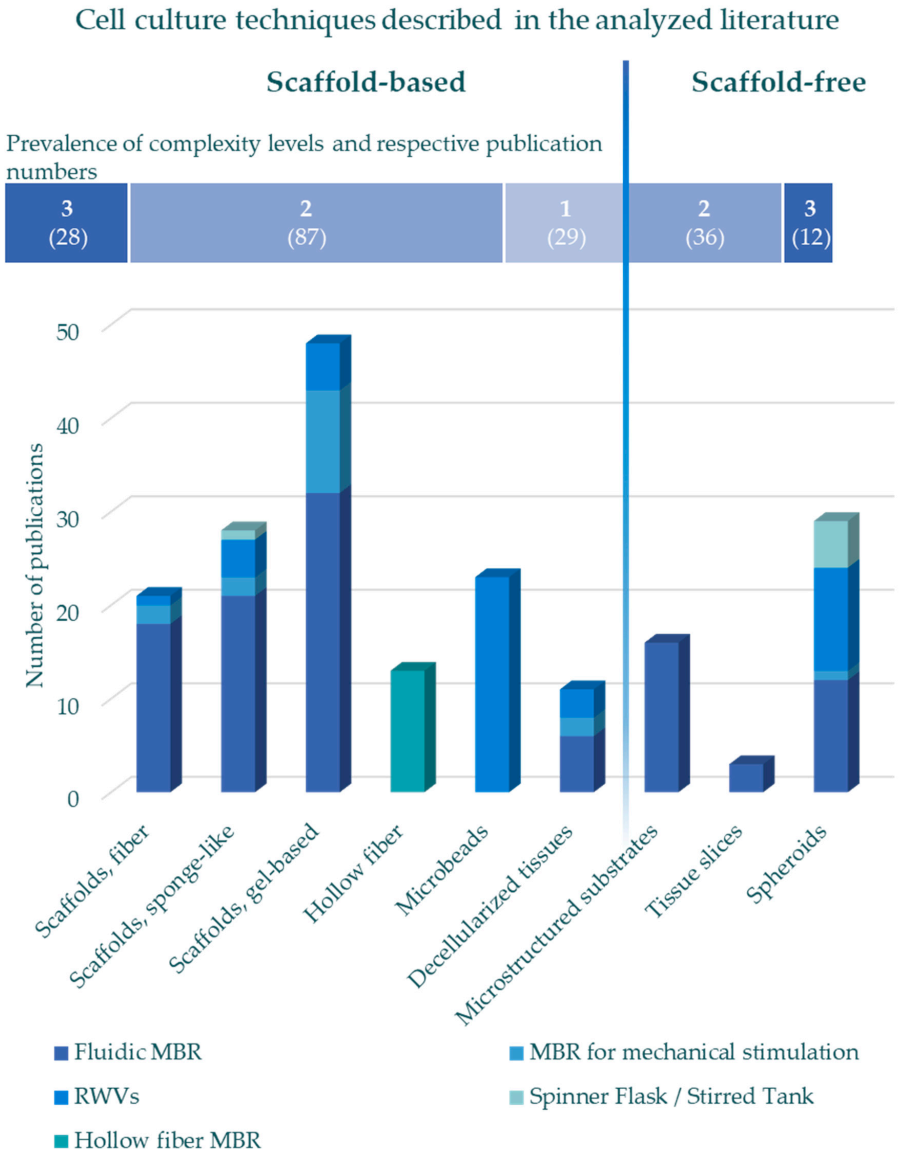

Figure 6. 3D cell culture techniques and their application described in the literature under study. The techniques were organized with regard to their complexity level, the use and nature of the scaffolds and their application in the various MBR types. The numbers in parentheses refer to the number of corresponding publications.

