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# Review of three-dimensional spheroid culture models of gynecological cancers for photodynamic therapy research

RWK Wu [a,*], JWM Yuen [b], EYW Cheung [c], Z Huang [d], ESM Chu [c,*]

[a] Department of Biological and Biomedical Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Glasgow, Scotland, UK
[b] School of Nursing, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong Special Administrative Region of China
[c] School of Medical and Health Sciences, Tung Wah College, Hong Kong Special Administrative Region of China
[d] MOE Key Laboratory of Photonics Science and Technology for Medicine, Fujian Normal University, Fuzhou, China

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ABSTRACT

Photodynamic therapy (PDT) is a specific cancer treatment with minimal side effects. However, it remains challenging to apply PDT clinically, partially due to the difficulty of translating research findings to clinical settings as the conventional 2D cell models used for *in vitro* research are accepted as less physiologically relevant to a solid tumour. 3D spheroids offer a better model for testing PDT mechanisms and efficacy, particularly on photosensitizer uptake, cellular and subcellular distribution and interaction with cellular oxygen consumption. 3D spheroids are usually generated by scaffold-free and scaffold-based methods and are accepted as physiologically relevant models for PDT anticancer research. Scaffold-free methods offer researchers advantages including high efficiency, reproducible, and controlled microenvironment. While the scaffold-based methods offer an extracellular matrix-like 3D scaffold with the necessary architecture and chemical mediators to support the spheroid formation, the natural scaffold used may limit its usage because of low reproducibility due to patch-to-patch variation. Many studies show that the 3D spheroids do offer advantages to gynceologcial cancer PDT investigation. This article will provide a review of the applications of 3D spheroid culture models for the PDT research of gynaecological cancers.

## 1. Introduction

Gynaecological cancers refer to cancer that starts in a woman's reproductive organs. One of the challenges to the conventional treatments is the preservation of fertility. Patients who received treatments might experience an increase in the abortion rate. Studies also revealed the high recurrent rate of gynaecological cancers after receiving conventional treatments [1]. Therefore, there is an urgent need to develop novel treatment strategies for gynaecological cancer patients. Photodynamic therapy (PDT) is an FDA-approved cancer treatment for several malignant diseases, including skin cancer, lung cancer, oesophageal cancer, and head and neck cancer [2]. It is also used for gynaecological cancer, pre-cancer and HPV infection treatment, with no serious adverse effects reported [3]. PDT offers advantages over surgery, chemotherapy, and radiotherapy including non-invasive, highly specific, minimal side effects, low systemic toxicity, limited loss of function after treatment, re-sensitize resistant cells to conventional treatments, and could be used multiple times [4,5]. PDT could also be used for palliative intention for cancer patients who have exhausted all treatment options. PDT is a light-based technology that uses a combination of photosensitizing agents (PS), visible light, and molecular oxygen to selectively destroy the biological target. Either the PS, visible light (usually with wavelength 600 – 800 nm for better tissue penetration), or molecular oxygen alone is not toxic to cells, but when combined can generate reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{-\bullet}$), and hydroxyl radical (HO•). PDT initiates photo-destruction of biological targets and causes direct cell disruption through apoptosis, necrosis, autophagy, tumour-associated vasculature disruption leading to tissue ischaemia, inflammation, and immune modulation [5,6]. PDT effect depends on the accumulation and localization of PS, molecular oxygen concentration, and the energy and wavelength of light illumination. The abnormal tumour stroma and the lack of lymphatic drainage favours the accumulation of PS in cancer cells [7]. The ROS generated by light activation of PS with a short lifetime, which limits its diffusion in cells. The phototoxic effect of PDT thus highly depends on the properties and intracellular location of the PS and the precise light activation at the

* Correspondent author.
*E-mail addresses:* ricky.wu@gcu.ac.uk (R. Wu), elliechu@twc.edu.hk (E. Chu).

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desired site and generates ROS to damage biological targets but preserves normal surrounding cells [8,9].

## 2. PDT anti-tumour mechanisms

PDT can eradicate cancer cells via apoptosis, necrosis, and autophagy. In most cases, PDT induces a mixture of these to eradicate cancer cells [10]. Apoptosis could be activated by two pathways, named the extrinsic pathway and the intrinsic pathway. Studies demonstrated that PDT triggers apoptosis via both pathways, but mostly via the intrinsic pathway. The mitochondrion is one of the molecular targets for most PS [11,12]. ROS generated in the mitochondrion causes its destruction and results in DNA degradation, leading to apoptosis [13]. PDT could also induce apoptosis via alternating the signal transduction pathways including Bcl-2 and caspases pathways [14–19]. Necrosis is a rapid form of cell degeneration that results in the typical characteristic changes including cell swelling, and destruction of organelle and plasma membrane. PDT could induce necrosis by the direct destruction of the plasma membrane [20]. Autophagy is a catabolic process initiated in cells to remove damaged organelles and recycle cellular components [21]. However, the constitutive activation of autophagy can promote cell death as excessive self-destruction of cellular organelles results [22]. Studies demonstrated that PDT could induce autophagy formation induced by the oxidatively damaged organelles or the destruction of the mitochondria [23–26].

