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Advances in screening hyperthermic nanomedicines in 3D tumor models

Joana F. Soeiro, ^{ab} Filipa L. Sousa, ^a Maria V. Monteiro, ^a Vitor M. Gaspar, ^{ab} Nuno J. O. Silva ^{ab} and João F. Mano ^a

Hyperthermic nanomedicines are particularly relevant for tackling human cancer, providing a valuable alternative to conventional therapeutics. The early-stage preclinical performance evaluation of such anti-cancer treatments is conventionally performed in flat 2D cell cultures that do not mimic the volumetric heat transfer occurring in human tumors. Recently, improvements in bioengineered 3D *in vitro* models have unlocked the opportunity to recapitulate major tumor microenvironment hallmarks and generate highly informative readouts that can contribute to accelerating the discovery and validation of efficient hyperthermic treatments. Leveraging on this, herein we aim to showcase the potential of engineered physiometric 3D tumor models for evaluating the preclinical efficacy of hyperthermic nanomedicines, featuring the main advantages and design considerations under diverse testing scenarios. The most recent applications of 3D tumor models for screening photo- and/or magnetic nanomedicines will be discussed, either as standalone systems or in combinatorial approaches with other anti-cancer therapeutics. We envision that breakthroughs toward developing multi-functional 3D platforms for hyperthermia onset and follow-up will contribute to a more expedited discovery of top-performing hyperthermic therapies in a preclinical setting before their *in vivo* screening.

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1 Introduction

Hyperthermia for cancer therapy is continuously evolving as a valuable strategy compared to standard chemotherapeutic treatments owing to its potential for heating tissues to induce cancer cell death and increase the immune response in a more controlled and localized mode.^{1,2} As cancer cells are more susceptible to heat damage than normal cells, hyperthermia

^a Department of Chemistry, CICECO – Aveiro Institute of Materials, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal.

E-mail: vm.gaspar@ua.pt, nunojoao@ua.pt, jmano@ua.pt; Tel: +351 234370733

^b Department of Physics, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal



Joana F. Soeiro

Joana Soeiro is a PhD student in the Department of Chemistry of the University of Aveiro. She obtained her MS in Biomedical Engineering from the University of Aveiro in 2021. Her PhD research is focused on studying the theranostic ability of magnetic nanoparticles for optical hyperthermia, as contrast agents, and as cellular thermometers for MRI.



Filipa L. Sousa

Filipa L. Sousa graduated and received her Master degree from University of Aveiro, Portugal in 2000 and 2003, respectively. Then she moved to the University of Bielefeld, Germany where she got her PhD degree in 2010. Afterwards, she joined University of Aveiro first as a postdoctoral researcher and later as Auxiliary Researcher at the Chemistry Department and CICECO. Her research interests included polyoxometalate-based materials for biomedical applications, as well as the development of magnetic nanoparticles applied to nanosensing.



can induce cancer cell death with minimal injury to the healthy tissue surrounding the tumor.^{3,4} The damage caused in cells depends on the achieved temperature and the duration of the procedure.⁵ Three ranges can be defined: 40–42 °C (mild hyperthermia), 42–45 °C (moderate hyperthermia), and ≥ 50 °C (ablation).⁶ Thermal ablation is usually performed for short periods and causes irreversible cell damage by inducing apoptosis.^{6,7} Mild and moderate hyperthermia are performed for longer periods, inducing changes in the blood perfusion and oxygenation of the tissue, causing protein denaturation and aggregation. Furthermore, mechanisms of DNA repair and cell proliferation can be inhibited, ultimately altering the physiology of the tumor.^{7,8} Recent evidence pointing toward

hyperthermic activation of immune cells and increased resistance against secondary tumors further supports the validity of such hyperthermic strategies.⁹

In recent years, different types of nanomaterials approved by the Food and Drug Administration (FDA) for hyperthermia have been explored in the clinical setting (e.g., Aurolase[®], Nanospectra Biosciences, Inc., Houston, TX; and NanoTherm[®], MagForce AG, Berlin, Germany) since they can be engineered to accumulate in the desired area and generate heat upon an external stimulus, typically an electromagnetic wave or an alternating magnetic field (AMF).¹⁰ Thus, nanomaterials can improve selectivity to prevent major injury in the tissues surrounding the tumor.¹¹ Despite relevant developments,



Maria V. Monteiro

Maria V. Monteiro holds a MSc in Biotechnology where she developed novel stratified pancreatic cancer 3D models that accurately recapitulate the human pancreatic cancer signatures. Currently, she is a PhD student in Biotechnology at the COMPASS Research Group based in the Associate Laboratory CICECO – Aveiro Institute of Materials. Her currently PhD work focuses on the development of decellularized pancreatic cancer matrix-based hydrogels to be integrated

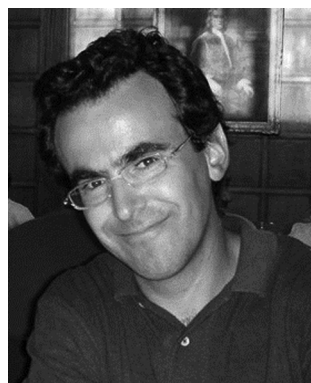
with novel stratified hydrogel-based platforms for pancreatic cancer modelling. Additionally, she is working on the development of chemical modified biopolymers, 3D bioprinted constructs and bioprinted cancer-on-chip devices for advanced tumor modelling and high-throughput screening assays.



Vítor M. Gaspar

Vítor M. Gaspar is an Assistant Researcher at the Chemistry Department of the University of Aveiro and member of COMPASS Research Group, in the Associate Laboratory CICECO – Aveiro Institute of Materials. He holds a PhD in Biochemistry and currently his research interests include the precision chemical modification of ECM-biomimetic and tissue-derived biomaterials, particularly decellularized extracellular matrix. He has been focusing on the use of these matrices

for the bottom-up engineering of cell-biomaterial living 3D microtissues and organoids engineering. He has been leveraging advanced manufacturing techniques such as 3D/4D bioprinting for processing cells and materials toward tissue engineering and in vitro disease modelling applications.



Nuno J. O. Silva

Nuno J. O. Silva is a Principal Researcher at the University of Aveiro. He studied physics at the University of Porto and University of Aveiro, where he got interested in the magnetic properties of nanoparticles and aqueous ferrofluids. He is now exploring the magneto-thermal properties of nanoparticles for imaging, supported by a ERC grant.



João F. Mano

João F. Mano is a full professor in the Chemistry Department of the University of Aveiro, Portugal, and director of the COMPASS Research Group from the Associate Laboratory CICECO – Aveiro Institute of Materials. His research interests include the use of biomaterials and cells toward the progress of transdisciplinary concepts to be employed in regenerative and personalized medicine. He has applied biomimetic and nano/micro-technology approaches to polymer-

based biomaterials and surfaces to develop biomedical devices with improved structural and (multi-)functional properties, or in the engineering of microenvironments to control cell behavior and organization, to be exploited clinically in advanced therapies or in drug screening.



upgrade this therapeutic methodology. It is envisaged that advances from upgraded preclinical validation models will contribute to changing the current approaches for the validation of innovative hyperthermic nanomedicines, opening new avenues for accelerating their translation toward the clinical scenario.

Conventionally, the preclinical validation of candidate hyperthermic nanomedicines has been routinely performed in gold-standard flat 2D cell cultures.^{14,15} However, these are unable to recapitulate the 3D architecture of the human tumor microenvironment (TME), as well as the complexity of its cellular (*i.e.*, cancer, stromal, immune cells, *etc.*) and non-cellular elements (*i.e.*, extracellular matrix (ECM)),¹⁶ ultimately leading to a sub-optimal *in vitro/in vivo* correlation that impacts human clinical trial validation stages and ultimately translation into the market.^{17,18} Exploring 3D *in vitro* tumor models as alternative preclinical platforms for hyperthermic nanomedicines validation opens the possibility to recapitulate: (i) human solid tumors gene expression profiles associated with heat/drug resistance (*i.e.*, heat-shock proteins – Hsp70/Hsp90,^{14,19} drug resistance mechanisms – ABC transporters²⁰), (ii) the modulation of the secretion of key exosomes,²¹ as well as (iii) the establishment of hypoxic/necrotic regions and volumetric pH gradients,²² in an approach that is significantly more similar to that found *in vivo*.²³ The coexistence of multiple cancer-stroma cellular populations and the dynamic cell-cell or cell-TME interactions in 3D can also be explored to better contribute to evaluating hyperthermic therapeutics performance.^{24–28} Upgrading from the conventional use of monolayers toward volumetric tumor models also enables researchers to specifically evaluate the influence of solid tumors on heat transfer mechanisms and nanomedicines penetration in 3D in a more biomimetic set-up.^{18,26,29} The latter is particularly relevant, since a sub-optimal and non-homogeneous distribution of hyperthermic nanomedicines within the tumor volume may impact the overall therapeutic outcome. Leveraging 3D models and high-throughput/high-content imaging approaches to improve the selection process of top-performing hyperthermic nanomedicines at preclinical stages also contributes to reducing the use of laboratory animals and surpasses major ethical and economic issues associated with these models.²⁴

Despite the promising advantages of advanced 3D tumor models, these have yet to be broadly adopted during hyperthermic nanomedicine design and performance screening.³⁰ Aiming to shed light on recent advances, the key aspects of hyperthermia nanomedicines validation in advanced 3D *in vitro* models are herein addressed. An informative discussion focusing on the importance of heat transfer simulations in 3D and real-time temperature evaluation strategies during treatment is also provided, considering the highly required advances in technologies to monitor hyperthermia nanomedicines in a non-invasive mode. State-of-the-art examples leveraging on the use of 3D *in vitro* tumor models for screening and validating nanohyperthermia technologies will be showcased and discussed, considering their contribution to further consolidate and