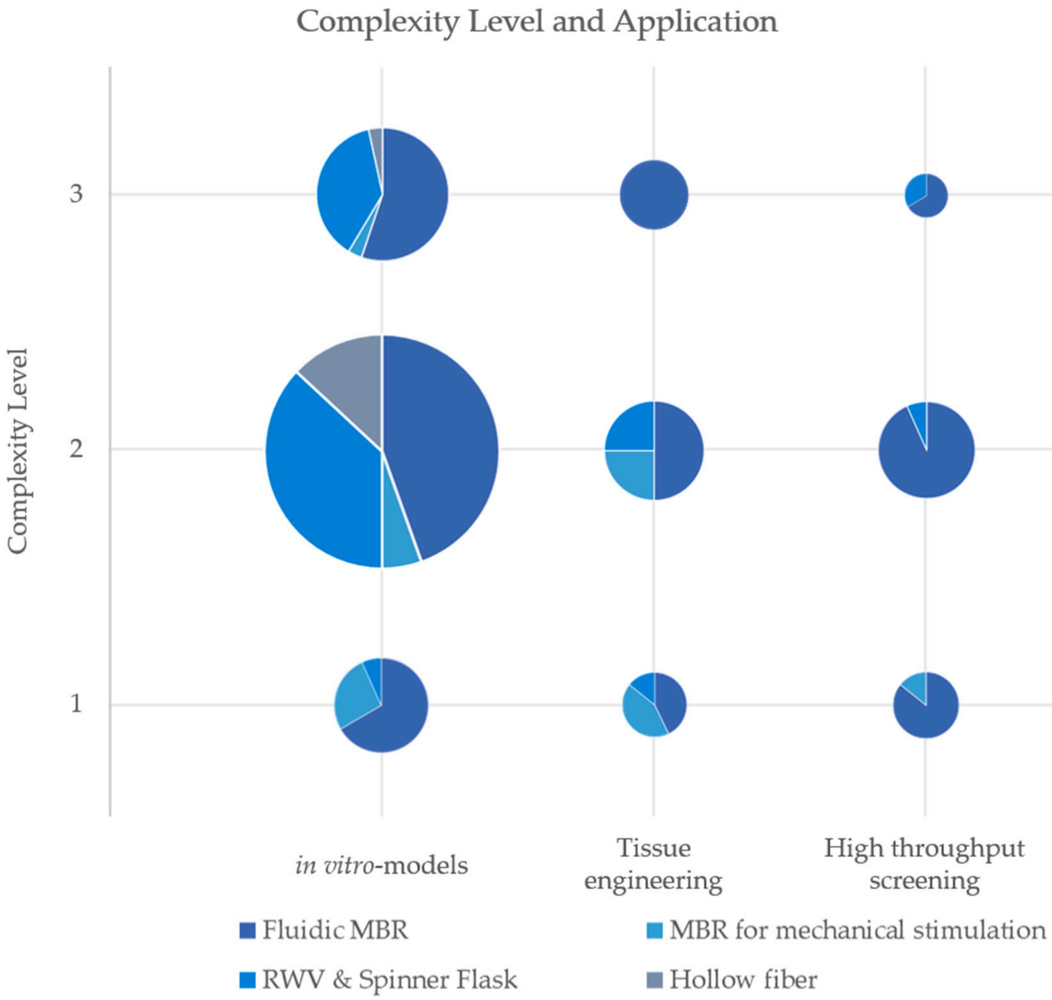

Figure 7. Application fields of the MBRs described in the analyzed literature with respect to the complexity levels. The dimensions of the pie charts reflect the number of publications in the respective application group and complexity level subgroup. Number of publications: 192.

