Gel models to assess distribution and diffusion of reactive species from cold atmospheric plasma: an overview for plasma medicine applications

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Abstract. The emerging field of plasma medicine opens new therapeutic opportunities with the use of Cold Atmospheric Plasma (CAP) as a versatile tool for the treatment of tissues in various medical indications. Yet, the complexity of this very reactive medium combined with a high dependence on its environment and generation parameters make it difficult to predict and optimize such treatment. To this end, a simple yet robust and accurate tissue phantom allowing to study the penetration and distribution of plasma action in simulated *in vivo* conditions has been developed by several groups. It combines a hydrogel-based matrix closely resembling tissues and chemical reporters incorporated in the gel to measure the delivery of Reactive Oxygen and Nitrogen Species (RONS) by the plasma. This paper reports the use of these models in the literature to give an overview of the state of the art, their capabilities and the further research required to improve it. First the hydrogels composition (i.e. gelatin and agarose) is discussed, as well as the parameters allowing to fine tune the model. In particular, we show that modifying mass fraction has been reported to mimic several types of tissues and that different model configurations allow to test different treatments conditions, including the barrier effect of skin or the direct treatment of a tissue bulk. The role of other critical parameters is highlighted, including manufacturing, diffusion, electrical characteristics but also liquid composition, thickness, aging and temperature influence. Secondly, RONS reporters used in the plasma medicine literature (colorimetric/fluorometric dyes) are summarized. The analysis techniques are discussed and the dyes characteristics (i.e. wavelength, specificity, concentrations) are reported. Finally, the influence of medium and time on these measurements are covered. For both sections, the limitations of these current models are presented and linked to potential improvements and further research.

Keywords: tissue model, hydrogel, agarose, gelatin, plasma medicine, RONS, probes, reporters.

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Contents

1 Introduction 4

2 Hydrogels 7
2.1 Material . . . 7
2.1.1 Nature . . . 7
2.1.2 Mass fraction . . . 8
2.2 Model types . . . 11
2.3 Manufacturing . . . 14
2.4 Diffusion . . . 15
2.5 Electrical characteristics . . . 16
2.5.1 Electrical characteristics of real tissues : . . . 16
2.5.2 Matching hydrogel electrical parameters : . . . 17
2.5.3 Tuning conductivity : . . . 17
2.5.4 Tuning permittivity : . . . 18
2.6 Important parameters . . . 18
2.6.1 Liquid medium . . . 18
2.6.2 Thickness . . . 19
2.6.3 Aging . . . 19
2.6.4 Temperature . . . 19
2.7 Limitations . . . 20
2.8 Model Improvement . . . 20

3 Reporters 22
3.1 Species of interest . . . 22
3.1.1 Nature . . . 22
3.1.2 RONS lifetime . . . 22
3.2 Measurement . . . 24
3.2.1 Dyes . . . 24
3.2.2 Spectroscopy . . . 25
3.2.3 Imaging . . . 26
3.2.4 Colorimetric dye reporters . . . 26
3.2.5 Fluorescent dye reporters . . . 30
3.3 Important parameters . . . 32
3.3.1 Specificity . . . 32
3.3.2 Sensitivity and concentration . . . 32
3.3.3 Medium . . . 33
3.3.4 Alternative reporters . . . 33
3.3.5 Influence of time . . . 33
3.4 Limitations . . . 34

4 Discussion and future work **35**
4.1 Achievements 35
4.2 Proposition of experimental protocol for the manufacturing of hydrogel-reporter model 37
4.3 Conclusion 37

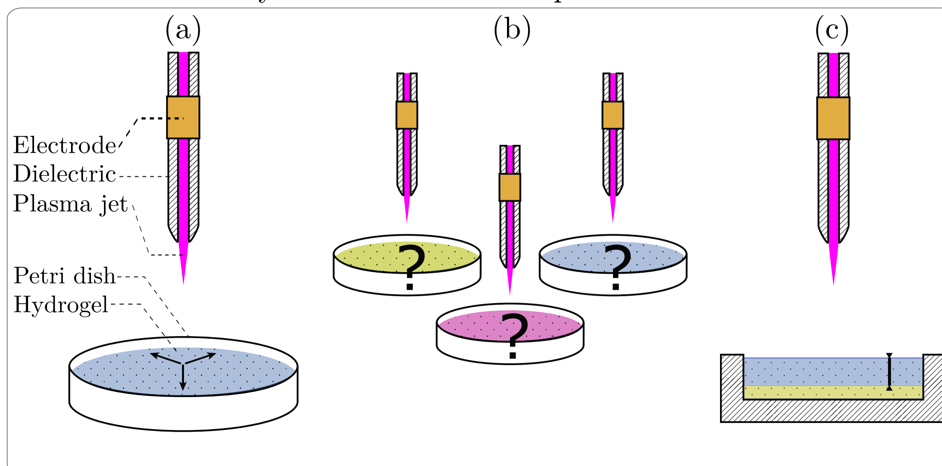
1. Introduction

The use of Cold Atmospheric Plasma (CAP) in medicine draws increasing attention worldwide and the so-called Plasma Medicine field has rapidly grown in the last two decades [1–4]. The combination of electric field, Reactive Oxygen and Nitrogen Species (RONS) and the several other actors it contains has been shown to elicit a large range of biological effects [5] from sterilization to tissue regeneration and cell death. It could thus represent a powerful tool for medicine and technology-dependent procedures such as therapeutic endoscopy [6]. Its effect is however very complex and further research is needed to improve its use to the maximum of its capabilities, and in a safe manner. Indeed, CAP is an extremely complex medium exhibiting a high number of interplaying actors and is strongly influenced by its environment [7]. In addition, each plasma source is different (in terms of geometry, generation and operational parameters) and the plasma itself considerably depends on various parameters of the setup. In this frame, it is highly challenging to predict the effect of CAP on living tissues, which is critical to ensure a safe and efficient treatment. While the obvious solution for testing remains *in vivo* testing, it comes with several disadvantages among which high time consumption, high cost, low repeatability (because of high dependence on the specimen), with no possibility to tune the target or easily use it in a chemistry laboratory environment, not even accounting for ethical issues. However, this approach remains the closest to a real treatment and it is a necessary step in the development of plasma-based medical devices but should come at the last step of testing. For more fundamental studies and to be able to study multiple plasma configurations during the development of a plasma source and/or a plasma treatment, a simpler model can be of great use, as illustrated in Figure 1.

In order to study the effect of CAP on tissues in general, and of specific plasma devices or parameters in particular, different models of escalating complexities have been developed over the last 20 years, summarized in the work of Lu et al. [8]. It encompasses water and liquid media (water being the major component of main tissues), gels and soft matter (allowing to have a solid fixed medium with high water content) and dead animal tissues. Water-based gels (namely hydrogels) constitute a more complex and convenient model than liquid models, while remaining more tunable and comparable than real tissues, and have elicited a growing interest in the last decade. Although differing from living tissues in terms of composition and structure complexity, hydrogels have been shown to closely resemble tissue in terms of propagation and penetration by Dobrynin et al. [9], later confirmed by other teams [10–13]. In addition, they allow to incorporate chemical reporters (colorimetric or fluorescent dyes changing color through reaction with RONS) to easily assess the effect of CAP, such as membrane rupture [14] or more importantly the action of RONS, which are the major drivers of plasma effect [3] and thus the most investigated effect. Although relatively simple, this model remains a good approximation of the chemical composition (i.e. mostly water and collagen) and conductivity of the target. In this review, the gels and associated chemical reporters used

up to this date are summarized and discussed to present an overview of the possibilities offered by this model and help the interested reader in the development of his own.

Fundamental study on distribution and penetration of various RONS



Quick, easy and reproducible step in a plasma medicine study workflow

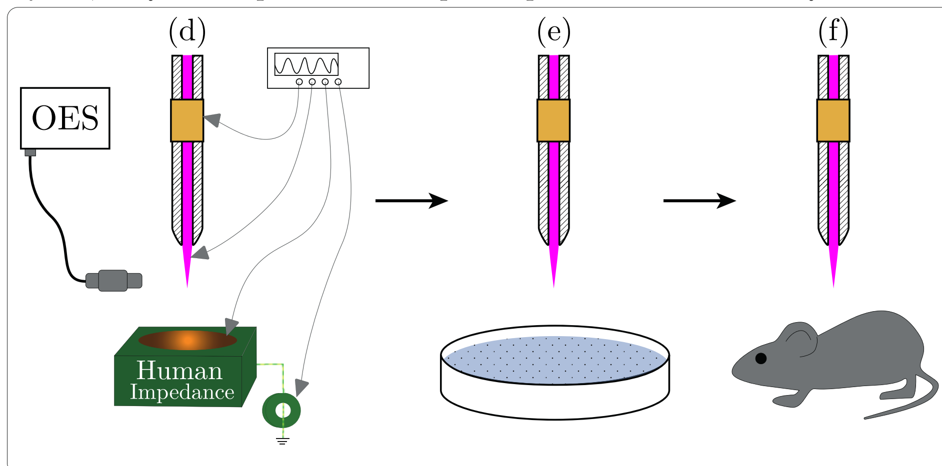


Figure 1. Use of hydrogels in [top] fundamental studies for (a) assessment of RONS distribution, (b) detection of specific RONS and (c) penetration of RONS, [bottom] development of plasma-based medical device from (d) plasma setup characterization (chemical and electrical characterization with human electrical model [15]), (e) modeling of plasma effect on tissues, (d) in vivo/real tissue experiment

This overview first presents the hydrogels used as tissue models, going through the different materials and configurations, their manufacturing, diffusion properties, electrical characteristics and important parameters to take into account in their preparation. Limitations of these gels and potential improvements are also presented. Secondly, the chemical reporters introduced in these gels to report the plasma action are presented, starting with a discussion on species of interest and the types of dyes used in the literature. Measurement of these reporters after treatment are then considered, as well as key factors impacting this analysis (sensitivity, specificity, influence of time), to end again with the limitations and potential improvements of these reporters.