2 Hyperthermia technologies

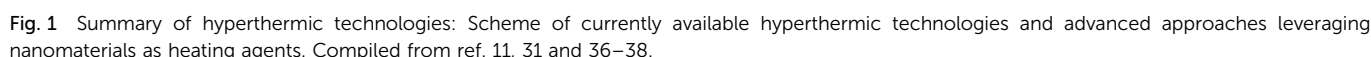
Conventional hyperthermia for cancer management is performed by exploiting: (i) electromagnetic waves: radiofrequency (RF), microwaves (MW), near-infrared (NIR) light, and (ii) mechanical waves, namely ultrasound (US).^{31,32} Even though these methods are efficient in increasing the temperature of tissues, they fail to target only the desired tissue.¹¹ This targeting can be dramatically enhanced using nanosized heating agents that are more efficient than tissues in absorbing the incoming wave, leading to an increase in temperature in a specific region. Hyperthermia has been rapidly emerging owing to its higher precision and therapeutic versatility, particularly in the case of photothermal therapy (PTT) and magnetic hyperthermia (MH), complementing the available toolset of hyperthermia technologies.^{33–35} Fig. 1 highlights examples of traditional hyperthermia methods and examples of nanomaterials used as heating agents in hyperthermia procedures.^{11,31,36–38}

2.1 Photothermal nanotherapy

In PTT approaches, nanosized photothermal agents (nano-PTAs) are leveraged to convert electromagnetic radiation, usually NIR, into thermal energy to heat cancer cells.³⁹ The interest in NIR-II-absorbing materials (NIR-II: 1000–1350 nm⁴⁰) has recently increased since they show a reduced light scattering, enable a higher spatial resolution, and higher signal-to-background ratio compared to NIR-I-absorbing materials (NIR-I: 650–900 nm⁴⁰) which have been the most explored for PTT.^{41,42}

To date, a wide range of inorganic (*i.e.*, carbon based-nanomaterials, noble metal nanoparticles (NPs), metal-oxide NPs), and organic nanomaterials (*i.e.*, conjugated polymers, semiconducting polymers, organic dyes, *etc.*) have been exploited for PTT methodologies.^{11,43,44} Table 1 reports examples of nano-PTAs recently used for hyperthermic nanotherapy screening in 2D *in vitro* models. Both classes of nano-PTAs present advantages and disadvantages, with most inorganic nano-PTAs being non-biodegradable and exhibiting limited biocompatibility, while organic nano-PTAs (*i.e.*, biodegradable, and biocompatible) commonly exhibit lower photothermal conversion efficiency (PCE) and reduced photostability.^{45–47} The rationale selection of nano-PTA should thus account for several factors, including the type of cancer, the tissue/organ being treated, the location of the treatment, and the optimal light wavelength.

Carbon-based materials, including graphene,^{56–58} carbon nanotubes (CNTs),^{59,60} fullerenes,^{61,62} and carbon dots,^{63,64} have been increasingly used as nano-PTAs as they display high absorption in the NIR region, suitable biocompatibility, and can generally accumulate in the tumor site due to their nano size.



The efficiency of the MNPs in MH depends on their coating, size, morphology, and magnetic properties (*e.g.*, saturation magnetization (M_s), magnetic susceptibility, and magnetocrystalline anisotropy).^{34,88,94,95} The size and morphology of the MNPs are key features to be considered due to their influence on cellular uptake, which will be further discussed.⁹⁶ Moreover, the size of the MNPs will influence the mechanism responsible for generating heat within the tumor: hysteresis losses due to domain rotation/reconfiguration are more significant in multi-domain NPs, and Brownian and Néel relaxation losses are dominant in single-domain NPs.^{90,97,98} The size distribution of MNPs should be uniform to contribute to a homogeneous distribution of heat, and the MNPs should be well dispersed in small concentrations.^{87,88,90} Usually, larger particles have a higher M_s , which is proportional to the heating efficiency of the MNPs. So, a compromise between the size of the NPs must be found to maximize their heating capacity and cellular uptake.^{87,99,100} Besides being important to guarantee a high M_s and magnetic susceptibility, MNPs should have a suitable volume and magnetocrystalline anisotropy, whose product is the energy required to change the orientation of the magnetic moment of the particles, to match the characteristic relaxation



Table 1 Examples reporting the performance of nano-PTAs for 2D *in vitro* PTT

Heating agent	Core	Coating	Size w/coating (nm)	PCE ^a (%)	Heating conditions	2D tumor model	Dose (μg mL ⁻¹)	T reached (°C)	Cell death (%) (time after treatment, technique)	Ref.
SCNTs and MCNTs loaded with therapeutic siRNA	SCNTs and MCNTs	Peptide lipid and sucrose laurate	length: 0.5–2 μm; diameter: 30 nm; SCNTs: length, 1–3 μm, diameter: 24 nm, TEM	SCNTs ~ 59	808 nm, 1 W cm ⁻² , 5 min	HeLa	30	42–45	SCNTs: ~30 ^a	48
				MCNTs ~ 58					MCNTs: ~40 ^a (PTT alone) (24 h, MTT)	
MCNTs	MCNTs	Amide	Length: 591 nm, Diameter: 29 nm, TEM	N.D.	1064 nm, 3 W, 5–45 s	HMLER ^{shEcadherin} , HMLER ^{shControl}	50	49	~40 ^a (for both cell lines) (24 h, MTT)	49
DPP NPs	DPP NPs	TPA	Technique: N.D. ~76, DLS	~35 (80 μg mL ⁻¹)	660 nm, 0.5 and 1 W cm ⁻² , 5 min	HeLa	20	N.D.	0.5 W cm ⁻² ; ~30 ^a ; 1 W cm ⁻² ; ~70 ^a (48 h, MTT)	50
Black mesoporous silicon NPs	Black mesoporous silicon NPs	PEG	156, DLS (w/o coating)	~33 (100 μg mL ⁻¹)	808 nm, 1 W cm ⁻² , 10 min	CT26	75	N.D.	84 (12 h, FC)	51
Chitosan-based hydrogels with nanosized {Mo ₁₅₄ }	Chitosan-based hydrogels with nanosized {Mo ₁₅₄ }	—	3.4 ^c {Mo ₁₅₄ }, technique: N.D.	N.D.	808 nm, 0.8 W cm ⁻² , 10 min	M21	0.092 wt%	N.D.	~98 (after irradiation, CellTiter Glo)	52
Gellan Gum and Ca ²⁺ hydrogels with AuNRs	Gellan Gum and Ca ²⁺ hydrogels with AuNRs	PEG	N.D.	~21 (100 μg mL ⁻¹)	(1) 808 nm, 0.5 W cm ⁻² , 2 min; and (2) 660 nm, 50 mW cm ⁻² , 5 min	HeLa and MCF-7	50	66.2	~44 and ~66 for HeLa and MCF-7 cells, respectively (12 h, CCK8)	53
Ag NPs	Ag NPs	PVP	~93, DLS	N.D.	970 nm, 3 W, 70 s	MDA-MB-231	12.5	52.4	>85 (72 h, MTT)	54
Au25 nanoclusters conjugated with ICG	Au25 nanoclusters conjugated with ICG	Gluta-thione	~3.4, DLS	N.D.	808 nm, 0–1 W cm ⁻²	MCF-7	30 μM	N.D.	0.5 W cm ⁻² ; ~35 ^a 0.8 and 1 W cm ⁻² ; ~100 ^a (after irradiation, MTT)	55

^a Value extracted from graphs. ^b The conditions that are not indicated are the same as the heating conditions; N.D. – non described; PCE: photothermal conversion efficiency; MCNTs: multiwalled carbon nanotubes; SCNTs: single walled carbon nanotubes; DPP: diketopyrrolopyrrole; TPA: triphenylamine; PEG: polyethylene glycol; FC: flow cytometry; NRs: nanorods; PVP: polyvinylpyrrolidone. ^c Value from the literature indicated in the article.

time and the AMF frequency, aiming to maximize the heating efficiency.^{88,101,102}

Functionalization of MNPs with hydrophilic and low toxic materials is usually performed to increase both the dispersibility of NPs in water and colloidal stability.⁹⁴ Moreover, it improves biocompatibility and tumor target selectivity, prevents MNPs from agglomerating, and increases the NPs' circulation time.^{87,95,103,104} The material of the surface coating, its thickness, and the size of the core influence the heating efficiency of the final particle. Usually, a thinner coating and a larger core size contribute to a higher SAR, while a thicker coating and a smaller core size contribute to a lower SAR by inhibiting Brownian relaxation. Moreover, MNPs with a low dispersibility behavior require a thicker coating to improve their stability and prevent aggregation.^{88,105} So, a compromise between the thickness of the coating and the size of the core must be found to maximize the SAR, dispersibility, and colloidal stability in aqueous medium.⁸⁸ Coating MNPs with appropriate materials such as poly(lactic-co-glycolic acid) (PLGA), polyvinyl alcohol (PVA), poly(vinylpyrrolidone) (PVP), polyethylene glycol (PEG), or dextran has been shown to improve NPs-cell interactions and also prevent toxic side-effects.^{106,107} Lipid-based nanomaterials, such as liposomes^{108,109} or niosomes,^{110,111} have also been used as coatings for MNPs due to their biocompatibility, flexible design, and surface modification capacity. Both polymeric and lipid coatings allow for a controlled release of the inner NPs/drugs upon the use of different stimuli (*e.g.*, temperature, pH, light, magnetic or electric fields, *etc.*), which is advantageous, for example, for target drug delivery and for MRI contrast.^{112,113} Recently, hybrid systems consisting of polymer and lipid coatings have emerged as promising approaches capable of improving NPs' stability, biocompatibility, and drug release kinetics.^{114,115}

Superparamagnetic iron oxide NPs (SPIONs) have been the most used for MH as they show valuable size-dependent magnetic properties, suitable biocompatibility, reduced toxicity, high surface-to-volume ratio, proper stability in aqueous suspension, can be easily functionalized, and are FDA approved.^{116–118} Moreover, these particles have been shown to have the potential to induce heat by optical stimulation, which, in combination with MH, can contribute for increasing the efficiency of candidate anti-cancer treatments.¹¹⁹ Table 2 summarizes the most recent studies exploring MNPs for hyperthermic nanomedicines screening in flat 2D *in vitro* models. The use of MNPs has several advantages since, in addition to being used as heat sources, they can also be used as contrast agents, non-invasive temperature thermometers, or for complementary targeted drug delivery.^{120–122} However, it is still a challenge to guarantee that the MNPs are homogeneously distributed in the target region, achieve uniform heating, and to ensure that the magnetic properties of the NPs/administered concentration are adequate to promote an effective outcome.^{119,123}