As described earlier, parameters of the 3D microenvironment, such as gradients of signaling molecules, cell/cell- and cell/ECM-interactions, determine the cellular behavior in vitro. Hence, in order to induce or maintain tissue specific cell differentiation and functions, various cell culture techniques have been established which were classified in three complexity levels in the present review as described in Section 2 (Figure 2A). The techniques can further be divided in two groups, namely techniques using scaffolds for the immobilization and organization of the cells and scaffold-free techniques (Figure 6). Both techniques utilize the self-organizing, polarizing, and adhesion capabilities of the cells to form multicellular aggregates within scaffolds or without supporting structures. To give an overview of the distribution of the different techniques in the literature under study, we analyzed the described cell culture techniques with regard to complexity level, the use and nature of the scaffolds and their application in the various MBR types (Figure 6). The results from our literature analysis reveal that in the scaffold-based group the majority of studies used cell culture approaches with a complexity level of 2, i.e., multicellular aggregates of one single cell type (Figure 6). In the scaffold-free group, cell aggregates with one cell type (complexity level 2) also comprise a greater proportion when compared to complexity level 3 but is less strongly represented than compared to the scaffold-based group. If we take a look at the application of the specific cell culture techniques in the different MBR systems, our analysis demonstrates that gel-based scaffolds, composed, amongst others, of collagen, hyaluronan, polyethylene glycol, or Matrigel®, are most frequently used for 3D culture in microfluidic MBR systems, followed by sponge-like and fibrous scaffolds, and microstructured substrates. Microbead-based scaffolds were exclusively described in studies using RWVs and thus could only be assigned to this type of MBR, whereas spheroids are commonly applied in RWVs and spinner flask/stirred tank systems. This distribution can be explained by the immobilizing and structuring function of the scaffolds and hollow fibers, which is a prerequisite for proper perfusion or superfusion in microfluidic and hollow fiber MBRs. In contrast, the concept of RWV systems is to provide the nutrient and gas supply of the cells by stirring or rotating movement of the 3D-cell constructs such as microbeads and spheroids. Regarding the techniques used frequently in MBRs for mechanical stimulation, gel-based, fibrous, and sponge-like scaffolds, as well as decellularized tissues play a relevant role (Figure 6) because they allow for transmission of the applied mechanical forces to the cells.