The tumour microvasculature is another common target of PDT as PS could accumulate in the endothelial cells similar to that of cancer cells. Through precise light activation, PS accumulated in vascular endothelial cells could generate ROS and destroy the vascular walls. The destruction of the tumour vasculature system could interrupt the nutrient supply to tumour and result in cancer cell death [27]. The PDT effect on the vascular system could be further enhanced by applying the short drug-to-light interval providing a good partition of PS accumulation in vascular endothelial cells (maximize) and in surrounding normal cells (minimize) [28,29].

Recent studies demonstrated the effect of PDT to induce an immune response. It has come to light that PDT-mediated ROS activities can trigger local and systemic inflammatory responses [30]. PDT-induced necrosis and vasculature destruction results in the release of immune mediators and cytokines including NF-kB, AP-1, tumour necrosis factor-alpha (TNF-α), and granulocyte colony-stimulating factor (M-CSF), IL-1, IL-6, IL-8, and IL-10 [31]. Amongst those, IL-8 and M-CSF are known as chemo-attractants while IL-1, IL-6, and TNF-α are known as pro-inflammatory factors. Additionally, potent pro-inflammatory factors such as membranous lipids and arachidonic acid metabolites were stimulated following photo-oxidation [32]. The release of such immune mediators and cytokines alters the tumour microenvironment and initiates an acute inflammatory response. PDT could also induce the release of chemo-attractants, which attract the infiltration of immune effector cells including neutrophils, mast cells, macrophages, and dendritic cells to the tumour stroma. Upon arrival, these immune cells perform phagocytosis to engulf the damaged cancer cells and present specific antigens to the helper T lymphocyte and activate the cytotoxic T lymphocyte, which initiates an adaptive immune response [33]. These activated cytotoxic T lymphocytes not only work locally on the tumour stroma but also work systemically through circulating the body to provide the specific anti-tumour immune response [34].

## 3. Challenges of *in vitro* PDT study using 2D cell culture models

The high tumour selective property of PDT and its advantages over chemotherapy and radiotherapy attract the investigation of PDT therapeutic roles in cancer. Different pre-clinical *in vitro* and *in vivo* models have been developed to study the PDT efficacy and the underlying PDT bio-mechanisms on various cancer origins [35]. Amongst these, the conventional 2D monolayer cell culture model is the most popular culture model used in PDT *in vitro* studies. Our group also applied 2D cell culture models to determine the PDT responses and elucidate the anti-tumour mechanism involved in PDT in different types of cancers, such as gynaecological cancer [36], uterine sarcoma [37], and nasopharyngeal carcinoma [38–42].

Although 2D cell culture offers advantages including low cost, simplicity, reproducibility, and less time required compared to animal models, it is accepted as not physiologically relevant to a solid tumour as it usually with an adequate, steady, and uniform supply of nutrients, oxygen, and other essential components such as growth factors, which is not the case in a solid tumour [43]. The 2D monolayer cell culture is too simple and fails to mimic the complex or true natural tumour microenvironment. The 2D cell culture models also lack cell-to-cell and cell-to-extracellular matrix communications. As a result, the gene and protein expressions, cell behaviour, and functions between a 2D cell model and the three-dimensional (3D) tumour fed by blood circulation are proved to have significant differences [44]. It is also difficult to optimize the PDT dosimetry based on a 2D cell model as the diffusion distance of PSs, light penetration, and the intracellular oxygen concentration may vary from a 3D solid tumour. It is also impossible to study the effect of PDT on vasculature disruption, immune cell infiltration, and the epithelial-mesenchymal transition by using the monolayer 2D cell models [45]. These variations between 2D cell culture models and solid tumours limited the value of bench-side research as findings are difficult to translate into the clinical setting. In this connection, a physiologically and pathophysiologically relevant 3D cell culture model is needed for the study of PDT therapeutic roles in cancer.

## 4. The use of 3D spheroid cell cultures in gynecological cancer PDT studies

The use of the 3D spheroid culture model could partially address these limitations. The 3D spheroid cell culture model is currently accepted as a bridge to fill the gap between conventional *in vitro* cell culture models and *in vivo* animal models. The 3D spheroid model is an *in vitro* system that mimics the *in vivo* tumour microenvironment and the natural development of cells. It allows cells to aggregate and create their microenvironment by forming tissue spheroids or embedding cells on the defined scaffold that mimics the extracellular matrix and exhibits features that are closer to the complex *in vivo* conditions [46]. These features are particularly useful for PDT studies as 3D spheroids provide a better evaluation of PS distribution and PDT efficacy. We identified 44 studies using 3D spheroids for gynaecological cancer PDT investigations from 1998 to 2023 (Supplementary Tables 1 and 2). Griffiths et al. group first used the 3D ovarian spheroid model to study PDT efficacy (Cell viability) [47]. Since after, there is an increased interest in using 3D spheroids for PDT studies with significant growth in publications being seen in the last decade from 2 studies in 2013 to the highest of 14 studies in 2020 shown in Fig. 1. The use of scaffold-free and scaffold-based methods for cervical and ovarian cancers are shown in Fig. 2.