MH and PTT are complementary, in the sense that while PTT is more suitable for surface and near-surface applications, MH is applicable in deep tissues, since the penetration of the AMF exceeds the penetration depth of light.^{94,124,125} Consequently, MH can be used to trigger hyperthermia in deeper tumors than PTT.⁸⁷

Table 2 Examples reporting the performance of MNPs for 2D *in vitro* MH

Heating agent		Size w/ coating (nm)	Heating conditions	SAR ^c (W g ⁻¹)	M _s (emu g ⁻¹)	2D tumor model	Dose (μg mL ⁻¹)	T reached (°C)	Cell death (%) (time after treatment, technique)	Ref.
Core	Coating									
Fe ₃ O ₄ Fe ₃ O ₄	Aminosilane CA	110, DLS 46, TEM	557 kHz, 300 Gauss, ~24 kA m ⁻¹ , 40 min 60 kA m ⁻¹ , 30 min	338 2380 ^b (325 μg mL ⁻¹ , 83 29 kA m ⁻¹)	N.D. 83	C6 A549	100 ^b 46 ^b 85.5 ^b 177.5 ^b	44 43.6 45.95 49.67	~70 (N.D.; FC) ~20 ^a ~80 ^a ~100% (24 h, AB)	126 127
	Fe ₃ O ₄	PCL	21, TEM	318 kHz, ~32 kA m ⁻¹ , 15 min 313 kHz, ~47 kA m ⁻¹ , 15 min	153 (10 ³ μg mL ⁻¹) 201 (10 ³ μg mL ⁻¹)	64	HepG2	100	43.2 46.1	~39 ~60% (24 h, MTT)
Fe ₃ O ₄	PVP	145, DLS	~333 kHz, 170 Oe, 15 min	160 (10 ⁴ μg mL ⁻¹)	72	MDA-MB-231	500	42.5	75 (24 h, MTT)	129
CoMn-Fe ₂ O ₄	PEG-PCL	~79, DLS	420 kHz, ~27 kA m ⁻¹ , 30 min	1237 (w/coating)	93 (w/o coating)	ES-2	50	55 ^a	~99 (48, calcein AM)	130
GGO-Fe ₃ O ₄	—	20, XRD	236 kHz, ~4 kA m ⁻¹ , 10 min	70 ^a	50 ^a	HeLa	1000	N.D.	40 (2 h, MTT)	131
MnFe ₂ O ₄	—	31, TEM	765 kHz, 300 Oe, (1) 4–6 min followed by (2) 18–23 min after 2 days	~300 ^a	~70	Saos-2	250 500	(1) ~45 ^a (2) ~43 ^a -for both doses	(1) 25 ^a ; (2) 75 ^a (1) 30 ^a ; (2) 90 ^a (N.D.)	132 132

^a Value extracted from graphs. ^b Dose of Fe. ^c The conditions that are not indicated are the same as the heating conditions; N.D. – non described; FC: flow cytometry; AB: Alamar Blue; CA: citric acid; PCL: polycaprolactone; PVP: polyvinylpyrrolidone; GO: graphene oxide.

When designing nanomedicines for hyperthermic applications, it is important to consider the factors that affect their penetration in tumors that is influenced by NPs' properties (*e.g.*, size, morphology, or surface chemistry/functionalization), and by physical and biological barriers (*e.g.*, uptake by the immune system, shear stress under circulation, renal filtration, interstitial fluid pressure, or tumor desmoplasia).^{133,134} In general, NPs bigger than 200 nm are easily accumulated in the liver and spleen, and NPs smaller than 6 nm are usually filtered by the kidney, not being able to accumulate in the target site.^{95,135} Moreover, coating the NPs with neutral polymers generally reduces clearance mediated by the immune system.¹³³ Since some tumor types have a leaky vasculature with increased permeability and poor lymphatic drainage, nano-sized agents are more likely to be passively internalized and retained by the tumor after systemic administration, which is referred to as the enhanced permeability and retention (EPR) effect.^{95,136} In fact, the internalization of nano-agents as drug carriers in tumors can exhibit over a 10-fold increase in effectiveness compared to free-drugs, even though only 10 to 15% of injected NPs successfully accumulate within the tumor.¹³⁷ Approaches aiming to improve the accumulation and retention of NPs in tumors include, for example, the use of ligands or antibodies to bind with specific malignant cell receptors to promote a targeted delivery.^{138–140} Regardless of their physicochemical features, the preclinical validation of such systems is a requirement before they are considered for clinical applications.

To date, the preclinical performance and validation of such engineered nano-heating agents for localized heat generation has been mainly performed in 2D cell monolayers and/or mice models.^{141,142} Recently, the use of 3D *in vitro* models in the design and validation stages of hyperthermic nanomedicines has been rapidly emerging as an alternative strategy, owing to its potential to overcome the issues of 2D cultures, for significantly reduce animal model usage, and for accelerate the identification of top performing nano-heating agents.

To fully explore the potential of 3D *in vitro* tumor models for evaluating the performance of hyperthermic nanomedicines, it is important to discuss the relevance of the third dimension in what relates to NPs' penetration and distribution in the complex TME, as well as heat transfer mechanisms, that are otherwise more difficult to be modeled in 2D cell cultures.

3 *In vitro* tumor models for nanomedicines screening

3.1. Mimicking the tumor microenvironment (TME): 2D versus 3D models

The complexity of tumors and their interactions with the surrounding microenvironment has prompted researchers to develop more advanced and sophisticated 3D tumor models that better recapitulate different tumor hallmarks in a preclinical setting when compared to the limited 2D flat cell monolayers methodologies.¹⁴³ Owing to the inherent spatial differences, 2D

and 3D tumor models recapitulate the TME components and its biological hallmarks differently in an *in vitro* setting.

In the native TME, the tri-dimensional existence of the surrounding stroma plays an important role in tumor progression, invasion, and metastasis, for example by producing enzymes to degrade the ECM, by supplying biomolecular cues to promote cancer cell growth and angiogenesis, by suppressing the immune response, or by recruiting healthy cells.^{144–146} The stroma is comprised by the ECM and other cell types, such as endothelial cells, cancer-associated fibroblasts (CAFs), mesenchymal supporting cells, cells of the vascular/lymphatic system, and immune system cells.^{147–149} In malignant tissues, the ECM undergoes significant alterations resulting in *de novo* deposition of ECM, where proteins (*e.g.*, collagen, fibronectin, laminin, *etc.*) are upregulated and an enzyme-mediated (*e.g.*, lysyl oxidase) stiffness increase occurs, which represents a physical barrier to therapies.^{27,150,151} Each cancer-associated cell type has its own function on tumor progression, making it important to consider the rationale addition of these living units in the design stage of a 3D *in vitro* tumor model.¹⁴⁴ For example, fibroblasts contribute to ECM *de novo* deposition and remodeling of ECM proteins, facilitating the invasion of cancer cells into neighboring tissues, while immune cells can also have tumor promoting capacity after being recruited by other cancer cells.^{149,152}

In conventional 2D platforms, cells grow as monolayers in adherent conditions. This results in a flat and homogeneous environment that deprives cell–cell and cell–ECM interactions and leads to changes in cellular morphology and function. Moreover, 2D models were shown to modify cellular polarity, morphology, secretion, gene expression, and signaling. These models generally only focus on one cell type (monotypic), making it difficult to co-culture different cellular components, which often neglects the essential contribution of stromal components to tumor development. 3D tumor models provide a far more realistic volumetric architectural rearrangement and cellular representation of the TME by allowing the inclusion of stromal components and enabling to easily recapitulate cellular diversity and heterogeneity.^{14,26,141,153}

Since cancer cells are highly proliferative, the angiogenic process (*i.e.*, formation of vasculature) cannot generally keep pace, leading to the disorganized formation of leaky and branch vessels with irregular sizes and increased permeability, in some cancers.¹⁵⁴ This, in turn, results in an increase in the interstitial pressure. Consequently, the inner cells of the tumor mass become deprived of nutrients and oxygen, leading to the formation of a hypoxic and acidic environment with gradients of oxygen, nutrients, and metabolites.^{152,155,156} 2D *in vitro* testing platforms, where cells are cultured in an air–liquid interface, are unable to mimic these gradients or allow to recapitulate the formation of vessel-like structures, elements that are essential to better understand tumor behavior.²³

The hypoxic microenvironment leads cancer cells to adapt their metabolism to promote survival, invasion, and metastasis. These adaptative changes are further supported by proteomic and genomic alterations.¹⁵⁷ Interactions between the stroma



In essence, 3D tumor models represent a testing platform that more closely resembles the architectural complexity,

3.2 3D tumor models for preclinical screening of therapeutics

In the pursuit of better alternatives to conventional 2D monolayered models, researchers have been focusing on developing different types of 3D tumor models, with a particular emphasis on spheroids, organoids, organ-on-chip platforms, and 3D bioprinted models (Fig. 2), all of which are addressed in detail in the following section.