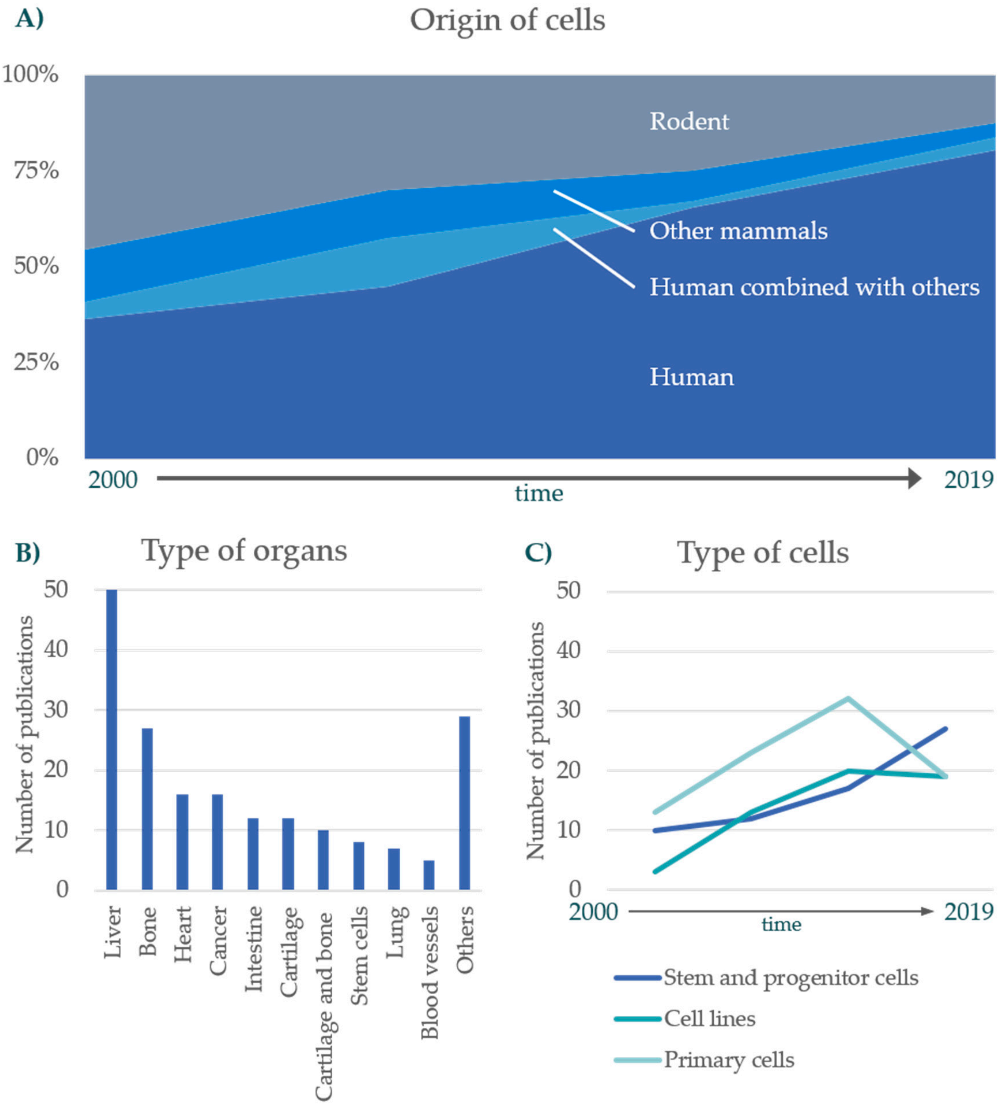

Figure 8. Distribution of the cells used in the publications under study with respect to the origin of the cells (**A**), the target tissues/organs (**B**), and the type of cells (**C**). Others: bladder, placenta, muscle, endometrium, skin, vaginal epithelium, retina, kidney, pancreas, nerves, connective tissue, and brain. Number of publications: 192.

Since 3D culture techniques have proven useful in many applications we grouped the papers into the three most common fields of application that could be extracted, namely "in vitro models", "tissue engineering", and "high-throughput-screening (HTS)", split them according to the complexity level in subgroups and examined the MBR distribution within the respective subgroups. For this, we deduced the main application area indicated in the papers or categorized them by the soundness and impact of the results when no main application was specified, or more than one application possibility was given. The presented results may therefore not be considered as strictly as Figure 7 proposes since there are smooth transitions possible, especially between the groups "in vitro models" and "tissue engineering".

As presented in Figure 7, most of the analyzed studies could be assigned to the "in vitro models" group and were mainly done with cell cultures of complexity level 2, i.e., cell aggregates of one cell type. In this group, approaches that used two or more cell types for the in vitro model, i.e., complexity level 3, represented the second largest proportion or subgroup which was followed by complexity level 1 techniques using immobilized cells in (hydro-) gels. In the "tissue engineering" group again publications using complexity level 2 cell cultures predominate and the number of studies with complexity level 3 was lowest. The same trend holds true for the HTS group. The pie charts further reveal that microfluidic MBRs are most strongly represented throughout all groups, and prevail particularly in the HTS group. The latter might be due to the possibility to integrate analysis features into microfluidic MBRs which support data evaluation in HTS systems. The second largest share among the MBR systems are represented by RWVs and spinner flasks as they support the generation of uniform and complex cell aggregates which are often used for in vitro models. Table 1 lists publications that are included in our analysis.

Table 1. List of the 192 publications used for the analysis, broken down by complexity level and type of micro-bioreactor.