The scope of this overview was restricted to the specific literature using hydrogels for plasma medicine purposes. It was meant to include all relevant existing articles starting from the first reports in 2012 up to June 2021 included. Using the relevant keywords (tissue model, CAP, agarose, gelatin, hydrogel, plasma, etc.), authors names (researchers with several publications in the field and their co-authors) and their combinations on Google Scholar, plus cross-checking citations, around 50 relevant papers were found and analyzed. While this may seem a small number of references, it is however exhaustive to the extend of our knowledge and should give an in-depth overview of the field.

2. Hydrogels

2.1. Material

2.1.1. Nature A hydrogel is a network of hydrophilic polymer chains in which the swelling agent is water (illustrated in Figure 2). Hydrogel phantoms (i.e. a tissue substitute traditionally used in imaging) have been widely used in numerous fields related to medicine (imaging, dosimetry [16], acoustics [17], surgery [18]) as tissue models to mimic different properties of living organisms as their high-water content makes them good candidates to mimic tissues. In particular, agarose and gelatin are biological hydrogels used for decades as tissue-equivalent in several of these fields, such as imaging [19–21], ultrasound [17, 22, 23], optics [16], dosimetry [16], elastography [24] or even cancer-related studies about radiofrequency ablation [18,25,26] and radiotherapy [27].

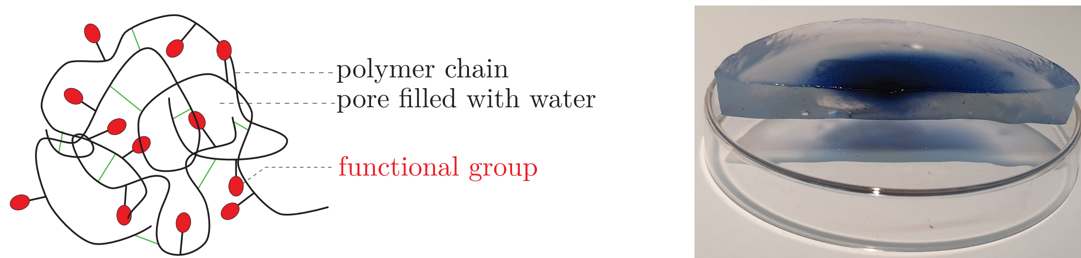


Figure 2. [left] Hydrogel structure and [right] example of an agarose hydrogel

Hydrogels are arguably the most used models in plasma medicine to study the distribution and penetration of the plasma action, because of the quasi solid “frozen” structure they provide as opposed to liquid phantoms. In addition, they are easy to shape (Park et al., for example, created an agarose phantom containing a tubular cavity to simulate bronchi [28]), can be easily tuned either to closely resemble tissues (i.e. using different mix, various chemicals or heterogeneities) or, most conveniently, to incorporate reporters enabling to locate and quantify plasma action. In addition, they are cheap, easy and quick to manufacture [8] and relatively stable (as discussed later), which make them a good candidate for laboratory experiments as a repeatable model, both in time and in experiments replicates. The materials used in plasma medicine are mostly agarose, gelatin and more rarely PolyVinyl Alcohol (PVA).

Agarose is a linear polysaccharide contained in agar, which is a component derived from seaweed. Agar constitutes the supporting structure of cell walls in some species of algae and is composed of a mixture of agaropectin and agarose (see Figure 3). This latter is massively used in biological routines for electrophoresis (sorting of macromolecules depending on their size [29]), which ensures it is an easily accessible, well characterized and controlled product.

Gelatin is a polymer composed of a mixture of peptides (see Figure 4) and proteins derived from partial hydrolysis of collagen, the main structural protein of connective tissues, skin and bones, from which they are usually extracted in cattle or pig. It is

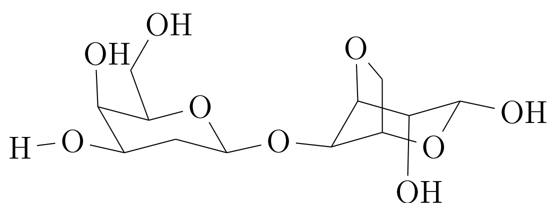


Figure 3. Chemical structure of an agarose monomer

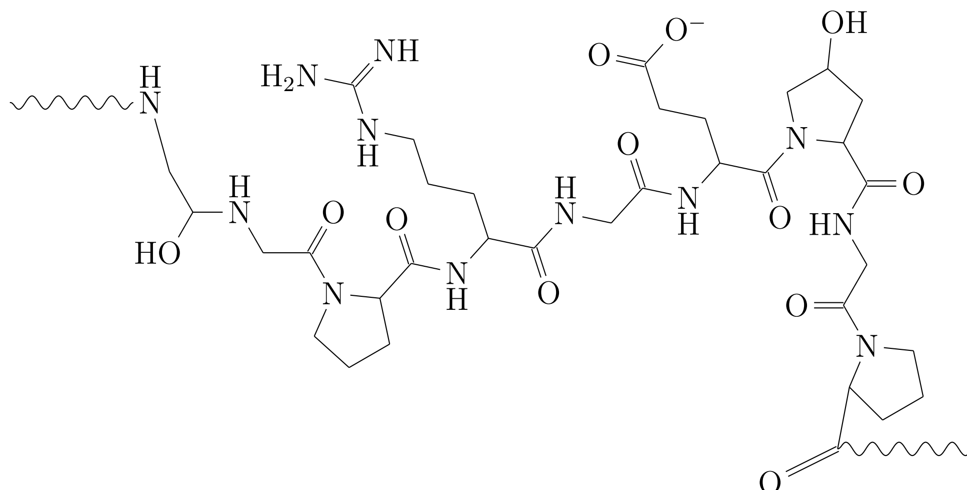


Figure 4. Chemical structure of a gelatin polypeptide

closely similar to collagen, and water-collagen solutions have been claimed to be a close simulation of human tissue [30].

PVA is a synthetic polymer also traditionally used to simulate human tissues [31, 32] but its non biological origin and its relatively long preparation time (around 24h [17, 33]) do not compensate for its higher stability, which probably explains its restricted [34] reported use so far in plasma medicine literature is limited. It will thus not be discussed more in depth in this paper.

Gelatin and agarose are thus by far the reference material for hydrogels in plasma medicine. With its collagen derived material and a water content similar to real tissues, gelatin might appear as a closer representative of human tissues compared to agarose, extracted from seaweed and showing much higher water content. However, this latter has been widely used for tissue phantoms in other applications and both gels have shown very good results as a model for tissue.

2.1.2. Mass fraction When designing an hydrogel model, the first parameter to select is the mass fraction of the dry component with respect to the swelling agent (i.e. water). It is often expressed as the weight of dry component per volume of swelling agent (weight/volume percentage or w/v%) or per weight of swelling agent (weight/weight percentage or w/w%). Depending on the mass fraction, the gel is supposed to mimic different kinds of human tissue. Gelatin gels usually contain 5% to 40% w/v dry gelatin

product where agarose range is situated between 0.5% and 5% w/v.

For gelatin, this percentage can easily be tailored depending on the targeted tissue simply by using the same water content, that can be found for example in Duke et al. tables [35] for different tissues. For example, skin has a water content between 58% and 72% [35] and gelatin gels of 30-40% w/v can be used to mimic them [8, 36]. He et al. used 15% w/v gelatin to model generic connective tissue such as blood [12, 37], which has a water content of 80% according to Duke et al. [35] or 85% in Davis et al. work [38]. Different relevant percentage ranges are presented in Figure 5. These percentages can also be tuned to represent even more accurately the final target of a potential plasma treatment. For example, several studies have shown an increase water content in tumors [36].

For agarose gels, the mass fraction determination is not so straightforward as its chemical composition is more different from living tissues than gelatin's collagen, and thus cannot be simply inferred from the water content of these tissues. However, it can also be tailored to model different tissues. Nebuya et al., for example, used an agar phantom to model a multi-layered skin and muscle phantom [39]. If the model needs to represent a specific tissue, the only reported solution up to this date is to determine this mass fraction from literature and/or empirically (through comparison of concentration, distribution, penetration of RONS between real tissue and gel, conductivity measurement, etc.). Low concentration agarose gel (0.2%-0.6%) were used as brain models by Chen et al. [27]. 1% agarose phantom, were shown to have a penetration depth similar to the one in water [40]. 1.5%-2.5% w/v agarose gels have been linked to muscle tissues in the frame of a MRI phantom development where Kato et al. computed the agarose mass fraction for numerous tissue types for each organ [21]. This was confirmed by the pioneering work of Dobrynin et al. [41] that found 1.5% w/v gel to be the closest to real muscle tissue. 2% agarose gel were also used to model breasts [17]. Finally, 4% agarose gels are reported closer to skin, notably because of their lower water content [40].

Figure 5 summarizes the mass fraction of gelatin and agarose gels used in plasma medicine literature (further detailed in Table 1). In parallel, the mass fraction ranges corresponding to different tissue types found in the literature are also displayed as a reference. While it gives a good overview of the commonly used mass fractions, it can be noticed that a large proportion of early studies used gels that seem to fit no reported existing tissues or tissues differing from their intended treatment target. This can probably be explained by the very exploratory nature of these first works and the lack of consistent data on the fitting of models to real tissues depending on mass fraction. This highlights the need of such studies to refine this model. Indeed, several works showed that the penetration depth of RONS decreases linearly with increasing mass fraction both for gelatin [12,37,42] and agarose [41,43]. This is probably consistent with real in vivo situation, with higher fractions of biological polymers (such as collagen) increasingly impairing the diffusion of small molecules.