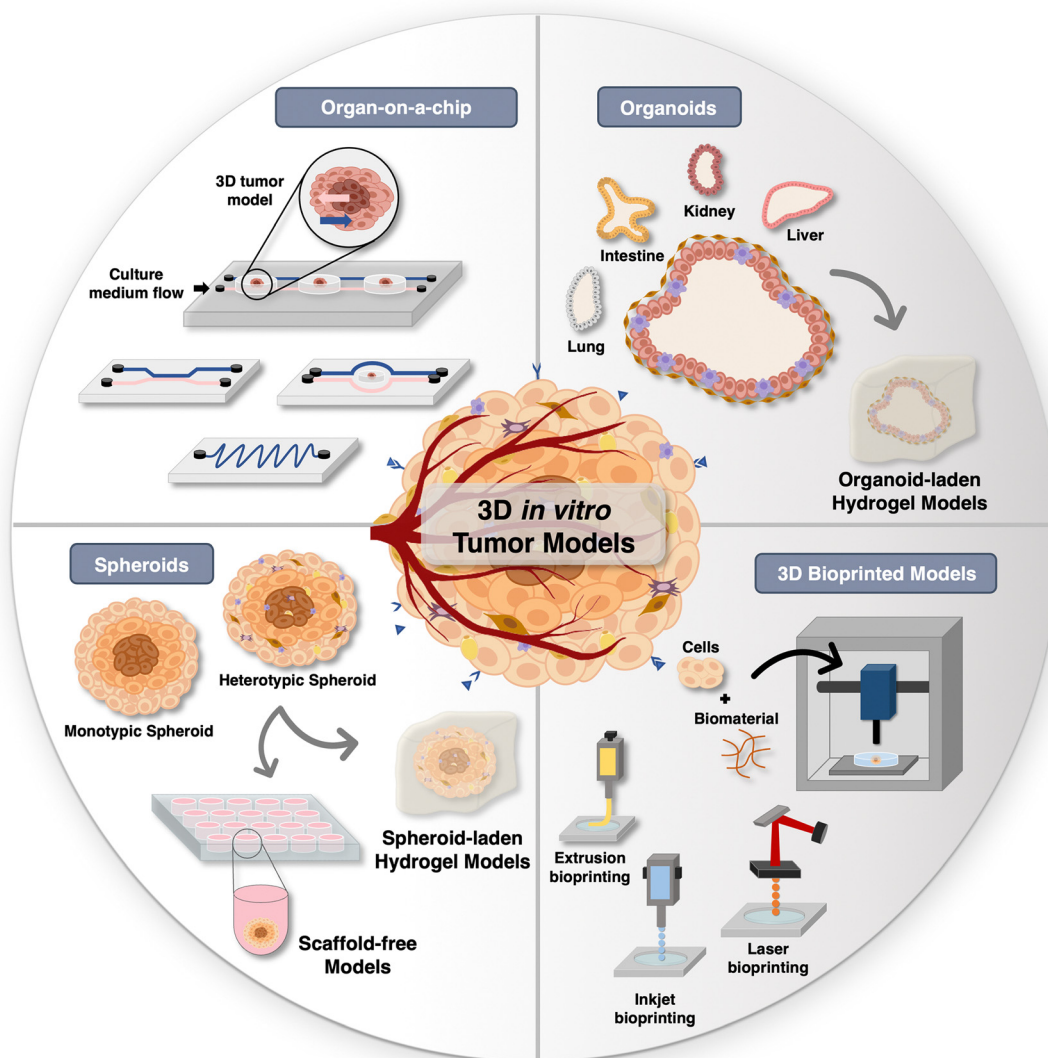


Fig. 2 Overview of *in vitro* tumor models: Schematic of diverse 3D tumor models for hyperthermic nanomedicines screening.

3D bioprinting has been used to assemble spheroids, organoids, and tumor-on-a-chip models. Moreover, attempts have

been made to implement vasculature in tumor models with this technique, particularly within microfluidic platforms.^{209,210} Adding to this, 3D bioprinting can be helpful to generate customizable 3D *in vitro* models with a user defined size that better mimics that of the human scale,²¹¹ which is advantageous for pre-clinical imaging techniques, such as magnetic resonance imaging (MRI), positron emission tomography (PET), or computed tomography (CT), that have limitations in sample size due to their spatial resolution.²¹²

In addition to the discussed differences between 2D and 3D *in vitro* tumor models, the latter may also offer the ability to manipulate the non-cellular microenvironment through the encapsulation of cells in ECM-mimetic hydrogels. These, so-termed scaffold-based 3D *in vitro* models, are highly valuable to study the influence of the biophysical and biomechanical properties of the ECM, particularly through the modulation of various parameters that exist in the tumors matrix (e.g., establishment of chemical gradients, shifts in stiffness/viscoelasticity, evolving biomolecular composition, mass transfer phenomena), thus enabling to more accurately mimic *in vivo* conditions.^{213,214} Furthermore, these scaffold-based *in vitro* models provide the possibility to model the influence of each parameter in different stages of tumor development.²¹³ In this framework, recent endeavors have focused on bioengineering ECM-mimetic hydrogel 3D tumor models with on-demand tailored mechanical properties to study tumor growth,²¹⁵ migration/invasion,^{175,216,217} or metastasis.²¹⁸ Adding to this, cutting-edge bioengineering methods, such as 3D bioprinting, offer the ability not only to generate *in vitro* models with controlled mechanical properties but also with a physiometric architecture of the microenvironment.^{214,219} In a complementary approach, the integration of 3D bioprinting with microfluidic technology has been proven to be crucial in integrating mechanical cues within 3D *in vitro* models, encompassing shear flow, gradients, and mechanical stimulus. These cues, in turn, can influence cellular signals, cell adhesion molecules, cytoskeleton dynamics, and activation of membrane transporters and ion channels.^{220,221} By manipulating these properties in 3D *in vitro* models, researchers can create a more realistic and tailored model to better predict cells' response to treatments.

3.3 Heat response and transfer mechanisms: probing dimensional impacts on heat response in 2D and 3D models

During stress, such as oxidative, pH, hypoxic, heat, or radiation, an overexpression of heat shock proteins (Hsp) is initiated to promote cell survival, especially involving Hsp70 and Hsp90. This includes binding to denatured proteins, preventing incorrect aggregations, assisting protein assembly, secretion, and degradation, as well as transporting those proteins through membranes.^{14,222} Hsp are mainly regulated by the heat shock factor 1 (Hsf1), which normally resides in the cytoplasm in an inactive state. In response to a stress situation, Hsf1 enters the nucleus and initiates the transcription process, resulting in the production of Hsp. Once the stress is removed, Hsf1 returns to its inactive state in the cytoplasm.^{19,223} This response enables cells to maintain their functionality, evade the signals that

trigger cell death, and mitigate the effects of drugs, leading to the development of resistance to treatments.²²⁴ Consequently, researchers have directed their efforts towards discovering strategies to target Hsp with inhibitors, aiming to increase cancer cells death upon treatment.^{19,225} Moreover, hyperthermia has shown to promote the infiltration of immune cells in tumors, enhancing immune response and modulating the TME. This dynamic interplay can improve tumor cell recognition and destruction by the immune system.^{6,226}

The response of cells to stress is influenced by the conditions of the culture. In 2D models, cells are simultaneously and uniformly placed under similar conditions, leading to a higher rate of cell death when compared to 3D cultures. This is primarily due to the additional architectural, cellular, and environmental components of 3D models.^{14,227} Such testing platforms are highly valuable to better understand and predict the performance of candidate hyperthermia treatments.

The temperature in tissues is the result of a balance of factors, including ambient temperature, heat generation as a result of metabolic activity, and heat transfer from hot to cold regions of the body by conduction and convection.^{228,229} It is worth noticing that convection is quite more efficient in transporting heat.²³⁰ According to a study, the size and shape of NPs, blood flow, and vessel geometry can all impact the distribution of NPs inside tumors, which in turn can affect the temperature distribution.²³¹ Moreover, nanosized particles are more efficient in penetrating deeper into tumors but have high elimination rates.^{231,232} Another study demonstrated that the rate of heat generation and its impact on surrounding tissues are influenced by the infusion rate, blood flow, and distribution of nanoparticles within the tumor. A lower infusion rate was shown to be more successful in raising the concentration of NPs in the TME, causing less heat to dissipate to the surrounding tissue which can damage it.²³³ Heat dissipation to the surrounding tissues is another important consideration, as it can have an impact on the safety and effectiveness of hyperthermia therapy. As the volume of the tumor grows, less heat is dissipated, resulting in a greater temperature differential between the tumor and the surrounding media, allowing for the selective heating of the tumor, as more heat is carried away by perfusion on the normal tissues.^{234–237} Due to all the complex factors that have to be considered for hyperthermia, advanced techniques and technologies for heat transfer modeling have been developed. The heat balance of the tissues was first described by the bioheat equation proposed by Pennes', which is now a standard model for studying the temperature distribution in tissues. This model states that the heat stored in the tissue is equal to the balance between the heat generated by metabolic activity and the heat dissipated by conduction and convection.^{238,239} Beyond the Pennes' bioheat model, others have been developed, such as the local thermal equilibrium (LTE) and local thermal non-equilibrium (LTNE) equations, and dual-phase-lag bioheat model.^{239,240} The LTE and LTNE models consider the existence of a porous medium that consists of a solid matrix (*i.e.*, tissue) and blood vessels that are in thermal equilibrium or non-equilibrium, respectively. The



LTNE model represents a more realistic situation since it considers a temperature gradient between both phases.^{239–242} Pennes', LTE, and LTNE models consider that the blood velocity is infinite (based on Fourier's law), meaning that when applying heat, the temperature of a tissue changes immediately, which does not happen in non-homogeneous tissues. The dual-phase-lag (DPL) bioheat model considers that the temperature and heat flux have a lag time. Consequently, this model focuses on the micro-structural interactions and considers that the tissues and blood can have different temperatures.^{239,240,243–245} Table 3 summarizes the main advantages and disadvantages of each model, as well as examples of the application of these models to predict heat distribution in tumors.

When predicting hyperthermia effects in 3D *in vitro* tumor models, the choice of the bioheat equation depends on the specific characteristics of the tissue. For instance, a study concluded that the DPL model can better predict heat transfer in tissues with larger blood diameters, but when considering tissues with micro-capillaries, the Pennes' model and the DPL model had similar performances.²⁴⁶ In another study, the performance of the LTNE model with variable porosity and the Pennes' bioheat model with variable perfusion was compared when simulating thermal ablation in tumor tissue, and it was shown that the LTNE model was more accurate in predicting heat distribution after comparing the results with *in vivo* experiments.²⁴⁷ Although, due to its simplicity, the Pennes' bioheat model has been the most used to study heat transfer in biological tissues.²⁴⁸ In a balance between simplicity and accuracy, the Pennes' bioheat equation has undergone some modifications over the years, aiming to predict heat distribution in tumors as close as possible to an *in vivo* situation. Different parameters, such as the direction of the blood flux, spatial and temporal variations of the velocity of the blood, size, geometry, and density of the vasculature, and the distribution of nanomedicines, have been considered.^{231,249–253} Fig. 3 shows the use of advanced tumor models to better understand heat distribution, particularly by varying the density of tumor vasculature (Fig. 3A1 and A2),²⁵⁴ and by using Fe₃O₄ MNPs for MH (Fig. 3B1–B4).²⁵⁵ Even though recent studies have been providing important information, it is difficult to resemble a perfect *in vivo* situation due to the complex and unpredicted variables, such as tumor size or the geometry and density of the vasculature, that have a huge influence on how the NPs and heat are distributed. Moreover, it is difficult to confirm the veracity of the obtained results due to the lack of experiments performed in clinical settings.²⁵⁴ Currently, researchers are dedicated to improving mechanisms to predict heat delivery in tissues by introducing variables such as the size and morphology of the NPs.²³¹ Significant advances in this direction are expected in the upcoming years, especially considering that 3D hyperthermic simulations are an important tool to accelerate the clinical translation of nano-hyperthermia.