Complexity Level	Type of Micro-Bioreactor				
	Spinner Flask or Stirred Tank	**RWVs and Derivatives**	**Fluidic Micro-Bioreactor**	**Hollow Fiber Micro-Bioreactor**	**MBR for Mechanical Stimulation**
1		[4,5]	[6–24]		[25–32]
2	[33–35]	[36–71]	[3,24,72–132]	[133–144]	[145–153]
3	[154–156]	[157–165]	[2,166–190]	[191]	[192]

Since our literature analysis revealed that most publications under study involve "in vitro models" we were next interested in the distribution of the applied cell types and target tissues/organs in the MBR field (Figure 8). With respect to the origin of the cells, the examination of the published literature between 2000 and 2019 revealed that at the beginning of the 2000s, human and rodent cells were the most common models, applied in 41% (human cells and combinations) and 45% of the studies, respectively (Figure 8A). By comparison, the share of publications using cells from other mammals, mainly bovine for bone and cartilage models, was at 14% and thus relatively low. During the period under review, the usage of human cells individually or in combination with cells of other origin increased to about 80% by 2019, whereas publications using rodent and other mammalian models declined to 13% and 4%, and thus play a minor role in this research field by now. The reason for this trend becomes apparent when the distribution of the target tissues/organs is analyzed (Figure 8B). From this follows that most published studies address the liver, with the primary objective to establish liver models for metabolism and toxicity testing (data not shown). Since meanwhile it is commonly agreed upon that rodent hepatocytes do not adequately reflect human hepatocyte functions with respect to the panel of detoxification enzymes and proteins, in vitro models based on rodent primary cells lost most of their importance. A similar situation may prevail for other tissues displayed in Figure 8B. Regarding the type of cells cultured in the MBR systems, our literature research demonstrates that in the early 2000s a higher number of studies used primary cells, i.e., differentiated and/or mature cells, followed by stem and progenitor cells, and to a lesser extent cell lines (Figure 8C). Interestingly, the application of primary cells considerably increased until 2014 and sharply decreased from 2015 to 2019 to the same level as cell lines which also increased to a lesser extent in the same period. The progress in the field of stem cell research however may lead to a moderate but constant increase of the publications using stem and progenitor cells, and thereby is gradually replacing primary cells and cell lines.

As mentioned at the beginning, it is not always easy to distinguish MBR approaches and organ-on-a-chip (OoC) systems, as definitions are not consistent and transitions are fluid. OoCs are defined as microengineered devices with microfluidic channels with at least one cell culture compartment in which functional units of organs are modeled [193–195]. Organ-on-a-chip systems do not only mimic human organs' in vivo physiology, for instance by providing an active flow, they also integrate actuators or sensors for further analysis.

To give the reader an idea of the number of published studies using OoC in comparison to MBR systems we conducted a second literature research with the term [("organ on chip" OR "organ-on-chip" OR "organ on a chip" OR "organ-on-a-chip") AND ("tissue engineering" OR "three dimensional cell culture" OR "3d cell culture" OR "3-d cell culture")]. As depicted in Figure 9 the number of recently published studies using 3D cell cultures in MBR has been almost constant in the last years, whereas the number of OoC-related publications has increased dramatically in the last 5 years. At second glance, however, the published systems do not always reach the complexity defined for OoCs, such as integrated sensors, respectively, actors or modular combination of different organ models. Although MBRs are often referred to as organ-on-chip systems [81], a distinction was made between OoCs and MBRs as described above. Since OoC systems are repeatedly considered to be among the top emerging technologies [196], it could be speculated that the use of this keyword is to increase the popularity of the results.