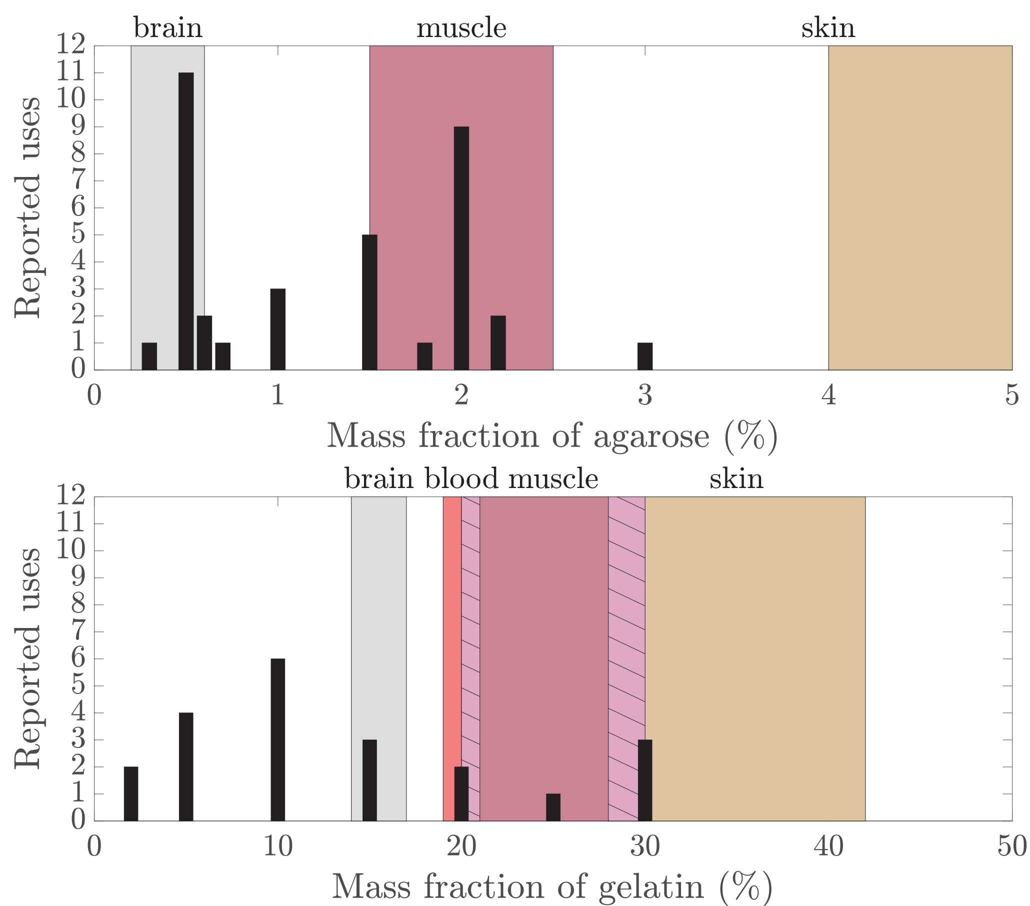


Figure 5. Reported uses of gelatin and agarose gels in the literature (from 2012 to 2020) per mass fraction. Several relevant values reported in the literature for corresponding tissues are superimposed as a reference (agarose ranges are referenced in the text and gelatin ranges are based on Duket et al. tables [35]).

2.2. Model types

In total, nearly 50 uses of gel as a tissue model for CAP treatment studies (all using plasma jets) have been reported in the literature, and are reported in Table 1. These gels were used in different setup configurations depending on the experiment, which have been classified in 5 categories (illustrated in Figure 6) :

(a) Gel matrix. This most simple model is similar to hydrogels used as phantoms in other fields. It uses no chemical reporters and is rather utilized as a support (thus the designation of gel "matrix") for others tests (e.g. bacterial invasion [44] or external reporters [45]) or as a barrier before a measurement setup.

(b) Gel reagent. This configuration is the most reported in the literature. It consists of the gel, simulating a living tissue bulk, and incorporating chemical reporters, thus the designation of gel "reagent", used by Kawasaki team [11]. It is used to study the distribution and penetration of RONS in the tissue volume, depending on different parameters either related to the plasma (i.e. gas mix, applied voltage, plasma generation, etc.) or its environment (nature of the gel, distance to the gel, treatment angle, etc.).

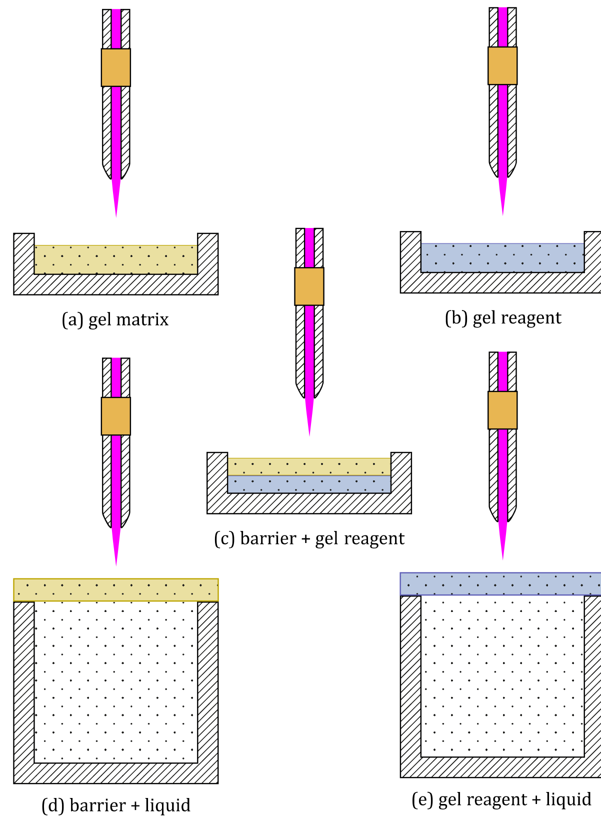


Figure 6. Illustration of the different model configurations

(c) Barrier + gel reagent. This configuration also comprises a gel reagent but is covered by a barrier (gel or liquid) to study the effect of RONS transport through it on the treatment of the gel reagent underneath. It allows to study the barrier effect of tissues (e.g. skin) and fluids on RONS distribution and penetration in contiguous tissues.

(d) Barrier + liquid. Similarly, this configuration uses gel as a barrier model to study the transport of RONS through an obstacle (typically skin) and the buffering action of this tissue in time by analyzing their delivery in a liquid (either with the same chemical reporters or with other techniques such as UV-VIS spectroscopy). This configuration is also widely used (see Table 1).

(e) Gel reagent + liquid. This configuration is similar to the previous one except that it has additional chemical reporters inside the barrier gel simulating to study the propagation of RONS inside it in parallel to their delivery in the liquid.

Table 1. Summary of models, materials, mass fraction and thicknesses used in the literature

Model	Material	Mass fraction (w/v%)	Thickness (mm)	Source
gel reagent	Agarose	0,5	2,5	[46]
		0,5	not specified	[11, 47, 48]
		0,5	14	[49, 50]
		0,6	not specified	[41]
		0,625	7	[51]
		1	1	[52]
		1,5	not specified	[9, 28, 41]
		1,5	2	[53]
		1,8	10	[54]
		2	2	[13]
		3	not specified	[41]
	Gelatin	2	6.5	[55]
		5	2	[10]
		10	not specified	[56, 57]
		10	3	[58]
		1-5-10-15-20-25-30	13	[12]
		10-15-30	3	[42]
gel matrix	Agarose	2	not specified	[44]
		2	3,2	[59]
barrier + gel reagent	Agarose	0,5	not specified	[60–63]
		0,5 (gel reagent)	4	[64, 65]
		1 (gel reagent)	3	[66]
		2,2 (barrier)	1	[64]
		2,2 (barrier)	0,5-1-1.5-2-3	[65]
gel reagent + liquid	Agarose	0,3	2	[67]
		1,5	2	[68]
	Gelatin	1,5 to 30	1	[12]
barrier + liquid	Agarose	0,7	2,2-3,5	[43]
		1	1-2,2-3,2	[43]
		1,5	2	[53]
		2	1,5	[69]
		2	3	[66, 70]
		2	4	[71]
		2	1,7-3,6	[43]
		2	1,3-2,5-5,8	[72]
	Gelatin	5	4	[73]
		5-10	1	[34]
		15	1	[37]
		10-20-30	1-2-3	[37]

Additionally, the literature using these models is classified according to the most relevant objectives of the study in Table 2, which have been divided in :

- Distribution of RONS : general study of RONS distribution and effect on the gel.
- Transport through barrier : study of RONS transport through the model.
- Plasma configuration : study of multiple plasma configuration parameters such as distance to target, target tilt angle, etc.
- Influence of gas : study of influence of gas flow, gas mix, etc. on RONS distribution and transport.
- Assess model : evaluation of the validity of the hydrogel model and comparison with other models or real tissues.
- Penetration depth : study of the RONS penetration depth in the gel.
- Correlation with biology : use in parallel with biological reporters (bacteria, cell lines).