Numerical simulation methods are a valuable resource for hyperthermia treatment planning. These methods can help to predict how the chosen treatment for the specific size and location of the tumor can affect both tumor and healthy tissue, and to adapt the heat and heating agents' doses needed for

Table 3 Advantages and disadvantages of the bioheat models used to predict heat distribution in tumors, compiled from references^{239–245,258,259}

Model	Description	Advantages	Disadvantages	Application of the model to predict heat distribution in tumors
Pennes' bioheat equation	The heat stored in the tissue is equal to the balance between the heat generated by metabolic activity and the heat dissipated by conduction and convection	Simplicity; consideration of blood perfusion	Assumes a homogeneous and isotropic tissue; constant blood perfusion; restricted spatial variability; do not consider the vascular geometry	247, 254, 255 and 257
Local thermal equilibrium equation	Considers the existence of a porous media that consists on a solid matrix (<i>i.e.</i> , tissue) and blood vessels that are in thermal equilibrium	Simplicity; can consider blood flow direction	Assumes that the temperature of the blood and tissue is the same; it can only be applied in scenarios with limited vessels diameter and blood velocities	260
Local thermal non-equilibrium equation	Considers the existence of a porous media that consists of a solid matrix (<i>i.e.</i> , tissue) and blood vessels that are in thermal non-equilibrium	Consider the temperature of tissues and blood; can consider blood flow direction; improved model compared to the one-equation model	Complexity; detailed information about thermal properties of tissues is required	247 and 260–262
Dual-phase-lag bioheat equation	Considers that there is a lag time in the temperature and heat flux	Consider the temperature of tissues and blood; consider micro-structural interactions effects	Complexity; detailed information about thermal properties of tissues is required	263–266



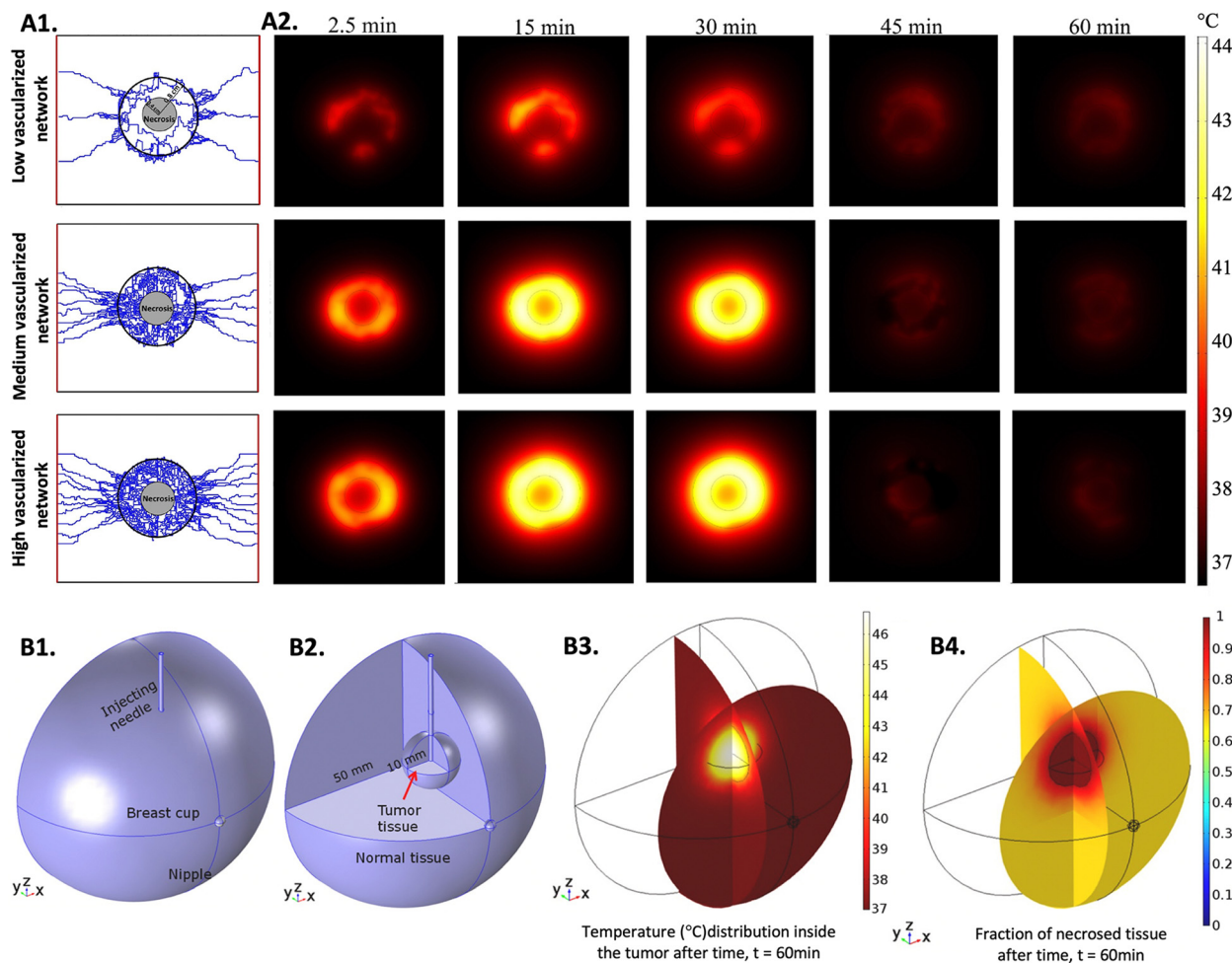


Fig. 3 Simulations of heat distribution in 3D tumor models: (A1) three capillary models with low, medium, and high vascularization used to perform the numerical studies, (A2) computational estimation of temperature distribution for three different tumor vasculature densities at five time points. The MNPs were injected at constant rate for 30 minutes. Reprinted from ref. 254, Copyright (2022), with permission from Elsevier and (B1) designed model representing the breast cup with the injecting needle, (B2) designed model enhancing the tumor and the normal tissue, (B3) 3D simulation of the temperature distribution in the breast cup and (B4) 3D simulation of the necrotic tissue. Reprinted from ref. 255, Copyright (2020), with permission from Elsevier.

personalized treatment. Moreover, by comparing the outcomes of these methods with the ones obtained from 3D *in vitro* models, it is possible to validate these theoretical techniques for modulating the tumor response to hyperthermia. These improvements, combined with the increasing computational power and the advent of artificial intelligence (AI) algorithms for simulation, are envisioned to become a major part of the preclinical optimization/validation process of hyperthermia therapies and of the research to predict their effect on healthy tissues.^{239,256,257}

4 Advances in preclinical screening of hyperthermic nanomedicines in 3D tumor models

The development of 3D tumor models has revolutionized the field of cancer research and encouraged their application for the screening of hyperthermic nanomedicines.¹⁴ By using these models, it is expected to have a deeper understanding of the

interactions between hyperthermic nanomedicines and cancer cells, as well as the influence of the TME on drug delivery, nanomedicines distribution in the tumor, and therapeutic response. With a clear understanding of these mechanisms, researchers can optimize treatment strategies and develop more clinically relevant hyperthermic approaches for cancer therapy.^{14,267,268}

This chapter aims to provide an overview of the recent advances in the preclinical screening of hyperthermic nanomedicines using 3D tumor models, with a prominent role in PTT, MH, and hyperthermia combined with conventional therapies. By examining the cutting-edge research in this field, it is expected to provide valuable insight into the potential of 3D tumor models as powerful tools for preclinical screening, allowing the identification of hyperthermic nanomedicines with optimal therapeutic efficacy and safety profiles.

4.1 3D tumor models for photothermal nanomedicines screening

The emergence of 3D *in vitro* tumor models represents a relatively recent development, and as such, numerous studies

are still dedicated to understanding the differences in the hyperthermic response between 2D and 3D models. Comparative studies evaluating the efficacy of hyperthermic nanomedicines within monolayers and tumor spheroids revealed that 3D models are more resistant to the treatment.^{269,270} Moreover, an additional study showed that 2D monolayers needed less NPs incubation time and had more efficiency in binding the NPs to cells than 3D models, enhancing the importance of replacing 2D with 3D models for treatment parameters optimization, aiming to obtain more relevant outcomes.²⁷¹ More complex models using microfluidic platforms to grow spheroids have been focused on understanding the penetration, distribution, and effectiveness of hyperthermic nanomedicines through more complex 3D models. As an example, a microfluid system consisting of PDMS was used to grow multicellular tumor spheroids to evaluate the PTT performance of hollow Au nano-shells modified with an anti-MUC1 aptamer (HGNs@anti-MUC1) (Fig. 4A1–A3).²⁷² Human lung epithelial carcinoma cells (A549) and human breast adenocarcinoma cells (MCF-7) were used to assemble the tumor spheroids. The tested nano-PTA

showed to be efficient in reducing the viability and size of tumor spheroids after irradiation, which was more pronounced with a double dose of irradiation with a 1 hour interval from the first one (Fig. 4A4). The study also tested the nano-PTA in spheroids of non-tumorous cell lines, and besides proving that the internalization of the nanomedicine was less effective compared to tumor spheroids, it was also observed that under the same treatment conditions, non-tumorous spheroids exhibited less pronounced effects, assuming the efficiency of HGNs@anti-MUC1 to specifically treat tumors. Furthermore, spheroids from different cell lines had different treatment outcomes, reinforcing the fact that the response to hyperthermia is tissue-dependent.²⁷²