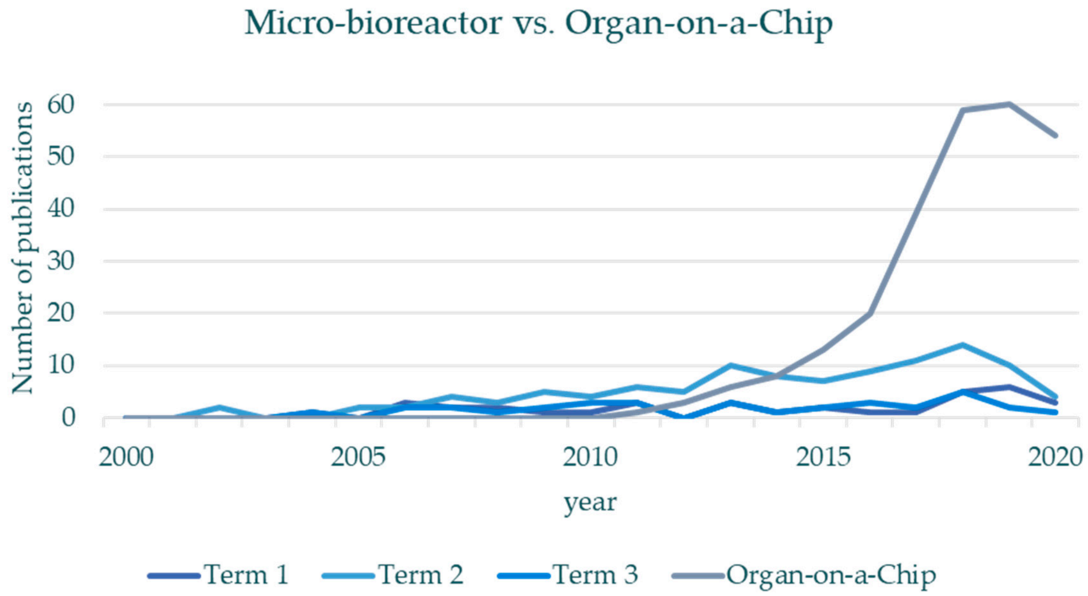

Figure 9. Search results in the database PubMed between 2000 and 2020 with the search terms 1–3 used in this review for micro-bioreactors (Term 1: 3D cell culture" AND "microbioreactor; Term 2: (bioreactor OR microbioreactor OR micro-bioreactor) AND ("three-dimensional cell culture" OR "3D cell culture" OR "3-D cell culture"); Term 3: (microbioreactor OR micro-bioreactor) AND "tissue engineering" compared to organs-on-a-chip (search term: ("organ on chip" OR "organ-on-chip" OR "organ on a chip" OR "organ-on-a-chip") AND ("tissue engineering" OR "three dimensional cell culture" OR "3d cell culture" OR "3-d cell culture"), status as per 27 October 2020).

4. Conclusions

The results of our systematic review revealed that MBRs became very popular between 2000 and 2014, with microfluidic MBRs being most strongly represented in the fields of fundamental research with in vitro models, tissue engineering, and HTS. With respect to the cell culture configuration used in the MBR systems during this period, 3D aggregates of the same cell type, consisting mainly of human primary cells or cell lines are the most frequently used technique in this period, irrespective of whether scaffold-based techniques are applied or scaffold-free ones. Our analysis further demonstrates that MBR systems established very early in bioreactor-based 3D culture and tissue engineering are still used until today, possibly due to the commercialization of the systems. In the last 5 years, however, a new trend can be observed towards more complex 3D culture systems with an increasing number of publications using 3D coculture models, i.e., cell cultures consisting of at least two cell types, and human cells. Within the group of human cells, stem and progenitor cells have become very popular. This trend towards more complex human stem/progenitor cell-based in vitro models requires however further miniaturization of the systems as cell/tissue sources, especially with respect to personalized medicine, are often limited or yield low cell amounts. Consequently, further developments in 3D cell culture techniques, such as unique media formulations for coculture models, and in cell function analysis of low cell numbers or even on a single-cell level will also advance in the next years. With respect to the bioreactor systems, this trend manifests by a decrease in the number of MBR publications which is accompanied by a pronounced increase in the organ-on-a-chip literature. This most likely reflects the progress made in manufacturing and/or cell culturing techniques as well as the increase in experience made by using MBRs.

In part II of the review (Systems and Applications) we will have a closer look at the micro-bioreactors and the specific applications that they have been used for.

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