Table 2. Summary of main study objectives in plasma medicine literature

Distribution of RONS	Transport through barrier	Plasma configuration	Influence of gas	Assess model	Penetration depth	Biology correlation	Reference
✓	✓		✓				[60]
✓	✓				✓		[63]
✓	✓	✓			✓		[64]
✓	✓	✓					[61,65]
✓		✓		✓			[28]
✓		✓					[50]
✓		✓		✓	✓	✓	[68]
✓		✓	✓				[49,58]
✓		✓	✓		✓	✓	[54,55]
✓			✓			✓	[46]
✓			✓				[47]
✓				✓			[57]
✓						✓	[45,51,52]
✓							[11,56,67]
	✓	✓			✓	✓	[66]
	✓	✓					[43]
	✓		✓				[62]
	✓					✓	[73]
	✓						[34, 48, 53, 59, 69–71]
			✓				[42]
				✓	✓		[72]
				✓			[9,41]
					✓		[12,37]
						✓	[44]

2.3. Manufacturing

The manufacturing of these hydrogels is time-efficient and convenient. The material, which comes as powder of different purity grades, is simply weighed to obtain the desired mass fraction, poured in a container and mixed with the dispersion medium (water or water-based solvent) to 100% by weight. The polymer chains of agarose and gelatin, yet hydrophilic, need to be crosslinked to provide a three-dimensional solidity in the form of a real gel. Crosslinking can be performed either through physical crosslinking (e.g. thermal heating) or chemical crosslinking (using crosslinking agents such as formaldehyde or glutaraldehyde) [74]. Physical crosslinking provides non-covalent bonds between chains (typically hydrogen bonds and chains entanglement) and is reversible whereas chemical crosslinking provides covalent bonds between chains, resulting in a more permanent, skeleton-like polymerization [74], as illustrated in Figure 7. The type and degree of crosslinking influences the mechanical properties, structure, permeability and swelling properties of the gel [75, 76]. Only physical crosslinking of hydrogels is reported in plasma medicine literature, which could probably be explained by the fact that mechanical properties are not critical for these tissues models and that chemical crosslinking agents will interfere with plasma chemistry and can be toxic to cells if incorporated [75].

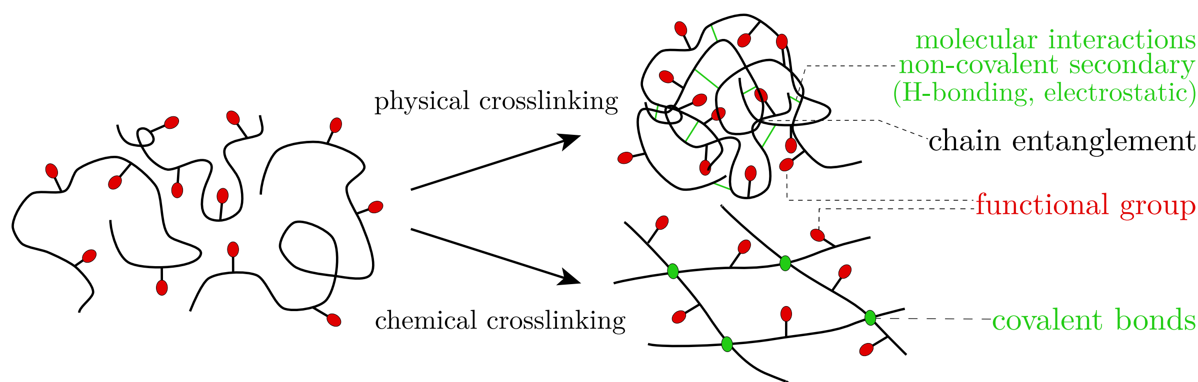


Figure 7. Hydrogel crosslinking process

This physical crosslinking is mostly achieved through thermal heating by bringing the solution to the boiling point, for example in a microwave [30] or a hotplate [17,20,77]. Using a hotplate also allows to stir the liquid to ensure a good homogeneity, which must turn from cloudy to clear (meaning that the powder has transitioned to molecular solution). After the transition (approx. 10-20min [20]), the solution can be kept warm for another 10min to ensure its completion [20], then poured in the designated container (which will act as a cast to give it its final shape) and left to cool until solidified (10 to 60min).

2.4. Diffusion

Diffusion in hydrogels has received very few interest in the frame of plasma medicine, especially compared to other fields (e.g. electrochemistry or biochemistry). It however appears to be a critical parameter to consider. Indeed, hydrogels are used to mimic the distribution of species in real tissues because they remain simple while exhibiting the two most important features of these latter, i.e. high water content and the ability to restrict the diffusion of a solute with biopolymers. Diffusion is thus largely responsible for the accuracy of the model since it conditions treatment depth and spatiality of the treatment. These are arguably the major outcomes of this model and will thus determine its fitting to real tissues.

The solute transport in water-based hydrogels occurs mainly in water-filled regions. Different mathematical models exist to describe this diffusion, reviewed in the work of Amsden [78]. Several factors usually impact the diffusion of a solute in the gel [78, 79] :

- Temperature, as discussed in subsubsection 2.6.4.
- Solute size.
- Solute chemical nature.
- Gel structure : Free volume (i.e. pore size), degree of crosslinking, polymer chain mobility, average polymer chain radius).
- Existence of charged groups on the polymer chains which may bind to the solute.

Depending on the type of hydrogel, Amsden has selected the most suitable models in his study. Agarose and gelatin are categorized as "heterogeneous" hydrogels because of the strong interactions between polymer chains, resulting in a low degree of mobility. In this type of gels, the pores can be considered constant in size and location [79]. Amsden's work showed that models based on the obstruction theory were the best to describe diffusion in such gels. This theory considers the polymers chains as impenetrable obstacles acting as a sieve to the solute, increasing path length. Several of these models are summarized in Amsden review. More recently, sophisticated models integrating several mechanisms (including obstruction) have been developed to even better predict and describe diffusion in gels [80]. However, unravel diffusion in tissues to reproduce it appears to be challenging and potentially out of the scope of hydrogels which are meant to be simple models. To some extent, these diffusion models could however allow to improve hydrogels used in plasma medicine, to better represent real tissues. Unfortunately, there is no literature yet directly discussing the tuning of hydrogels diffusion properties depending on the living tissue to be modeled, but rather empirical data as presented in subsubsection 2.1.2.

In addition, while many works have studied the diffusion of various components in gels, such as DNA, RNA and proteins for electrophoresis in biology, the knowledge about diffusion of RONS in hydrogels is clearly insufficient. This could bias the use of

hydrogels to model diffusion in tissues and research in this direction would be greatly valued.

While the diffusion of RONS in gels is not fully modeled and characterized, the hydrogel model has still been validated empirically by comparing diffusion in gels and tissues, as described in subsubsection 2.1.2, which is ultimately an indirect assessment of diffusion.

2.5. Electrical characteristics

The electrical behavior of a material consists in its ability to propagate an electric signal and is characterized by their conductivity σ (i.e. its ability to conduct electrical current, in Siemens per meter $S.m^{-1}$) and relative permittivity ϵ_r, also called dielectric constant (i.e. its insulating properties, in Farad per meter $F.m^{-1}$). These values determine the impedance of a material and depend on the frequency of the applied signal.

It is commonly accepted that the target impedance has a strong influence on plasma treatment. Several recent studies have shown its importance in the scope of plasma medicine and highlighted how to tune the target electrical characteristics to better simulate the final treatment [15, 81, 82]. This supports the need to adapt the impedance of hydrogel model to better represent reality by adjusting its conductivity and permittivity, although very few studies have considered this aspect. To this end, the electrical characteristics of the targeted biological tissue, at the frequency of the CAP treatment signal, should be determined and reproduced in the gel model.

2.5.1. Electrical characteristics of real tissues : Biological tissues are composed of cells which, electrically speaking, can be considered as ionic conductors (i.e. the liquid phase, named cytosol) surrounded by an insulating membrane (i.e. phospholipid bilayer). Depending on their cell composition and arrangement, living tissues will thus have different values of σ and ϵ_r. Electrical characterization is complex as tissues are inhomogeneous, can be anisotropic, and present varying physiological states. It implies that the mobility of charges inside a tissue depend on different parameters such as time, tissue orientation and frequency of the applied signal [83]. Empirical data can be found in the literature reviews of Gabriel et al. [84–86] or in reference books such as Grimnes work [87]. These works report conductivity and permittivity of most tissues (such as blood, cortical and cancelous bone, fat, grey and white matter, skin, muscles and various organs) depending on frequency. As an example, these values are given in Table 3 for a radiofrequency plasma (very commonly used in plasma medicine) of 3kHz as described in the works of Kawasaki group [47, 61, 62, 64].

Unfortunately, the literature does not include all tissues and frequency ranges, for example in the case of diseased tissues, which are precisely the targeted tissues of CAP. For example, Peyman et al. showed significant differences when measuring the electrical properties of healthy and cancerous tissues [88]. In this case, these values can be measured, as described in the next paragraph.

Table 3. Range of conductivity σ and permittivity ϵ_r at 3kHz. Given as an order of magnitude rather than exact values based on [84, 87]. Some tissues(e.g. brain matter, kidney, liver, etc.) are not characterized on the whole range of frequencies and have thus not been included

Tissue	σ $(S.m^{-1})$	ϵ_r $(F.m^{-1})$
Blood	10^0	10^3
Cortical bone	10^{-2}	10^3
Fat	10^{-2}	10^3
Lung	10^{-1}	10^5
Muscle	10^{-1}	10^6
Skin	10^{-5}	10^4

2.5.2. Matching hydrogel electrical parameters : σ and ϵ_r should be determined to tune the hydrogel model to match electrical characteristics of real tissues. Approximate values can be found in the literature, for example in previous plasma medicine hydrogels studies [46, 48, 58, 81] but the most accurate method is arguably to measure them. Indeed, it will ensure that the values used correspond to the exact tissues at the frequency used in the study.

There are different tools to measure values of σ and ϵ_r of both the hydrogel and biological tissues, including impedancemeter, network analyzers [88–91] or other data acquisition systems. Most common methods, depending on the frequency used, include two-electrodes measurement [92], improved to 4-electrodes [93, 94] to avoid electrode polarization at low frequency and coaxial probe measurement at higher frequencies (used both for tissues [88] or gels [91, 95]). These methods allow to measure the conductivity and permittivity of the gel, and tissues if needed. This will consequently allow to adjust these values by modifying the gel composition.