Treating brain tumors presents significant challenges as it involves the risk of damaging surrounding healthy tissue, which can lead to serious complications and low survival rates. The non-invasive nature of hyperthermia, its potential for selective tumor treatment, and the challenges posed by the complexity of the brain TME and the blood–brain barrier have encouraged the exploration of this therapy in 3D brain tumor

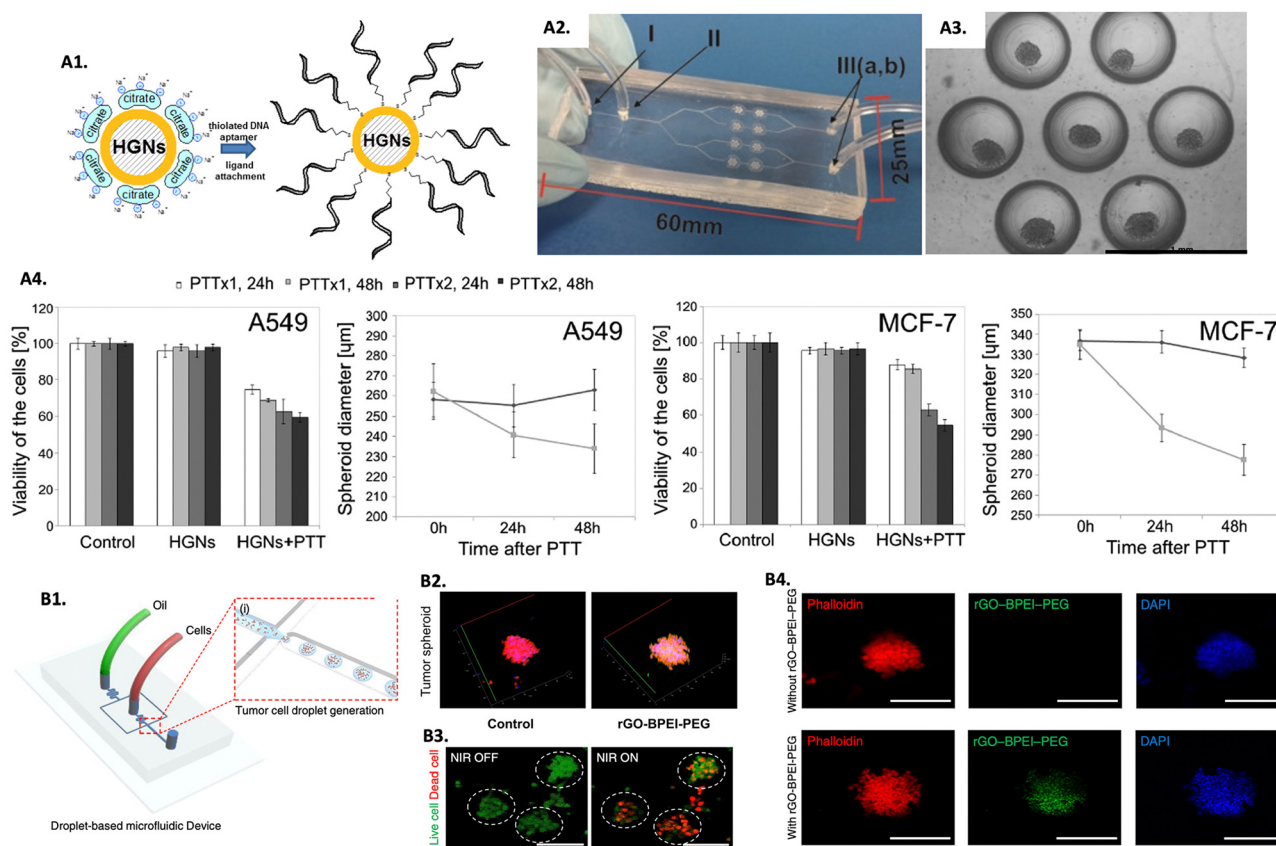
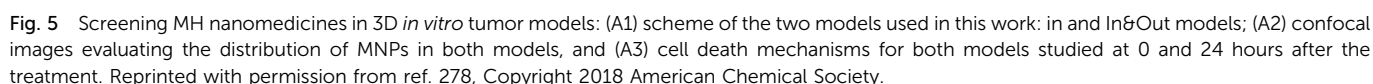


Fig. 4 Screening PTT nanomedicines in 3D *in vitro* tumor models for PTT: (A1) scheme showing the modification of HGNs with the anti-MUC1 aptamer, (A2) microfluidic system used to implement the spheroids (I, III – inlets/outlets and II – vent hole), (A3) image of a chamber of the microsystem containing spheroids, and (A4) spheroids viability and diameter submitted to one (PTTx1) and two (PTTx2) NIR doses, after 24 and 48 hours. Reprinted with permission from ref. 272, Copyright (2019), with permission from Elsevier; (B1) scheme of the generation of 3D tumor spheroids using the drop-based microfluidic device, (B2) Z-stack confocal laser scanning microscopy image of 3D tumor spheroids treated with rGO-BPEI-PEG (scale bar = 100 μm), (B3) live/dead images of brain tumor spheroids before and after PTT (scale bar = 100 μm), and (B4) fluorescence microscopy images of brain tumor spheroids with and without rGO-BPEI-PEG internalized, reprinted with permission from ref. 274, Creative Commons (CC) License 4.0 (<https://creativecommons.org/licenses/by/4.0/>).





Beyond demonstrating the feasibility of using PTT to replace traditional methods by being suited to perform treatment in tumors localized in deeper and sensitive parts of the body, such

MH allows a high penetration depth and selectivity, which has many benefits when the tumor is localized in sensitive tissues, such as the brain, or more deeply in the body.⁸⁷ Ongoing research and development efforts are focused on optimizing MNPs' design and targeting strategies. As mentioned before, iron oxide MNPs have been the most explored for MH, which is why they are the most used MNPs for hyperthermia screening in 3D tumor models. To explore the influence that the location, amount, and heterogeneity of iron oxide MNPs have on MH, a study was carried out to evaluate their performance for MH in 3D cell culture gels based on collagen of a murine macrophage RAW-264.7 cell line.²⁷⁸ MNPs distribution within the model was manipulated, namely: (i) the In Model had MNPs homogeneously localized only inside the cells, while (ii) the In&Out model had MNPs heterogeneously localized inside and outside the cells (Fig. 5A1 and A2). Results showed that AMF exposure promoted the uptake of MNPs, that could be related to an increase in collagen permeability. Furthermore, it was shown that the cell death mechanisms triggered after treatment were dependent on the iron concentration inside the cells. Additionally, the heterogeneous distribution of particles in the In&Out setup was

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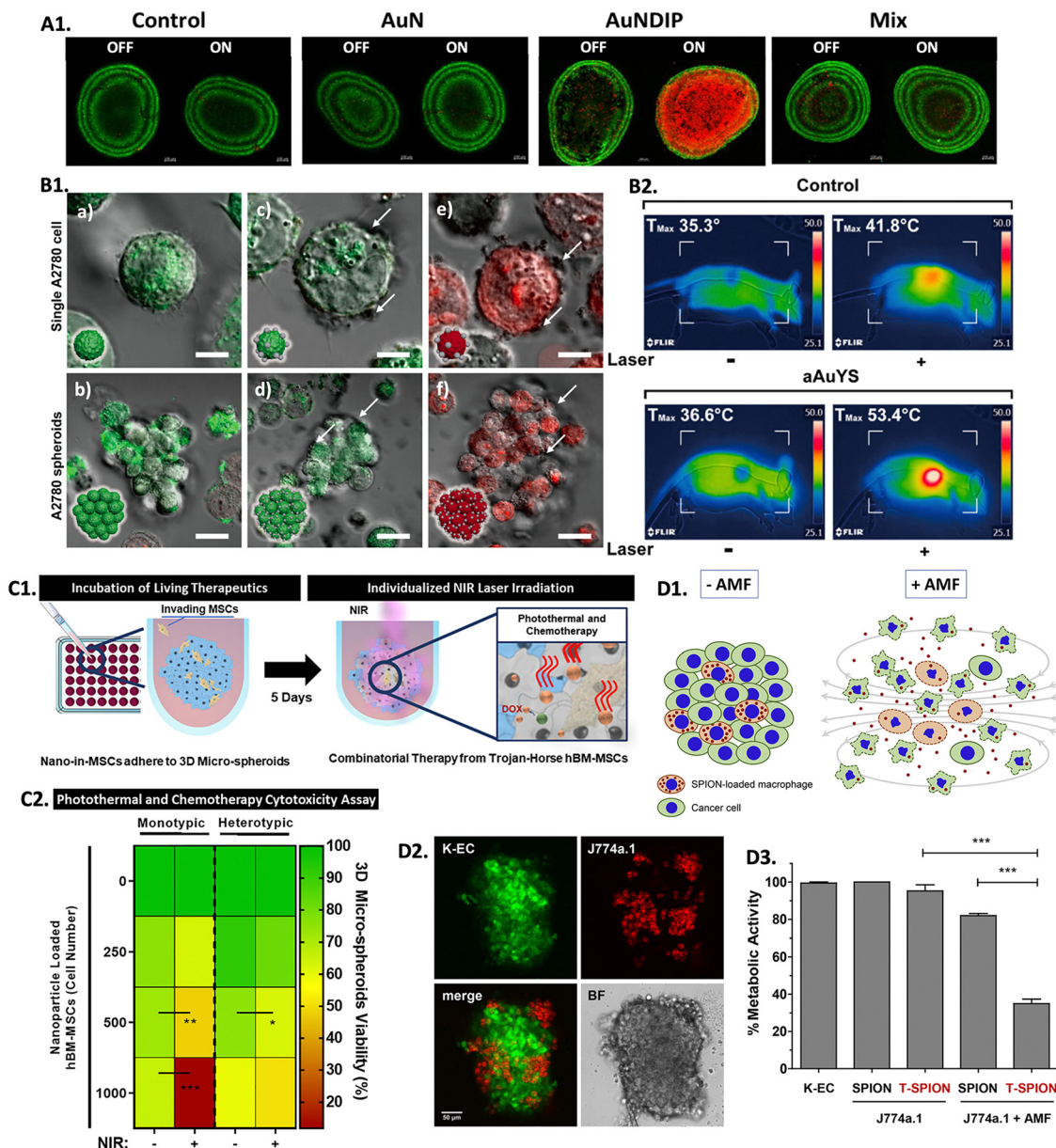