### 4.1. 3D spheroid models for PDT research

Compared with the 2D monolayer cell culture, the 3D spheroids are usually characterized by a lower proliferative rate, a higher resistance to anti-cancer treatments, a higher hypoxia core, different gene and protein expressions, and different cell-to-cell and cell-to-extracellular matrix (ECM) interaction, due to the complex cell populations and the 3D architecture of spheroids [48–51]. Different cell populations usually being identified in the 3D spheroids depend on the spheroid size, including proliferating, quiescent, apoptotic, hypoxic, and necrotic cells (Fig. 3) [52].

Proliferating cells usually present at the outer layer that has an adequate supply of nutrients and oxygens, and could mimic cancer cells close to the capillaries *in vivo*. The quiescent cells usually appear at the middle layer as the increase in diffusion distance progressively decreases

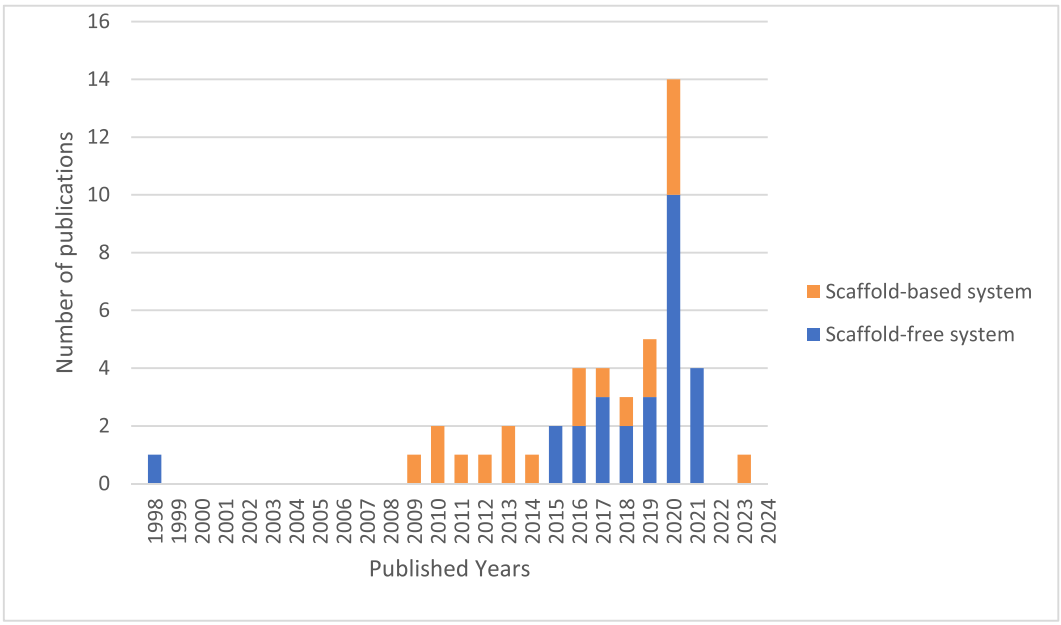


**Fig. 1.** This figure demonstrated the number of publications on photodynamic therapy studies using 3D spheroids generated by different methods from 1989 to 2023 May.

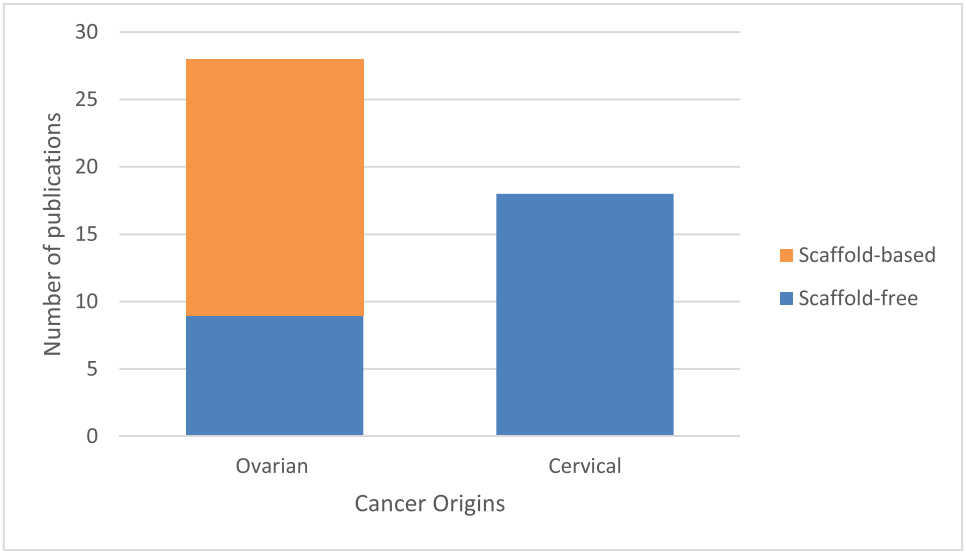


**Fig. 2.** This figure demonstrated the number of publications on photodynamic therapy studies using 3D spheroids generated by different cancer origins.