2.5.3. Tuning conductivity : Various chemicals can be used to modify the gel conductivity, NaCl being the most straightforward and popular choice. To illustrate this process, the work of Kandadai et al. is a very good example where agar, agarose and gelatin with various NaCl concentrations were compared in terms of conductivity [96]. Nebuya et al. built a multilayer phantom by using different concentrations of NaCl to match the conductivity of the different layers [39]. In the frame of plasma medicine, He et al. added 1% NaCl to investigate the change of target conductivity [58], with little impact however in this particular setup. PBS has also been used as a partially conductive medium (15–20 $mS.cm^{-1}$) as compared to water, showing a different plasma discharge (closer to a glow discharge) and a different gel treatment in terms of distribution and concentration of RONS [46]. This highlights the importance of conductivity, since Kawasaki et al. reported a value as low as 10 $S.cm^{-1}$ for a non tuned liquid target with

reporters [48]. Other products could also be tested and measured using the method proposed before. It is also important to mention that conductivity depend on other parameters of the gel. For example, Pomfret et al. showed that conductivity decreases with mass fraction in agarose gels [97].

2.5.4. Tuning permittivity : Permittivity has received much less to none attention compared to conductivity in the frame of plasma medicine. Chien et al. measured the permittivity of a PBS-agarose barrier and obtained relatively low values [66]. It would certainly be interesting to further study its impact on plasma treatment and consequently adjust it in gel models. To tune the capacitive behavior of the gel, additional dielectric materials have been used in gel phantoms, such as plastic powder/flakes [92, 98, 99] or oil [91, 91].

Obviously, the matching of electrical characteristics between hydrogels and tissues will never be perfect since tissues contain imperfections, anisotropies, various fluid flows and are time-dependent. Empirical values of σ and ϵ_r thus only allows to approach its electrical behavior.

2.6. Important parameters

Hereafter are detailed other important parameters to take into account when designing and using a hydrogel model; the liquid used with the agarose/gelatin powder to produce the hydrogel, the effect of gel thickness, the aging of the gel and finally the influence of temperature.

2.6.1. Liquid medium Most studies use deionized or distilled water. Other uses include Phosphate Buffered Saline (PBS) [34, 56, 57] or NaCl [13, 53] solutions and in some cases specific liquids such as NaNO2 solution [37] or HEPES (a cell culture buffer used in Marshall et al. [10]). The composition of this liquid is critical. Indeed, the plasma chemistry is strongly influenced both by its surrounding, notably in terms of conductivity (as discussed previously), and by the species it contains. Indeed, the RONS generated by the plasma will react with the components in the solution, as demonstrated for PBS by Girard et al. [7], who showed that treating different media leads to different RONS concentrations. Recognizing this fact, different liquid compositions will drastically impact the effect of the plasma treatment on the tissue model depending on the composition of the solution, its pH (and buffering ability) or its conductivity. It is thus arguably more stringent to use deionized water and optionally add the desired chemicals to tune the liquid characteristics in a controlled manner to represent the target tissue in a more accurate and repeatable fashion or if possible use a medium close to real tissue conditions.

2.6.2. Thickness Gel thickness will also have an influence on plasma treatment, obviously when used as barrier (thicker gels will slow down RONS penetration across the barrier) but also when used as a gel reagent, in terms of impedance. Indeed, thicker gels will show higher impedance as the current path from plasma to ground will be extended (Nebuya et al. used a conductivity per cm for their model [39]). This thickness is generally comprised between 0.5-5mm, as reported in Table 5. In terms of RONS transport, Szili et al. showed that the delivery of RONS in a liquid through a gel barrier is markedly affected by the barrier thickness [43,72], both in terms of time lag and total RONS concentration in the underlying liquid. Similarly, Kawasaki et al. showed the same phenomenon with a *gel barrier + reagent* model where both RONS concentrations and distribution were drastically changed when varying the thickness from 0.5mm to 3mm [65]. Additionally, it will have an influence on the absorbance measurement used to analyze reporters, as discussed in subsection 3.2.2.

2.6.3. Aging Gelatin and agarose being biological and high-water content hydrogels, their durability is limited and particularly dependent on handling and storage. The most restrictive threat is a change in water content and gel desiccation due to water evaporation. This can however be avoided to some extent by sealing the gel in airtight enclosures (containers and/or liquids) and preferably storing it at low temperature. The other threat is fungal or bacterial growth. The polymerization process including boiling the gel, it usually sterilizes it but resistant germs or a later external intake can happen. This can be avoided through the same storing procedure and/or the addition of preservatives such as formaldehyde [20]. For example, Zell et al. claim the agarose gel remains stable for several weeks when stored at 4°C under water [17]. Storage in oil has also been shown to preserve water content [16]. In order to avoid bias and considering the quick preparation of the gel, it could also be prepared before each experiment (as its preparation is very quick) or days before and properly stored [59,69].

2.6.4. Temperature The last important parameter affecting hydrogels is temperature as it will affect RONS chemistry and diffusion. Indeed, temperature changes the gel viscosity, affecting its structure and thus the diffusion of RONS. Gelatin has notably been shown to behave as a Newtonian fluid, with its viscosity decreasing with increasing temperature [100]. In addition, temperature changes the thermal velocity of the RONS [78]. These changes in viscosity and diffusion coefficient result in a diffusion rate increasing with temperature [79]. Temperature also affects other parameters such as conductivity [96].

Firstly, the general temperature of the gel should be considered. In order to best reproduce the real in vivo conditions in terms of chemistry and diffusion, thermalizing the gel at 37°C could lead to more accurate results. Along with the study of diffusion discussed previously, the influence of temperature on RONS diffusion in hydrogels should be investigated. Secondly, the localized temperature elevation that may occur at the point of contact between the plasma and the gel can have an impact on the gel that

may not represent real in vivo conditions, although this elevation could also happen on real tissues but with different consequences.

2.7. Limitations

Despite the numerous aforementioned advantages of using a gel model for the study of plasma effect on tissues, it obviously shows limitations and it is relevant to list them to understand the limits of the model and identify the potential improvements:

- The model does not account for complex cells characteristics such as the phospholipid bilayer or cell-to-cell communication.
- The model only reports the penetration of RONS but does not account for the cell-dependent effect it causes (apoptosis, necrosis, cell-cycle alteration) and its biological outcomes [101].
- The penetration is assisted by other driving forces (such as electric field, compressive stress, pressure wave penetration) that could potentially have a different effect *in vivo* [101].
- The complexity of real tissue is not well represented in terms of structure, macroscopic irregularities and composition. This will impact diffusion, conductivity, permittivity and change distribution and penetration, potentially leading to overestimation of treatment in the gel as compared to real tissues [102].
- Although conductivity and permittivity can be tuned to some extent, the model does not perfectly matches the impedance and complexity of real living tissues.
- The model is static and does not account for *in vivo* movements such as blood flow or heartbeat as pointed by Szili et al. [72].
- The model does not interact with other structures (veins and arteries, other organs, etc.).
- The presence of air bubbles in the gel.
- The lack of longevity and relative fragility of the gel.

2.8. Model Improvement

In order to compensate for these limitations, several suggestions have been proposed in the literature:

To better represent the role of cells in vivo :

- Instead of directly detecting RONS in the gel bulk with reporters (detailed in the next section), the group of Szili [10, 14, 103] developed sophisticated reporter models to better account for RONS action. This tissue model uses artificial phospholipid membranes containing the reporters to investigate membrane lysis and/or penetration of RONS inside these cell models (see Figure 8). A similar model was used by Ki et al. [104].

- Introduce live cells either to alone as such or in parallel with reporters [44, 45, 52, 105, 106]. These studies investigated the effect of CAP directly on cells, hence with a plasma effect supposedly closer to real *in vivo* conditions, and providing a tissue-like structure in the form of the gel as compared to regular *in vitro* testing. This allows to assess distribution and penetration of the effect on the cells, and optionally to compare it with incorporated reporters.

Structurally :

- Use chemical crosslinking to increase crosslinking ([16] and improve mechanical and thermal properties [74]).
- Experiment hybrid material (not reported yet for plasma medicine experiments), for example mixing gelatin and agarose [21, 107] or using other materials (fibrin for better structuring [108], to improve mechanical properties [109] and/or the adequacy of the model with real tissue.
- Design multilayer models [39].
- Control air bubbles number and size in the gel, for example using yeast [97, 110].

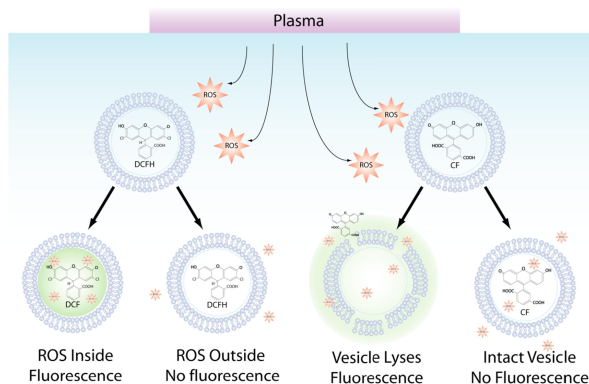


Figure 8. Artificial vesicles containing reporters (reproduced from [14])

Electrically :

- Measurement of conductivity and permittivity of the targeted tissues at the appropriate frequency
- Tuning the gel characteristics to match tissue properties, using dedicated chemicals (NaCl, plastic powder, oil, etc.)
- Use of an external system to reproduce the human impedance, connected to the gel to simulate the electrical behavior of the body [15, 82].

Other :

- Enable a fluid flow to simulate blood flow through a pumping system [72]
- Use of preservatives to improve durability [16]

3. Reporters

The combination of the hydrogel tissue model described in the previous section with chemical reporters to measure plasma action is the major strength of this model. Indeed, on one side, hydrogels provide a matrix simulating the distribution and penetration of plasma action in tissue, and on the other side, chemical reporters allow to locate and identify these action. In addition, reporters also are quite easy to handle, quick to prepare and often relatively inexpensive. These reporters are soluble in water and can thus be directly incorporated in the gel or the liquid. They change in color/fluorescence when reacting with RONS, thus allowing to detect one or more specie either very quickly in a qualitative manner (examining the size and depth of the colored area) or quantitatively by spectroscopy (fluorescence/absorbance) and imaging. Other analysis techniques without reporters have been used (mainly UV–vis spectroscopy [43,67,69,72] but also Electron Paramagnetic Resonance (EPR) spectroscopy, mass spectrometry [59]) but will not be detailed here as they are out of the scope of this paper.