Fig. 6 Screening combinational hyperthermia and chemotherapy in 3D *in vitro* tumor models: (A1) live/dead confocal images of control untreated C6 spheroids, and spheroids treated with AuN, AuNDIP, and Mix (DOX-ICG and B-anti G), with and without laser exposure (scale bar = 100 μ m). Reprinted from ref. 287, Copyright (2022), with permission from Elsevier. (B1) Confocal images of A2780 cells and spheroids: (a) and (b) non-treated with aAuYS under laser irradiation; (c) and (d) treated with aAuYS without laser irradiation, and (e) and (f) treated with aAuYS under laser irradiation (scale bars in the upper and lower panels represent 6 and 20 μ m, respectively), and (B2) infrared thermal images of a mice treated with DOX and aAuYS submitted to NIR laser, Reprinted with permission from ref. 288. Copyright 2021 American Chemical Society; (C1) scheme representing the performed procedure of hBM-MSCs NPs delivery in 3D spheroids followed by laser irradiation; (C2) heat map enhancing the influence of the number of MSCs loaded with PDA-ICG-DOX NPs in 3D spheroids viability, 3 days after NIR laser treatment (irradiation (NIR +), and without irradiation (NIR -)). Reprinted with permission from ref. 174, Copyright (2021), with permission from Elsevier; (D1) scheme representing the incorporation of macrophage loaded with SPIONs into cancer cells and its effect when submitted to an AMF; (D2) confocal microscopy showing the integration of J774a.1 macrophages (red) in K-EC spheroids (green) (scale bar = 50 μ m); and (D3) metabolic activity of re-cultured spheroids containing T-SPION loaded macrophages with or without AMF application after 48 h of treatment. Reprinted with permission from ref. 292, Copyright (2019), with permission from Elsevier.

that the heterotypic spheroids were more resistant to treatment than the monotypic spheroids and that the use of MSCs as nanocarriers was more efficient in reducing the viability of tumors than the introduction of free nanomedicines in the 3D tumor models.¹⁷⁴ These findings are important for understanding how a heterotypic environment can confer

more resistance to treatment, mimicking a more *in vivo*-like scenario.

Recently, a study intending to understand the feasibility of macrophages in transporting SPIONs coupled with a toxin (T-SPIONs) to KSHV-infected human endothelial cells (K-EC) spheroids for a combination of MH and chemotherapy (MH-CHT) was





Table 4 Examples screening the effectiveness of 3D tumor models in predicting the efficiency of nano-heating agents for human and hyperthermia

Heating agent		Core	Coating	Size w/coating (nm)	Heating efficiency ^c	Technique, heating conditions	Tumor model	Dose ($\mu\text{g mL}^{-1}$)	T reached during treatment ($^{\circ}\text{C}$)	Cell death (%) (time after treatment, technique)	Ref.
DOPA-rGO	Thiol-terminated poly(2-ethyl-2-oxazoline)			100–200, DLS	PCE N.D.	PTT (808 nm, 1.7 W cm^{-2} , 5 min)	MCF-7 monolayers and spheroids	75	N.D.	2D model: 97% (24 h, AB) 3D model: 70% (48 h, AB)	270
Au nanoshells	Anti-MUC1 aptamer			N.D.	PCE N.D.	PTT, 808 nm, 5 min, single (PTTx1) or double irradiation (PTTx2), 1 h interval	A549 and MCF-7 spheroids in MD	100 μM	N.D.	A549: 31% (PTTx1) and 41% (PTTx2) MCF-7: 15% ^a (PTTx1) and 50% ^a (PTTx2) (48 h, AB)	272
rGO	BPEI-PEG			50–60, AFM	PCE N.D.	PTT, 808 nm, 1 W cm^{-2} , 10 min	U87MG spheroids in MD	60	N.D.	~45% (after treatment, live/dead)	274
Black phos-ohorus nanosheets	HA coating with loaded cypate			187, DLS	PCE ~ 49% (at 0.5 W cm^{-2})	PTT, 808 nm, 1 W cm^{-2} , 3 min	4T1 monolayers and spheroids	25	N.D.	2D model: 60% (24 h, MTT) 3D model: ~75% (7 days, FC)	276
Fe ₃ O ₄ NPs	PMAO functionalized with glucose			48–67, DLS	SAR = $253 \text{ W g}_{\text{Fe}}^{-1}$ ($10^3 \mu\text{g}_{\text{Fe}} \text{ mL}^{-1}$, 20 $\text{W g}_{\text{Fe}}^{-1}$, 829 kHz)	MH, 13 $\text{W g}_{\text{Fe}}^{-1}$, 377.5 kHz, 30 min	ATCC TIB71 culture gels	200	N.D.	In model: ~40% In & out model: 38% (24 h, FC) ~96%, both models (48 h, FC)	278
Fe ₃ O ₄ NPs	Aminosilane			100, N.D.	SAR ~ $115 \text{ W g}_{\text{Fe}}^{-1}$ ($10^4 \mu\text{g}_{\text{Fe}} \text{ mL}^{-1}$)	MH, 300 Gauss, 305 kHz, 10 and 30 min	C6 spheroids in MD	$10^{4,b}$	41–43	10 min: 20% 30 min: 100% (after treatment, live/dead)	295
Fe ₃ O ₄ NPs	TPP			20, N.D.	SAR = $619 \text{ W g}_{\text{Fe}}^{-1}$ ($340 \mu\text{g mL}^{-1}$)	MH, 30 A, 300 kHz, 10 min	HepG2 monolayers; 3T3 and HepG2 spheroids	50^b	N.D.	2D model: ~50% ^a 3D model: ~20% ^a (after treatment, trypan blue)	279
SPIONs	Phospholipid bilayer			100, DLS	SAR = $406 \text{ W g}_{\text{Fe}}^{-1}$	MH, 40–47 $\text{W g}_{\text{Fe}}^{-1}$, 270 kHz, 30 min	PANC-1 monolayers and PDAC organoids	225 ^b	N.D.	2D model: 27% 3D model: 48% and 13% (2 h, 24 h, CellTiter-Glo [®])	280
Gold nanoroses (AuNs) loaded with DOX and ICG	Peptide B-anti G			295, (just AuNs) DLS	N.D.	PTT-CHT, 808 nm, 2 W cm^{-2} , 10 min	C6 monolayers and spheroids	100	N.D.	2D model: ~85% 3D model: ~88% (24 h, MTT)	287
PDA loaded with DOX and ICG NPs	hBM-MSCs			328, DLS	PCE ~ 90%	PTT-CHT, 808 nm, 1.6 W cm^{-2} , 5 min	Monotypic (MDA-MB-231) and heterotypic (MDA-MB-231 and BCAFs) spheroids	500	N.D.	Monotypic spheroids: ~60% ^a Heterotypic spheroids: ~40% ^a (3 days, CellTiter-Glo [®])	174
SPIONs coupled with a toxin	Silica coating; loaded in mouse macrophages (J774a.1 cells)			N.D.	N.D.	MH-CHT, 4.8 $\text{W g}_{\text{Fe}}^{-1}$, 779 kHz, 40 min	K-EC spheroids	$30 \mu\text{g}/10^5$ cells	N.D.	~65% ^a (48 h, WST-1)	292

^a Value extracted from graphs. ^b Fe concentration. ^c The conditions that are not indicated are the same as the heating conditions; N.D. – non described; MD – microfluidic device; DOPA: dopamine; rGO: reduced graphene oxide; BPEI: branched polyethyleneimine; AFM: atomic force microscopy; HA: hyaluronic acid; PMAO: poly(maleic anhydride-*alt*-1-octadecene; TPP: triphenylphosphonium cation; PDAC: pancreatic ductal adenocarcinoma; PDA: polydopamine; hBM-MSCs: human bone-marrow derived mesenchymal stem cells; BCAFs: breast cancer-associated fibroblasts.

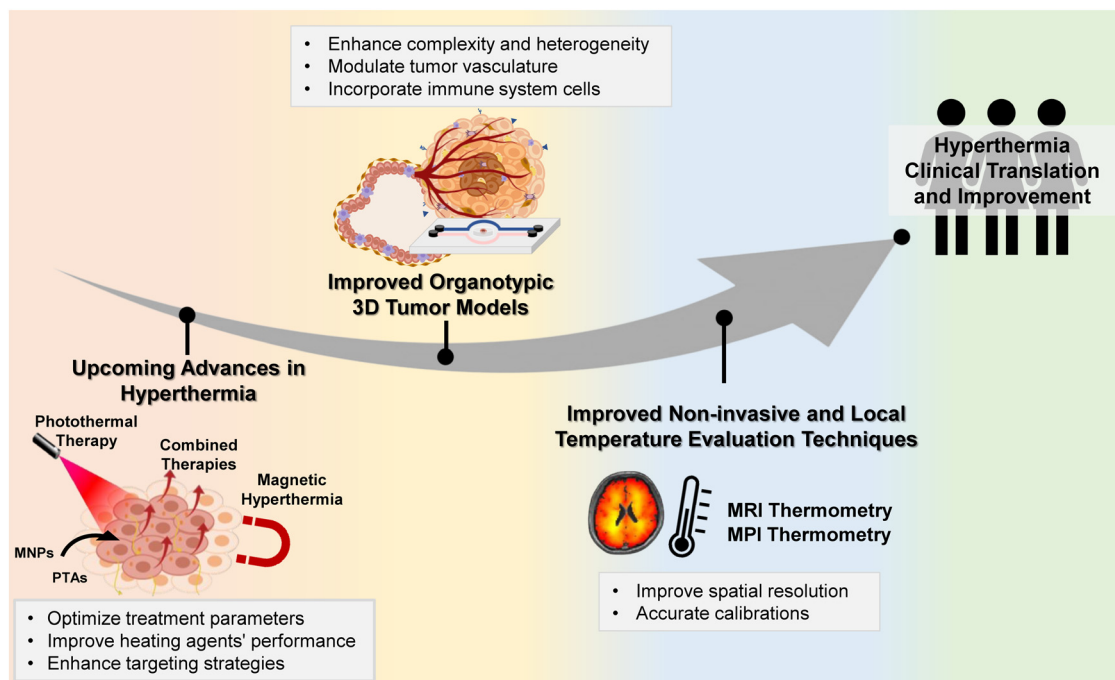


Fig. 7 Developments in hyperthermia for clinical translation: progressive advancements of hyperthermia treatments, 3D tumor models, and non-invasive thermometry techniques that can accelerate the translation of hyperthermia to clinical practice.