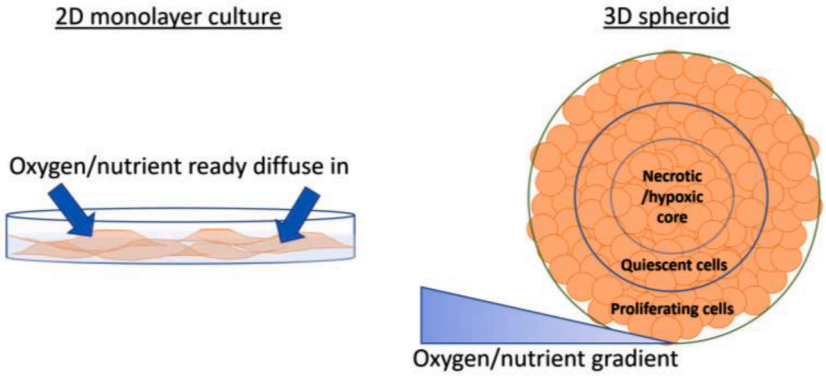


**Fig. 3.** This figure demonstrated different cell layers in 3D spheroids.

the supply of nutrients and oxygens and limits the cell metabolisms. Without the support of the vascular system, the core is usually the necrotic cells due to the hypoxic condition, low supply of nutrients and essential elements, and the accumulation of waste products [53]. These cell populations in 3D spheroids affect the drug diffusion rates, light penetration rates, and oxygen content across the spheroid, making it a suitable cell culture model for PDT studies. The majority of studies unitised the *in vitro* 3D spheroids to evaluate the uptake and bio-distribution of photosensitizers, PDT efficacy, PDT-induced changes to ROS level and hypoxia, PDT-induced gene and protein expression, and PDT-induced immune response (Supplement Tables 1 and 2).

As of today, there is no standardized method to generate 3D spheroids for PDT studies. Various techniques have been used for the development of 3D spheroids in PDT studies, which could be simply classified as the scaffold-free method and the scaffold-based method (Fig. 4). Table 1 summarized the characteristics of different methods used to generate 3D spheroids for PDT studies.

#### 4.1.1. *The use of scaffold-free methods for PDT studies*

The scaffold-free methods describe methods used to form 3D spheroids within a suspension without external support from a scaffold and the size of the spheroid could be controlled by factors including initial cell density, days of culture, percentage of FBS in medium, and types of culture medium used. It is the least complicated method making it suitable for 3D spheroid formation using different cancer origins. Different techniques could be used for scaffold-free spheroid formation, including the hanging drop method, the liquid overlay method with ultra-low attachment plate/agarose-base/other-bases, the spinner flask method, and the microfluidic method [54,55]. The scaffold-free methods remain one of the most popular methods used for gynceological PDT studies with more than 55 % of studies using these methods to obtain spheroids within 1 to 7 days with spheroid size/diameter commonly selected from 200 to 500 μm (Supplementary Table 1).

##### 4.1.1.1. *Hanging drop method for PDT studies.*

The hanging drop method is a technique based on the application of surface tension and micro-gravitational force to form spheroids. A small droplet of cell suspension with the desired cell density is placed on a reversed culture lid to facilitate cell aggregation. The hanging drop method offers researchers advantages including inexpensive, high efficiency, reproducible,