3.1. Species of interest

3.1.1. Nature Even if other cold plasma elements (UVs, electric field, charged species) have an effect on cells, the main plasma-cell interaction is mediated by RONS [1–4, 7]. In particular, these RONS are mainly the long-lived species O_3, H_2O_2, NO_2^- and NO_3^- [72,111,112]. These can be linked to more reactive and unstable forms such as the short-lived species OH° O_2° NO° or $ONOO^-$ [111,112]. These species are generated in the plasma in contact with air (then transferred in the biological target) and/or directly in the target (i.e. in the liquid phase). The vast majority of reporters used in hydrogels for plasma medicine thus target RONS. Although there detection of charged species in gels is not reported, electrons delivery is also important to measure.

3.1.2. RONS lifetime A critical parameter to consider when analyzing RONS effect on tissue or tissue models is their lifetime. Indeed, both long-lived and short-lived species have different effects on tissues [5]. While the first are easy to measure with their lifetime of several minutes to hours, the second must be detected either in real-time, either based on their action (i.e. the production of secondary long-lived species or a direct effect such as oxidation or triggering of cellular pathways). Advantageously, the hydrogels models allow to report *in situ* and in real time the action of RONS, including short-lived species and the long-lived species they produce. They do not account however for cell-dependent mechanisms such as membrane oxidation or cell signaling.

The estimation of RONS lifetime is however challenging in the case of hydrogels treatment. Indeed, while the half-life of RONS in gas phase can be approximated with good precision, the treatment of gels (mostly containing water) and living tissues occurs in the liquid phase. To estimate the lifetime of a specific specie in a liquid phase, the rate constants of its potential reactions are usually used. Since there are numerous reactions for each specie and that each reaction rate constant is strongly influenced by the medium

composition and conditions (temperature, pressure, shocks etc.), these rates may differ by several orders of magnitude depending on the plasma setup and the hydrogel model. It is thus difficult to precisely approximate this lifetime for the liquid state. However, Table 4 gives an overview of the orders of magnitude for relevant RONS, in the gaseous state and in physiological conditions, based on the work of Lu [8] and Graves [113]. These half-life values are also related to diffusion distances [113]. Alternatively, the interested reader can refer to rate constants tables (see Neta and Buxton works [114, 115]) to infer these lifetimes, or since it is often very complex, proceed either using simulation or empirically.

Table 4. Key RONS generated by plasma in the gaseous state and transported in liquids, based on [8, 113])

	Species	Half-life in gaseous state
ROS	O	nanoseconds
	O_3	minutes
	O_2^-	milliseconds
	$^1O_2$	seconds-minutes
	OH	nanoseconds
	H_2O_2	minutes
RNS	NO	seconds
	NO_2^-	minutes
	NO_3^-	minutes
	$ONOO^-$	milliseconds

3.2. Measurement

3.2.1. Dyes Depending on the objective, it will be relevant to study the type and concentration of different RONS in the gel. To that end, many chemical reporters exist. The reporters used in plasma medicine literature in hydrogels are summarized in Figure 9. Amongst the most prominent are the KI-starch system used by Kawasaki et al. to measure all ROS, the Amplex Ultrared reagent (and variations) to detect H_2O_2 or the Griess reagent to evaluate NO_2^-. No report of electron detection has been found in the hydrogel literature although it would probably be relevant in the frame of plasma medicine. It should be noted that while global reporters for RONS and ROS (i.e. reporters detecting all RONS/ROS at once) have already been used, no such global reporter has been employed for RNS in this field.

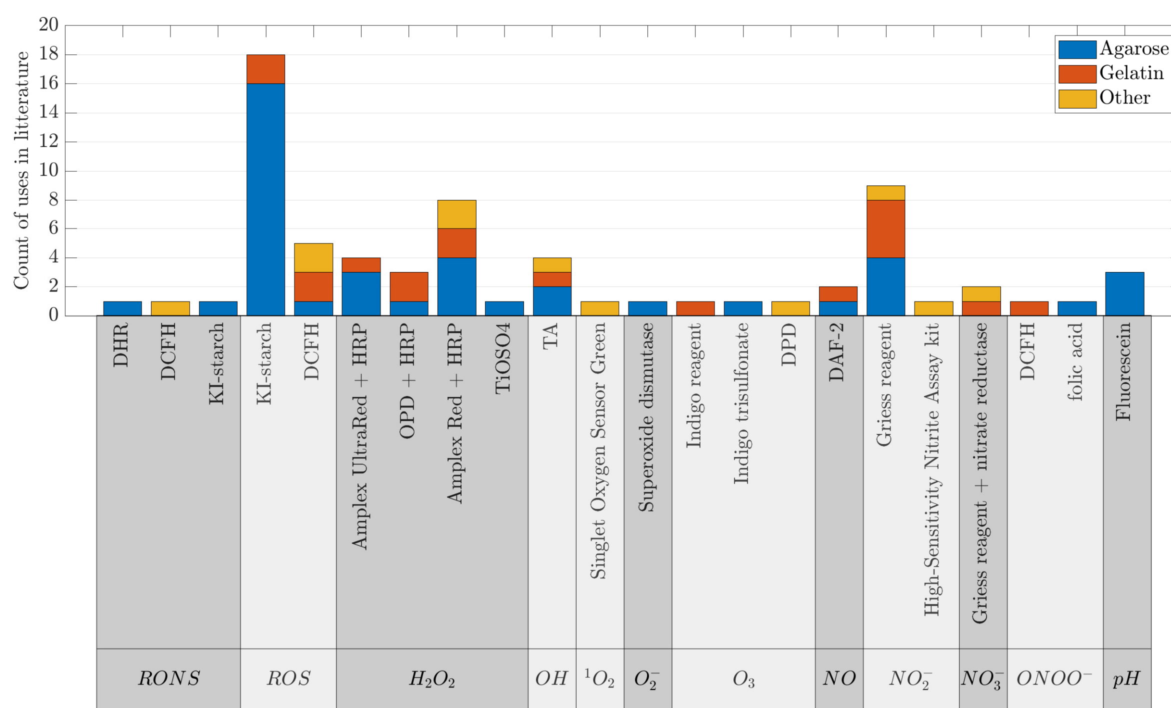


Figure 9. Summary of reporters used in plasma medicine literature for hydrogels, per specie

Most studies in plasma medicine use either fluorescent (35 uses) or colorimetric (37 uses) dyes and analyze the effect of plasma on these dyes through spectroscopy or imaging. Once treated, the reporter change in the hydrogel can either be measured qualitatively or quantitatively. Qualitative analysis can typically be used as a fast tool for discrimination between different plasma device setup or conditions, simply by comparing the size and intensity of the color change. Combined with the quick and easy preparation of the samples, this method is suited for fast setup analysis but not for reliable results. Quantitative analysis consists in using analytical instruments such as optical spectrometers or imaging tools to effectively measure the color change due to

plasma treatment.The majority of plasma medicine hydrogels studies use spectroscopy and imaging techniques.

In addition to the measurement method, a second challenge is to select what to measure, depending on the objective of the experiment. For example, the size of the treated area, the global intensity of the color change or its distribution, the depth of the treatment (which implies a cut of the sample) are different features analyzed in the literature.

3.2.2. Spectroscopy Optical spectrometry, or spectroscopy, is a technique allowing to study electromagnetic radiation across the visible spectrum, measuring its intensity as function of its wavelength. In this frame, two types of spectroscopies are used, absorbance spectroscopy for colorimetric dyes and fluorescence spectroscopy for fluorescent dyes.

A spectrophotometer allows to quantify the color change of a colorimetric dye inside a gel as a value of absorbance. This allows to directly quantify the concentration of the corresponding RONS through Beer-Lambert law by measuring the absorbance of a sample at a specific wavelength $\lambda_{absorbance}$ [116]. This $\lambda_{absorbance}$ is typically the maximum of the compound absorption spectrum to remain in accordance to Beer-Lambert law, obtain the best sensitivity and robustness between measurements.

Similarly, a fluorospectrometer allows to quantify the fluorescence change of a fluorescent dye inside a gel. Using a light beam of specific wavelength $\lambda_{excitation}$ and analyzing the subsequent emitted light at $\lambda_{emission}$, the fluorescence intensity of specific products is measured [116]. It thus also allows to quantify the corresponding RONS.

In both techniques, the treated sample is placed in the spectrometer sample compartment between a source of light on one side and a sensor on the other. The measurement is relatively quick and can be automated (some spectrometers also include microplate readers allowing to quickly analyze multiple experiments). The output is an absorbance or fluorescence value, and the exact corresponding concentration of the colored/fluorescent product can then be deducted using calibration curves. This is achieved by preparing solutions or gels containing the dye and treating those with solutions of predefined concentrations of the appropriate RONS. This latter is either measured in one point (for example to use this value as an average for the whole sample) or in multiple specific points (for example along a line to draw concentration distribution profiles such as in Kawasaki work [47]). The intensity and distribution of the color/fluorescence change can hence be correlated with the action of RONS. Penetration can be assessed in the same fashion by placing the sample sideways after cutting if needed. These techniques are very good tools with high accuracy and provide full quantitative measurements. They also show good specificity and sensitivity, ease of use and allow for good automation [116]. They however have some limitations to consider.

Firstly, regarding the detection of RONS, it is important to remind that concentrations of reactions products are not concentrations of corresponding RONS.