carried out (Fig. 6D1 and D2).²⁹² Even without being submitted to an AMF, after 48 hours in culture, spheroids containing T-SPIONs presented a significant loss of structure, whereas spheroids with only SPIONs or without SPIONs did not show any significant structural change. The spheroid's structure was even more compromised after treatment with AMF, being more evident when the toxin was coupled to the SPIONs, accompanied by a decrease in the metabolic activity of spheroids (Fig. 6D3).²⁹²

Hyperthermia before radiotherapy was shown to increase the efficiency of the treatment since heat can selectively target and sensitize cancer cells, making them more vulnerable to radiation-induced DNA damage and cell death. Consequently, hyperthermia can improve tumor response, overcome radio-resistance, and potentially reduce the side effects associated with high radiation doses.²⁹³ In an elegant approach, copper sulphide NPs (CuS NPs) were associated at the surface of upconversion NPs (UCNPs) due to manganese dioxide (MnO_2) coating, forming multifunctional nanoplateforms (UCCM).²⁹⁴ Formulated UCCMs were internalized in mouse colon (CT26) spheroids to evaluate internalization in a solid tumor model. It was observed that after 4 hours, CuS NPs were at the center of the spheroid. This allowed MnO_2 to release Mn^{2+} and oxygen, consequently reducing hypoxia and resistance to radiation. Furthermore, the released Mn^{2+} was shown to be a proper contrast agent for MRI, allowing real-time control of the tumor site that can be helpful for treatment guidance. The therapeutic effect of the UCCM NPs was then evaluated in mice bearing a human liver tumor, which revealed that the tumors shrank compared to non-treated tumors and that the normal tissues were minimally affected. Moreover, the combination of the

treatments was far more effective in destroying the cancer cells than the treatments alone.²⁹⁴

Studies using 3D *in vitro* models for screening nanomedicines for a combination of hyperthermia and radiotherapy are still a novel approach, thus accounting for the lack of literature reports on this subject. Recent studies continue to rely on standard 2D *in vitro* cultures,^{296–299} which is also important, but there is a need to embrace novel and innovative *in vitro* testing platforms that can more closely recapitulate human tissues response to treatments and ultimately contribute to understanding the underlying mechanisms that contribute to the observed synergistic effects that can accelerate translation to clinical practice. Table 4 showcases studies reporting the application of 3D tumor models as screening platforms to evaluate nano-heating agents for hyperthermia as a stand-alone or combinatory treatment. The examples reported in this section have provided valuable insights into the synergistic interactions between hyperthermia and traditional cancer treatments. This approach allows improved drug penetration, enhanced cancer cell killing, modulation of drug resistance mechanisms, and reduction of the radiation dose and/or chemotherapeutic agents, which is very important to guarantee the safety of healthy tissues. Optimizing treatment parameters, such as exposure time, and nano-medicine or radiation doses, remains crucial. Alongside, recent advances in the combination of hyperthermia with modern and more sophisticated therapies, such as immunotherapy, have been shown to provide better immune recognition and destruction of the tumor since the heat induced by hyperthermia can activate and boost immune cells' anti-tumoral activity.¹²⁵

Besides being used to predict the efficiency of the treatment, 3D *in vitro* models can be useful platforms to assist the design



process of the nano-agents for parameters such as size, morphology, coating, stabilization, and targeting optimization, in a specific and relevant tumor context. A study using colorectal cancer spheroids was carried out to understand how the internalization of NPs was affected by their size, surface, and bulk characteristics.³⁰⁰ The authors considered poly(styrene) NPs with different sizes and different coatings and concluded that unmodified NPs with smaller sizes had more efficiency in accumulating at the core of the spheroids. Regarding surface charge, it was shown that better penetration ability was achieved with positively charged NPs, due to the presence of negatively charged elements in the ECM that can repel negative charges. Moreover, it was discussed that lower molecular weight NPs have greater efficiency in penetrating tumors.³⁰⁰ In another study, the influence of the shape of the NPs on penetrating 3D HeLa tumor spheroids was assessed.³⁰¹ Sphere, long rod, and short rod-shaped NPs were studied, and the uptake efficiency in spheroids was shown to be strongly dependent on the length-to-width ratio. The authors concluded that NPs with higher length-to-width ratios had more difficulty penetrating tumors than those with lower length-to-width ratios. Additionally, the uptake efficiency was different when considering 3D tumor spheroids and monolayers.³⁰¹ The presented studies emphasize the importance of considering the physicochemical properties of NPs during the design phase, aiming to optimize their hyperthermic efficiency.

5. Conclusions and future challenges

With the developments achieved in the last few years, organotypic 3D models have emerged as feasible biomimetic platforms, capable of replacing the standard 2D models that continue to be widely explored for screening hyperthermic nanomedicines. These advanced biomimetic models enable a closer examination of the effects induced by heat within tumors, taking into consideration factors such as tri-dimensionality, size, composition of the TME, availability of nutrients and oxygen, intercellular signaling, and nanomedicines properties. The tissue-specific characteristics of 3D tumor models that allow the incorporation of several ECM components and other cell types can help in the design of cell-specific cancer platforms to optimize treatment outcomes. Due to the inherent unpredictability, complexity, and specificity of tumors for each patient, this approach allows for personalized treatment models that offer improved accessibility and predictive capabilities in accessing the response to hyperthermia therapies. Despite these improvements at the preclinical stage, the protocols of hyperthermic procedures must be standardized, as well as the characterization techniques.^{302,303} As discussed above (Tables 1, 2 and 4), highly relevant information regarding treatment procedures is lacking from literature reports, which may impede the establishment of standardized protocols.

It is pertinent to highlight the importance of considering the design of more complex cellular 3D *in vitro* structures (*i.e.*, regarding ECM components, cellular types, incorporation of vascularization and diffusion mechanisms, *etc.*) in the future,

aiming to provide more realistic screening platforms. As it was understood by the reported studies, spheroids are the predominant model as they are the most simple and cost-effective testing platforms. Certainly, organoids have gained increasing attention recently, offering more complexity and a more *in vivo*-predictive response to treatments. This development aims to facilitate the translation of hyperthermic nanomedicines into clinical practice. Furthermore, combining spheroids or organoids with tumor-on-a-chip platforms and 3D bioprinting techniques can increase the complexity of the models, as these allow to introduce components of the tumor vasculature and flow dynamics/mechanisms. Also, some studies reported here provided both 3D *in vitro* and *in vivo* assessment of the hyperthermic nanomedicines, which enables to confirm that these models can provide closer *in vivo* responses and consequently contribute to accelerating hyperthermic nanotherapies preclinical validation and subsequent transition to clinical practice.

Even though hyperthermia demonstrates notable efficacy in tumors, its widespread clinical implementation is being delayed by prominent limitations, including inadequate penetration and heterogeneous distribution of heating agents in tumors, and insufficient control over temperature regulation at the tumor site. As shown and discussed herein, researchers have been focused on studying different carriers and targeting agents capable of improving the internalization of heating agents in the tumor, which have been shown to provide a more localized treatment with better therapeutic outcomes. Approaches that involve focalized delivery are also envisioned to be further explored in the future.

Regarding temperature control, there is a need to have a reliable method for real-time assessment, thereby mitigating damage to healthy tissues around the tumor mass. However, invasive devices (*e.g.*, optical fibers and thermocouples) currently represent the prevailing method for temperature control in hyperthermia procedures.³⁰⁴ As noted in the examples documented in Table 4, a substantial number of studies did not present the temperature that the 3D models reached during the treatment, which can be due to the difficulty in controlling the temperature in real-time. Currently, non-invasive thermometry systems based on MRI,^{305,306} magnetic particle imaging (MPI),³⁰⁷ or fluorescence thermometry^{308,309} are being developed, representing an outstanding breakthrough in the field by allowing temperature control in real-time and in a minimally invasive way. However, 3D tumor models are challenging tools when it comes to obtaining temperature maps with adequate spatial resolution and for imaging. With increasing tumor size, light has less penetration ability, as it gets more scattered. Consequently, the ability to acquire temperature maps or high-resolution images declines, particularly considering deeper regions within the tumor. Furthermore, larger tumor sizes can be a barrier to prompt acquisition of images, thus compromising the real-time control of the heat therapy.^{23,304,310} To address these limitations, researchers have been exploring strategies to confer nanomedicines with theranostic capabilities, *i.e.*, the ability to perform an image-guided therapy.^{311–313}



By using nanomedicines for temperature control, the problem of spatial resolution can be addressed, but imaging techniques capable of capturing such fine-scale response are needed.³¹⁰ MRI has a spatial resolution in the order of the millimeters,³¹⁴ which can be crucial to provide high-resolution temperature maps, as the ones obtained in.^{315,316} By tuning the transition temperature of MNPs to room temperature, it is possible to use those nanomaterials as temperature sensors in the range of hyperthermia.³¹⁷ Consequently, these agents can be simultaneously used as contrast agents and thermometers for MRI during hyperthermia treatments. However, MRI-based thermometry still faces challenges with low signal-to-noise ratio and inaccurate temperature calibrations.³¹⁸

Integrating advancements in 3D *in vitro* tumor models with emerging hyperthermia techniques and non-invasive 3D thermometry systems could mitigate the disparities in readouts between preclinical and clinical trials for screening advanced anti-cancer therapeutics (Fig. 7). This convergence of technologies holds promise for enhancing the translational relevance of treatments, foreseeably facilitating their widespread use in clinical practice.

Conflicts of interest

The authors declare no competing interests.

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Review

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Review

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