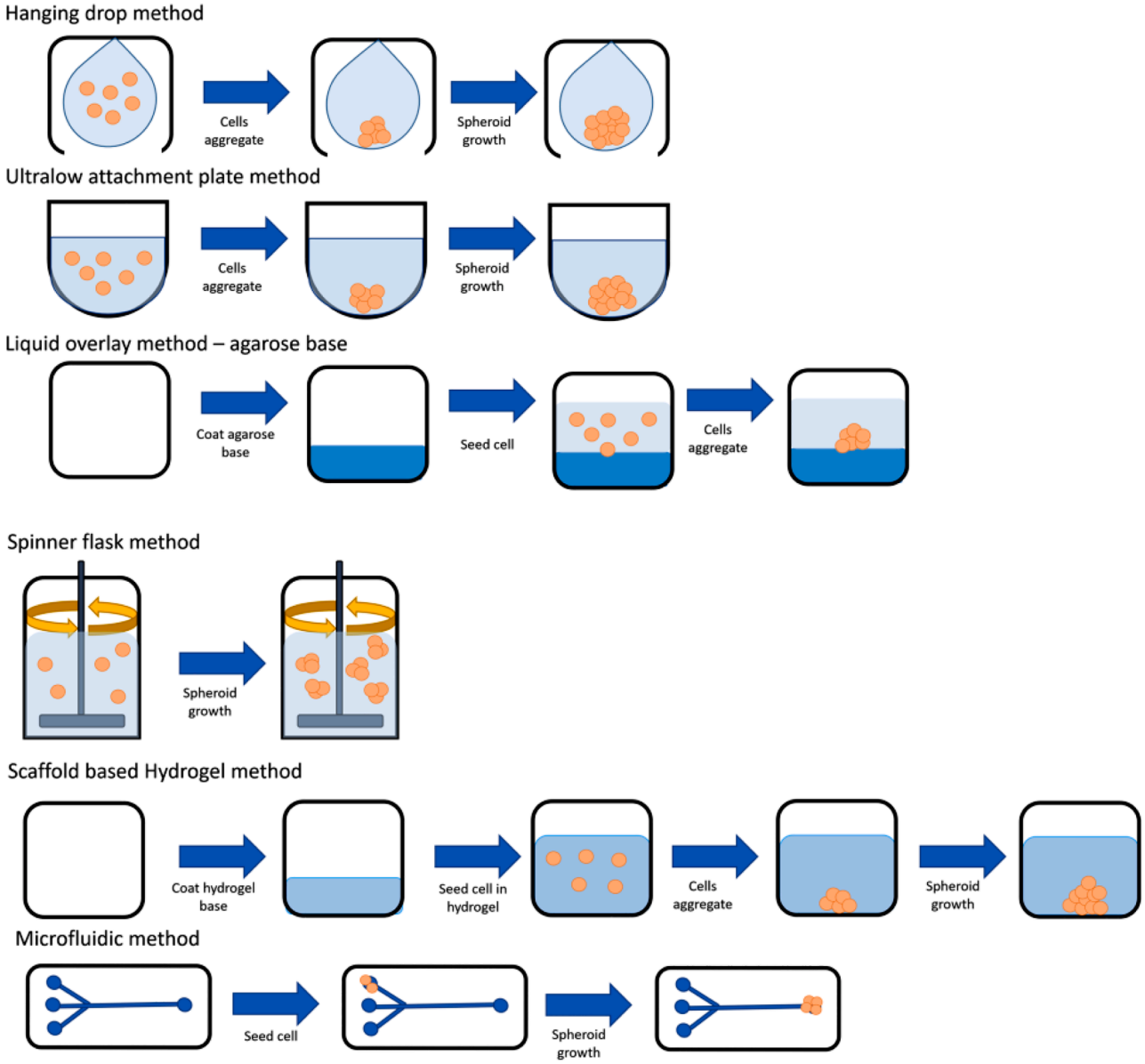


**Fig. 4.** This figure demonstrated common techniques used for the formation of spheroids for PDT studies.

**Table 1**
Summary of methods used to generate 3D spheroids for PDT studies.

| Key characteristics | Scaffold-free methods | | | | | Scaffold-based methods | |
|---|---|---|---|---|---|---|---|
| | Hanging drop method | Liquid overlay method – ULA plate | Liquid overlay method – Agarose/gel base | Spinner flask method | Microfluidic system | Matrigel | Collagen |
| Spheroid size | Uniform | Uniform | Uniform | Vary | Uniform | Vary | Vary |
| Number of spheroids obtained per reaction | Small | Small | Small | Large | Very small | Small | Small |
| The volume of solution retained | 50μL | 300μL/96well | 250μL/96well | – | Limited by the microfluidic system | 250μL/96well | 250μL/96well |
| Time to culture (day) | 1–4 | 1–7 | 1–4 | 10–15 | 2–7 | 2–13 | 1–7 |
| Reproducibility | ●● | ●●● | ●●● | ● | ●●● | ●● | ●● |
| Convenience (for PDT studies) | ●● (difficult to exchange medium/solution) | ●●● | ●● (culture plate needs pre-coat with agarose/gel) | ● (need to form cell aggregates before transfer to spinner flask for spheroid formation) | ● (high technical demand) | ●● (culture medium needs to be mixed with Matrigel) | ●● (culture medium needs to be mixed with Collagen) |

controlled microenvironment, and quick spheroids formation, usually with 1 to 4 days culture can results in spheroids diameters of 300 to 650 μm (Table 2). Gadzinski et al., Xiong et al. and Brand et al. used hanging drop spheroids to study the uptake and cytotoxic effect of PDT. Gadzonski et al. reported that spheroid is a more representative model of tumours for therapeutic study as the growth rate, drug diffusion distance and drug exits are different between spheroids and monolayer cultures. Their study demonstrated a slower photosensitizer uptake with Pc4 in spheroids and higher photosensitizer concentrations (Pc4) to achieve $IC_{50}$ in spheroids in comparison with the monolayer culture [56]. Xiong et al. also used hanging drop spheroids to study the PDT efficacy and penetration ability of photosensitizer APCs on spheroids. They reported a successful penetration of APCs (both at the core and the periphery of spheroids) with a high PDT efficacy on spheroids [57]. Brand et al. used hanging drop spheroids to study the penetration and phototoxicity of DARPin-IRDye 700DX conjugates. They reported a full penetration of photosensitizer and PDT efficacy on spheroids [58].

Although spheroids formed by this method are usually tightly packed, require no extra consumables, and are easy to set up, the limited volume of the drop (limited by 50 μL) restricted its application for PDT studies. The limited volume of the drop not only affects the size of spheroids' growth but also limits the initial cell densities used for spheroid formation, the days of culture (not suitable for long-term culture), and the volume of PSs added to the drop [55]. Our group used the hanging drop method to generate 3D spheroids for PDT studies and found the method labour intensive, time-consuming, with the technique required for the multiple washing steps, difficult to reproduce spheroids with similar size, and easy to disturb the spheroids in medium exchange during the steps of PS uptake and light activation [26]. The PDT experimental setup is also less favourable for the use of the hanging drop method as the multiple overturning of lids for medium exchange during the PSs admission and light activation increase the chances of disturbing spheroids, leading to experimental failure.