Although this is too often overlooked, several variables and phenomenons will interfere and bias the result, such as pH, temperature, saturation effect, various parallel reactions or interfering substances. Specificity and sensitivity in particular are further discussed is subsubsections 3.3.1 and 3.3.2. It is critical to consider these bias while interpreting the results and mitigate them when possible (e.g. determining the right concentration of dye to avoid saturation with preliminary tests). Secondly, regarding the measurement technique, the result strongly depends on the calibration curve (i.e. the selected range of concentration) and on the thickness of the sample. Indeed, the thickness of the sample corresponds to the optical path crossed by the light used in both spectroscopy techniques and will directly influence the absorbance value which is directly proportional to it. Hence, it should be kept constant between measurements. Thirdly absorption and fluorescence spectroscopy are not applicable at high concentrations, notably because of the restricted scope of application of Beer-Lambert law [116], limiting the dose of CAP that can be studied through this method.

3.2.3. Imaging The second most frequent technique to analyze the effect of plasma on reporters is the use of other imaging techniques, typically using camera pictures of the samples. The data are less robust (because the highly depend on both hardware imaging and software analysis tools), direct (image processing is required) and only semi-quantitative, since it provides only relative measurement as compared to spectroscopy. However, it remains an indirect measurement of absorbance and the color change hence remains proportional to the concentration of treated reporters. In addition, the measurement robustness can be improved using a color standard and preferably the exact same conditions between samples, i.e. camera settings, lighting, etc. Fluorescence imaging is also possible, for example using adapted microscopes. The use of various softwares such as ImageJ [13, 53] or Matlab then allows to analyze the image to extract semi-quantitative information such as color intensity or plasma treated area size. This method has the advantage of being very convenient for spatial analysis, as the measure of a whole area can be done easily with one picture, instead of triangulating the area with spectrometer measurements. Analysis can be achieved for example by reducing all values with respect to a maximum "100%" reference value, or using thresholds (a blue threshold allows to analyze KI-starch hydrogels as shown on Figure 10)). This enables to compare different plasma setups, plasma conditions, models, etc. but strongly depend on the choices made for the analysis (value of the threshold, software choice, etc.) and the hardware (camera, lighting, etc.).

3.2.4. Colorimetric dye reporters Colorimetric dyes react with specific species to provide a color change in the sample that is then analyzed by a spectrophotometer at a specific wavelength $\lambda_{absorbance}$. To illustrate this mechanism, the two most used colorimetric dyes, Griess reagent and KI-starch, are developed hereafter.

The Griess reagent is a a colorimetric dye based on the Griess reaction (represented in Figure 11). It includes two reagents; sulphanilamide, which reacts with NO_2^- to form

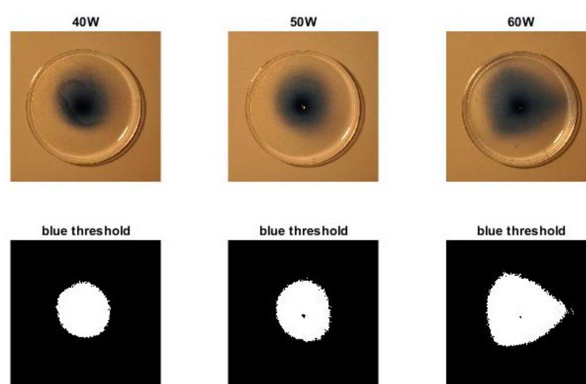


Figure 10. Example of a basic blue threshold using Matlab on KI-starch agarose samples treated by a plasma jet at different power described in [82]

a diazonium salt. This salt reacts with the second reagent, naphthyl ethylenediamine dihydrochloride in an azo coupling reaction to produce a magenta dye with an absorbance peak between 540-500nm [50, 52].

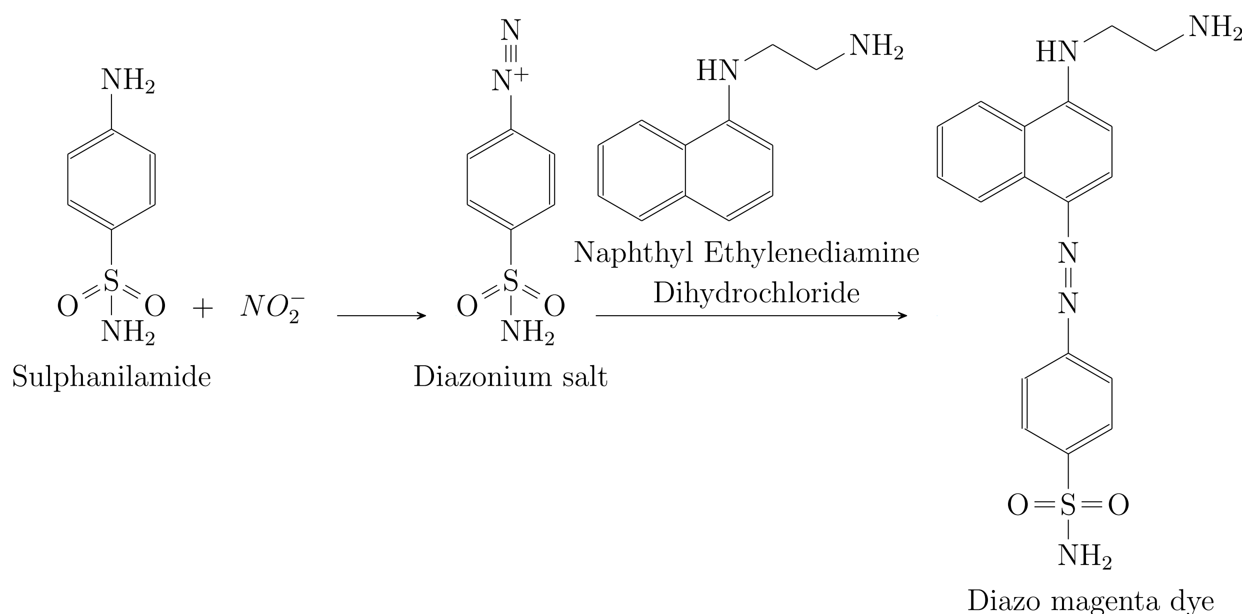
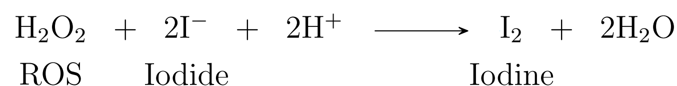


Figure 11. Griess reaction

The KI-starch reagent extensively used by Kawasaki et al. is a mix of starch and potassium iodide which allows to report several ROS at once: KI dissociates in water to form I^-, which can be oxidized to I_2 by ROS. It then reacts with excess I^- to form tri-iodide ion I_3^- which form a complex with starch, giving it its color [11]. The oxidation potential of this reaction being 0.54V, all the ROS with higher potentials will react and therefore will be detected, i.e. OH, O, O_3, H_2O_2 and HO_2 [60]. The color of the treated dye changes with the starch type as reported by Ghimire et al. which obtained brown and blue color using two different starches [67], where Kawasaki et al. reported a blue color with $\lambda_{absorbance}$ at 600nm [49] and 680nm [11].



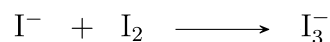


Figure 12. Example of KI-starch reaction with ROS

Table 5 summarizes the different colorimetric dyes used in plasma medicine in hydrogels with the reported $\lambda_{absorbance}$, as well as the specificity and concentration working range reported in the literature for these dyes. Interestingly, the choice of these wavelengths slightly varies between the studies, arguably because the absorption spectrum has several maxima (the best suited $\lambda_{absorbance}$ is then selected depending on the experiment) and/or because the medium itself interfere and shifts the absorption spectrum.

Table 5. Summary of colorimetric dyes and the corresponding targeted species, compared with the specificity and concentration ranges reported in the litterature for that specie, and their use in hydrogels (wavelength and reference)

Dye		Literature				Use in hydrogel	
Name	**Specie**	**Specificity**	**Interference**	**Concentration range (M)**	**Ref.**	$\lambda_{absorbance}$ **(nm)**	**Ref.**
DPD[a]	O_3	poor	all oxidants	ND**	[117, 118]	ND**	[119]
Griess reagent	NO_2^-	good	/	$10^{-7} - 10^{-5}$	[120–122]	540	[12, 37, 45, 52, 70]
						550	[50]
						492	[34]
Griess reagent + nitrate reductase	NO_3^-	good	/	$10^{-7} - 10^{-5}$	[123]	540	[12]
						550	[119]
Hydrogen Peroxide assay kit[b]	H_2O_2	NA*	NA*	NA*		570	[119]
Indigo trisulfonate	O_3	medium	$OH^{\bullet}$	$10^{-7} - 10^{-5}$	[118, 122]	600	[12, 52]
KI-starch	ROS	medium	RNS at low pH	$10^{-5} - 10^{-1}$	[124]	580	[46]
						680	[11]
						600	[49, 50]
						ND**	[47, 48, 60–62, 64, 65]
						imaging	[42, 51, 53, 58, 67]
OPD + HRP[c]	H_2O_2	ND**	ND**	ND**		450	[34, 57, 70]
TiOSO4	H_2O_2	good	specific compounds[d]	$10^{[-5,-3]}$	[125, 126]	407	[52]

[a] N,N-Diethyl-1,4 Phenylenediammonium
[b] Ortho-Phenylenediamine and Horseradish Peroxidase
[c] Not a dye per se but a mix provided by manufacturer
[d] e.g. formaldehyde
* Not Applicable
** Not Documented

3.2.5. Fluorescent dye reporters Fluorescent dyes are used extensively in biology. They contain compounds able to react with specific species to produce fluorescent molecules (called fluorophores) which can absorb light at a certain wavelength $\lambda_{excitation}$ and re-emit light at another wavelength $\lambda_{emission}$, usually higher. Fluorescence spectroscopy shows higher sensitivity and allows to measure larger concentration ranges than absorbance spectroscopy [116]. On the downside however, it is not as widely applicable since fewer chemical systems show sufficient fluorescence and is more prone to interference [116]. Amplex Red and its variants are the most used fluorescent dyes and is discussed or illustrative purpose. Amplex Red is a colorless fluorescent reagent which reacts with H_2O_2 to produce a highly fluorescent product called resorufin in the presence of its catalyst, HorseRadish Peroxidase (HRP) [127] as illustrated in Figure 13. This reaction occurs in a 1:1 stoichiometry and thus linearly measures the amount of H_2O_2 in the gel. Always according to Rhee et al. [127], resorufin presents the maximum $\lambda_{emission}$ at 587nm and a maximum $\lambda_{excitation}$ at 563nm and the sensitivity of the assay is high, allowing to detect concentrations as low as 50nM.