Several systems are now available to improve the experience of medium exchange and facilitate the transfer of spheroids for downstream assays for the hanging drop culture. These systems include Perfecta3D® hanging drop plate (3D Bioscience), Akura™ plus system (Insphero), and the hanging drop plate (SWISSCI). However, researchers are reminded that there is still a limitation of volume of the drop and with extra cost of consumables when using these hanging drop systems [26]. Table 2 showed the summary of using the hanging drop method to generate 3D spheroids for PDT studies.

*4.1.1.2. Liquid overlay method for PDT studies.* The liquid overlay method is the most popular method to generate 3D spheroids for PDT studies, with 2 to 7 days of culture usually resulting in spheroids diameters of 200 to 500 μm (supplementary Table 1). This method facilitates cell aggregation by preventing cells adhere to the surface of the culture flask or plate via the use of an ultra-low attachment U-bottom plate with a pre-coated surface, or any culture flask and plate manually coated with a layer of non-adherent inert substrates, which makes the method suitable for most cancer origins [59]. To enhance the success of spheroid formation, the cell culture plate could undergo centrifugation or orbit shaking after cell seeding to facilitate cell aggregation [26]. The liquid overlay method is easy to set up with spheroids formed usually with reproducible, regular size and morphology. Furthermore, this method allows researchers to modify the tumour cell microenvironment as a larger volume of solution could be retained. Zhang et al. and Karges

**Table 2**
Summary of PDT studies using the hanging drop method to generate spheroids.

| Cancer | | 3D spheroids | | | Photodynamic therapy (PDT) treatment condition/PSs condition for assays | | Ref |
|---|---|---|---|---|---|---|---|
| Cancer origins | Cell lines | Initial cell density (cells) | Growth time (days) | Spheroid size (μm) | Photo-sensitizers (PS) | Drug-to-light intervals/distribution (Hours) | |
| Cervical | CaSki, ME-180 | 10,000 | 1–4 | 300–650 | Pc4 | 24 | [56] |
| Cervical | HeLa | 4000 | 3 | 600 | Pyropheophorbide a (Aptamer-Pyro conjugates) | 1–2 | [57] |
| Ovarian | OV90, SKOV-3 | 15,000 | 2–3 | – | DARPin-IRDye 7000x conjugates | 2 | [58] |

N.B: (-) missing information from original papers.

et al. used the liquid overlay method to generate spheroids to study the effect of Ruthenium complexes-based PDT, with significant PDT cytotoxic effects observed [60,61]. Zhang et al. also reported that spheroids are accepted as valid 3D cancer models as they are heterogeneous cellular aggregates. They used 3D spheroids to study the combined therapeutic effect of photothermal therapy (PTT) and PDT, with results demonstrating that Ru complexes exhibit excellent PTT and PDT effects [60]. Our group also used this method to study the effect of hormonal modulation on the 3D spheroid culture systems by adding different concentrations of the hormone to the culture system at fixed time points to mimic the cancer hormonal microenvironment [62]. We found this method less complicated compared to the hanging drop method. It is also very difficult to establish this model using the hanging drop method as the method fails to retain a large volume of solution. Table 3 showed examples of using the ULA plate and agarose-based method to generate 3D spheroids for PDT studies.

*4.1.1.3. Spinner flask method for PDT studies.* The spinner flask method was first used by the Griffiths group to generate spheroids for the study of mTCMPC PDT on cell viability effect [47]. This method allows cells to keep in suspension and aggregate to form spheroids in a non-adherent cell culture flask via continuous stirring or rotation of the flask and is particularly common for colon cancer PDT studies. The advantage of using the spinner flask method is the possibility of generating a large number of spheroids in a single culture. However, the drawbacks are time-consuming (with usually 15 days for spheroid formation), with extra cost for the set-up of the spinner flask method, and the spheroids obtained usually with great differences in size and shape. Therefore, only a limited number of studies use the spinner flask method to generate 3D spheroids for PDT studies.

Griffiths et al. reported that the 3D spheroids are different from the monolayer cultures due to the complexity and the metastases, and used spheroids as a micrometastases model found in the ascites fluid in their study. Their study demonstrated that the penetration of m-TCMPC depends on the spheroid masses (negative relationship). They also reported that A2780 ovarian cancer spheroids were more sensitive to PDT than the monolayers [47]. Table 4 summarized the use of the spinner flask method to generate 3D spheroids for PDT studies.

*4.1.1.4. Microfluidic system for PDT studies.* The microfluidic system is known as the Lab-on-chip system, which uses microfabrication technology to design specific structures and scaffolds on a chip, allowing precise control of spheroid size, nutrient supply, and metabolic waste removal. With the help of hollow microchannels on the chip, the nutrients, drugs, and wastes can be steadily delivered or removed via continuous perfusion, resulting in a dynamic microenvironment for the study of the interaction between different cell types. Also, the microfluidic system with the advantages of high throughput, generates spheroids with uniform size, and allows monoculture and co-culture [63].

Flont et al. used a microfluidic system to generate spheroids with cellular multilayers that mimic the flesh and stroma in cancer tissue. They use this system to study the PDT efficacy of free and nanoencapsulated forms of meso-Tetrafenyporphyrin (TPP). Their findings demonstrated that cells in 3D spheroid can be cultured long-term and can mimic a fragment of cancer tissue. They also demonstrated that TPP-PDT can significantly reduce ovarian cell viability in a PDT dose-dependent manner [64]. Nath et al. also use a microfluidic system to study the effect of fluid shear stress on cancer treatment. They reported that BPD-based PDT caused a significant decrease in normalized viable tumour area under static and flow-induced shear stress conditions [65]. Although microfluidic systems provide researchers with better spheroid growth and better control of the fluidic environments, it is still not popular for PDT studies due to the high technical demand and resource implication for system development, and only accounts for a small number of the PDT 3D spheroids studies. Table 5 summarized the use of the microfluidic system method to generate 3D spheroids for gynaecological cancer PDT studies.

*4.1.2. Scaffold-based method for PDT studies*

The scaffold-based method requires the formation of a scaffold which provides the architecture, a variety of growth factors, and support that favour cell growth and aggregate three-dimensionally into spheroids within 1 to 13 days with size/diameter ranging from 100 to 700 μm (Supplementary Table 2). Hydrogel scaffolds are ECM-like 3D networks filled with a huge amount of fluids due to the presence of hydrophilic polymers, forming crosslinked networks. Hydrogels could be customized by changing their constituents and concentrations, providing a unique tumour microenvironment for different applications [46]. However, the scaffold-based method is less common because the data produced from this method are less reproducible. This is because the natural hydrogel collected from the mouse sarcoma cells usually contains unknown quantities of chemical compositions and with the batch-to-batch variations. Also, the effect of ECM composition on 3D spheroids formation is still not fully understood. Interestingly, the scaffold-based method with Matrigel attracts researchers' interest in PDT studies on ovarian cancer, with 68% of ovarian 3D spheroids studies using the scaffold-based method with Matrigel studying on PSs uptake, biodistribution, PDT cytotoxic effect, photobleaching, and mode of cell death (Supplementary Table 2). It is believed that the Matrigel scaffold provides better support with growth factors and hormones, which is of prime importance for hormone-dependent cancers [66,67]. The scaffold-based method is also suitable for the study of the PDT effect on the cell-to-ECM interactions as the hydrogel mimics ECM well.

Brand et al., Evans et al., and Rowlands et al. used the scaffold-based method to generate 3D spheroids for PDT studies. In Brand et al. study, they used the Matrigel-based spheroids to study the specificity of PDT targeting in a 3D environment and reported that only tumour cells were affected by PDT with a minimal bystander effect [58]. Evan et al. reported that the 3D spheroids provide many critical cell signalling cues that mimic the *in vivo* conditions. Their study demonstrated that BPD only diffused weakly into the nodules of the spheroid with a penetration depth of about 125 μm. Using the scaffold-based method Evans et al. also reported that the ECM barrier played a critical role in PS penetration. As a result, the BPD-PDT was observed to primarily kill cells at the exterior of large spheroids [68]. Similarly, Rowlands et al. reported the difficulty of Verteporfin in penetrating dense tumour spheroids and only the cells at the edges were well-treated with PDT [69]. Table 6 shows examples of

**Table 3**
Example of PDT studies using the ULA plate and agarose-based methods to generate spheroids.

| Cancer | | Scaffold-free methods | 3D spheroids | | | Photodynamic therapy (PDT) treatment condition/PSs condition for assays | | Ref |
|---|---|---|---|---|---|---|---|---|
| Cancer origins | Cell lines | | Initial cell density (cells) | Growth time (days) | Spheroid size (μm) | Photo-sensitizers (PS) | Drug-to-light intervals (Hours) | |
| Cervical | HeLa | Agarose base | 6000 | 3 | 400 | Ruthenium (II) complexes | 24 | [60] |
| Ovarian | A2780 | ULA plate | 4000 | 2–3 | 400 | Ruthenium (II) Complex (Ru (II)NP) | 12 | [61] |

N.B: (-) missing information from original papers.

**Table 4**
Summary of PDT studies using the spinner flask method to generate spheroids.

| Cancer | | 3D spheroids | | | Photodynamic therapy (PDT) treatment condition/PSs condition for assays | | Ref |
|---|---|---|---|---|---|---|---|
| Cancer origins | Cell lines | Initial cell density (cells) | Growth time (days) | Spheroid size (μm) | Photo-sensitizers (PS) | Drug-to-light intervals (Hours) | |
| Ovarian | A2780, CHO | $5 \times 10^5$cells in 10mL | – | 300 | meta-tetra(hydroxyphenyl)chlorin (mTHPC) | 24 | [47] |

N.B: (-) missing information from original papers.

**Table 5**
Summary of PDT studies using the microfluidic system to generate spheroids.

| Cancer | | 3D spheroids | | | Photodynamic therapy (PDT) treatment condition/PSs condition for assays | | Ref |
|---|---|---|---|---|---|---|---|
| Cancer origins | Cell lines | Initial cell density (cells) | Growth time (days) | Spheroid size (μm) | Photo-sensitizers (PS) | Drug-to-light intervals (Hours) | |
| Ovarian | A2780, HOF | $3 \times 10^6$cells/mL (A2780), $10^6$cells/mL (HOF) | 2 | – | TPP | 24 | [64] |
| | OCAR-5 | Microfluidic system | $0.5 \times 10^6$cells/500μl | – | Benzoporphyrin derivative | 24 | [65] |

N.B: (-) missing information from original papers.

**Table 6**
Examples of PDT studies using the scaffold-based methods-Matrigel to generate ovarian spheroids.

| Cancer | | 3D spheroids | | | Photodynamic therapy (PDT) treatment condition/PSs condition for assays | | Ref |
|---|---|---|---|---|---|---|---|
| Cancer origins | Cell lines | Initial cell density (cells) | Growth time (days) | Spheroid size (μm) | Photo-sensitizers (PS) | Drug-to-light intervals (Hours) | |
| Ovarian | OVCAR-3 + C5120 fibroblasts | – | 1 | – | DARPin-IRDye 7000x conjugates | 2 | [58] |
| | OVCAR 5 | – | 13 | >200 | EtNBS, BPD-MA | 1.5/4.5 | [68] |
| | OVCAR 5 | 18,600 | 6 | 100 | Verteporfin | 4 | [69] |

N.B: (-) missing information from original papers.

using scaffold-based methods to generate 3D spheroids for PDT studies.

### *4.2. Factors for the selection of 3D spheroid cell culture models for PDT studies*

The accumulation of photosensitizers is affected by the diffusion gradient of 3D spheroids. PSs are more ready to diffuse in small spheroids but are usually limited to the outer layer of large spheroids, which affects PDT efficacy [43]. Also, large spheroids with hypoxia core usually resist to PDT as the low oxygen concentration reduces the ROS level generated by PDT. On the other hand, the diffusion gradient also affects the recharge of oxygen that is consumed by the PDT reaction, which intensifies the tumour hypoxia. The hypoxia is usually associated with poor prognosis and treatment resistance as hypoxia could promote angiogenesis, tumour invasion, metastasis, and tumour immunosuppression [70,71]. The size of the 3D spheroids is another factor affects the light penetration, which a strong light is usually required for large spheroids for PSs activation. However, the use of strong light might cause oxygen depletion, which influences PDT efficacy [72]. Thus it is prime important to select a spheroid with appropriate size for PDT studies. Small spheroids (with <300 μm diameter) usually respond well to PDT because of good drug diffusion, light penetration, and adequate oxygen supply in the spheroid [73]. With the increase in the spheroid's size, the PDT efficacy drops because of poor drug and light penetration, and a decrease in oxygen content. The hypoxic cores which are commonly found in large spheroids (with spheroid size >500 μm diameter) further limited the PDT efficacy. Different factors affect the size of spheroids formed, including the techniques used for spheroid formation, the initial cell density, the percentage/type of FBS used, and the oxygen content for cell culture. The simplicity of medium exchange is another factor in consideration for the selection of 3D spheroid methods, as PDT studies require extra light activation and medium exchange steps. These extra steps increase the chances of disruption or loss of spheroids which leads to treatment failure. Taking all into account, we recommended using the scaffold-free ULA plate method to generate spheroids with small and large ranging from 100 to 250 μm and 500 μm diameter for PDT studies [52]. Yet researchers are reminded that there is still no single technique suitable to generate spheroids for all purposes of PDT studies. It is also very important to note the pros and cons of each spheroid culture method and to select the one to suits the research purposes.

## 5. Future perspectives and conclusions

Although the microfluidic system is with high technical demand, it offers the most precise control of the dynamic microenvironment (potentially offer the control of sex hormones in tumour microenvironment), the spheroid size (via control of initial cell density), and the possibility to control the oxygenation in 3D spheroids [74]. All these features make the microfluidic system one of the best culture systems for gynaecological cancer PDT study. However, there are limited studies using the microfluidic system to study PDT efficacy on gynaecological cancer, and to our best knowledge, none of these studies uses co-culture 3D spheroids with immune cells to study the PDT effect. We therefore believe that the development of the microfluidic system with 3D co-culture spheroids could be one of the novel tools that offer researchers a suitable model to evaluate PDT efficacy on varieties of gynaecological cancers.

In conclusion, available data and experience suggest that the 3D spheroids do offer many unique advantages to PDT investigation, particularly on the PSs uptake, biodistribution, PDT efficacy, and the PDT interaction with cellular oxygen consumption [47,56–58,60,61,64–69,75–106]. Amongst the methods discussed, the scaffold-free liquid overlay method offers lots of advantages, including high flexibility, low cost, short spheroids formation time, spheroids with appropriate size, can retain a large volume of solutions, and high reproducibility, and therefore is recommended as the simplest method to generate 3D spheroids for the start of new PDT study. However, there is no single spheroid formation method suitable for all purposes of PDT research and researchers still need to select an appropriate model and optimize the condition for spheroids formation for individual studies.

## CRediT authorship contribution statement

**RWK Wu:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **JWM Yuen:** Writing – review & editing, Methodology, Investigation, Conceptualization. **EYW Cheung:** Writing – review & editing, Conceptualization. **Z Huang:** Writing – review & editing. **ESM Chu:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

## Declaration of competing interest

This is to declare that the authors have no conflict of interest to disclose.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.pdpdt.2024.103975.

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