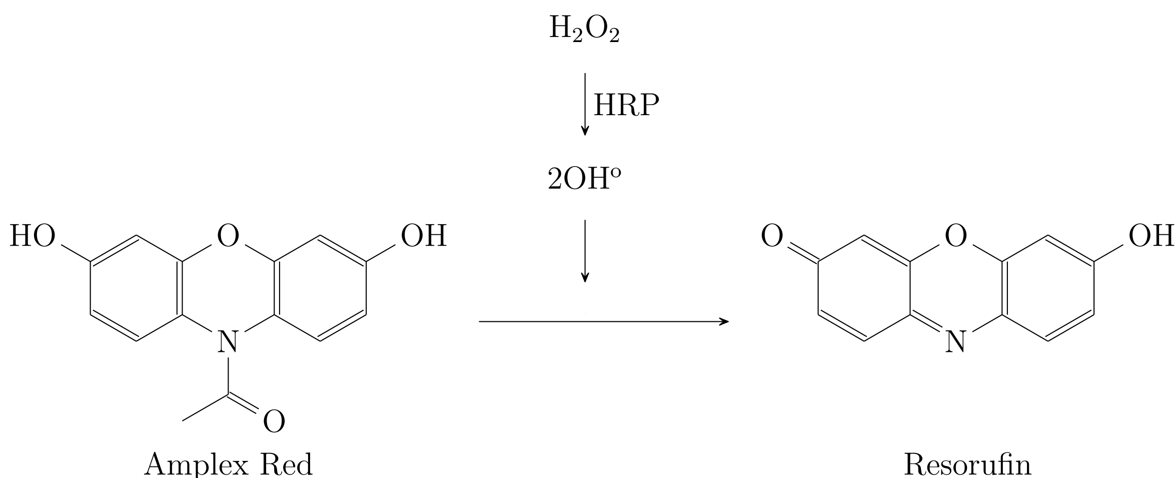


Figure 13. Reaction of Amplex Red with H_2O_2 in presence of HRP to produce resorufin [127]

It is also sometimes possible to measure fluorescent dyes change with a simple absorbance measurement (as for colorimetric dyes) such as in [50] which uses Invitrogen Amplex Red assay kit. Table 6 summarizes the different fluorescent dyes used in plasma medicine in hydrogels with the reported $\lambda_{excitation}$ and $\lambda_{emission}$, specificity and concentration working range. Again, the wavelengths differ between experiments, probably because they are adjusted depending on the spectrum maxima and the medium.

Table 6. Summary of fluorescent dyes and the corresponding targeted species, compared with the specificity and concentration ranges reported in the litterature for that specie, and their use in hydrogels (wavelength and reference)

Dye		Literature				Use in hydrogel		
Name	**Specie**	**Specificity**	**Interference**	**Concentration range (M)**	**Ref.**	$\lambda_{excitation}$ **(nm)**	$\lambda_{emission}$ **(nm)**	**Ref.**
Amplex Red + HRP	H_2O_2	good	one-electron oxidants[a]	$10^{-8} - 10^{-4}$	[128–130]	550	595	[12]
						531	605	[53]
						530	550	[50]
							other	[45, 66]
Amplex UltraRed + HRP	H_2O_2	good	less sensitive to pH than AR	twice more sensitive than AR	[131]	530	590	[9, 41, 56]
						ND**	ND**	[28]
Amplex UltraRed + Superoxide dismutase	O_2^-	good	less sensitive to pH than AR	twice more sensitive than AR	[131]	530	590	[9]
CF[b] in lipid vesicles	*Membrane rupture*	NA*	NA*	NA*		485	520	[14]
						ND**	ND**	[10]
DAF-2[c]	NO	medium	pH	$10^{-9} - 10^{-6}$	[132, 133]	485	538	[9, 56]
DCFH[d]	ROS	medium	RNS	ND**	[128, 130]	485	520	[72, 103]
						498	522	[57]
						502	523	[119]
						485	520	[34]
DCFH[d] in lipid vesicles	*ROS in cell*	medium	RNS	ND**	[128, 130]	485	520	[14]
DCFH[d]	$ONOO^-$	poor	$OH^\bullet$ $NO2^\bullet$ CO_3^-	ND**	[134, 135]	495	518	[56]
DHR[e]	$RONS$	poor	$OH^\bullet$ $NO2^\bullet$	ND**, better than DCFH	[128, 130, 136, 137]	485	535	[53]
Fluorescein	pH	NA*	NA*	NA*		473	520	[13]
						490	514	[41]
Folic acid	$ONOO^-$	good	/	$10^{-8} - 10^{-6}$	[138]	380	460	[52]
HSN Assay kit[f]	NO_2^-	NA*	NA*	NA*		365	450	[119]
SOSG reagent[g]	$^1O_2$	NA*	NA*	NA*		504	525	[119]
Terephthalic Acid	$OH^\bullet$	medium	O_2^-	$10^{-8} - 10^{-6}$	[139, 140]	310	425	[52, 66, 119]
						ND**	ND**	[34]

[a] but HRP catalysis is very strong
[b] 5(6)-Carboxyfluorescein
[c] Diaminofluorescein-2
[d] 2',7'-Dichlorofluorescein Diacetate
[e] Dihydrohodamine-123
[f] High-Sensitivity Nitrite Assay kit, not a dye per se but a mix provided by manufacturer
[g] Singlet Oxygen Sensor Green reagent, not a dye per se but a mix provided by manufacturer
* Not Applicable **Not Documented

3.3. Important parameters

3.3.1. Specificity While all studies using specific reporters claim them to detect a particular specie, they rarely discuss potential interference from other species in their measurements. In fact, these indicators are often used in biology in very controlled environments where the nature of reactive species is known and their number usually limited, allowing to easily forecast reactions with the reporters and achieve accurate measurement. However, most reporters are not entirely specific as illustrated in Tables 5 and 6 , i.e. they may react with other RONS or be influenced by various factors (pH, temperature, other reactions). This should be examined carefully in the frame of CAP, which contains a high number of interplaying actors (including multiple RONS) and elicits various reactions in both liquid and gas phases. For example, the removal of Amplex Red function to become resorufin, discussed previously, occurs normally through a very powerful oxidation but can be catalyzed by HRP to react with H_2O_2. This catalysis is thus used to detect H_2O_2 assuming that Amplex Red will not be degraded by oxidation, but in a very reactive medium such as CAP, this oxidation becomes likely to happen as well (as shown in Lefrancois et al. [141]), thus preventing to directly link between H_2O_2 concentration to resorufin concentration. In addition to the fact that not all reporter molecules will react with the targeted RONS, not all RONS will react with the reporters as they may be involved in other reactions such as destruction of reporter products by RONS, competing reactions, self-recombination, etc.

Unfortunately, few information about interfering reactions and/or species can be found in the manufacturers' datasheets and this should be investigated in future work. Several studies, such as Kalyanaraman work on fluorescent dyes [130] or the paper from Tarabova [122] on colorimetric dyes, give insights on such limitations. In the literature reviewed in this work, a few studies do discuss this issue, such as He et al. who tested the specificity of Amplex Red [12] adding potential interfering compounds and analyzing the result by fluorescence. This should be extended to all potential interfering RONS present in the plasma and other actors that could have an impact on the detection, with UVs and electrons being the most obvious candidates. Alternatively, broader reporters can be used to detect several RONS to circumvent biased results as Gaur et al. [34] did, switching from several specific reporters to a broad spectrum one (DCFH).

3.3.2. Sensitivity and concentration Measurements also obviously depend on sensitivity and concentration. Indeed, low sensitivity will result in bad accuracy and will not allow to reliably compare samples. On the opposite undersized sensitivity will result in saturation of the dye (meaning that all molecules will have reacted) and the total concentration of species will be impossible to measure. The concentrations involved depend on the experiment and the plasma setup and could require several pre-tests to adjust the hydrogel model to the experiment. In parallel, the sensitivity of a particular reporter is often specified in the manufacturers' datasheet. For example, the High-Sensitivity Nitrite Assay kit used by Duan et al. [119] is specified by its manufacturer

to have "an optimal range of 20–500 pmol nitrite, making it up to 50 times more sensitive than colorimetric methods utilizing the Griess reagent". The sensitivity of more universal reporters such as Griess reagent or Amplex Red have also been reported directly in the literature, as summarized in Tables 5 and 6.

3.3.3. Medium As discussed for the hydrogel, and in direct relation with the sensitivity of the reporter, the nature of the medium will strongly influence the detection. Indeed, the nature of the medium, including its buffering ability and its composition (with species that could react with RONS), as well as its pH need to be controlled. For an experiment using Griess reagent for example, nitrite/nitrate-free media should be used, such as DMEM (Dulbecco's Modified Eagle Medium) or simply distilled water. Manufacturers information often specify the optimal conditions, and these can also be tuned depending on the experiment.

3.3.4. Alternative reporters The dyes presented in this review are merely the few reported uses of a promising model in the rapidly growing field of plasma medicine. Numerous other dyes exist, for example in analytical chemistry and biology, and should be experimented. For example, Rhee et al. summarized the "Methods for detection and measurement of hydrogen peroxide inside and outside of cells" [127], including Amplex Red, but also 6 other detection methods that could be tested in hydrogels. Another very promising alternative is the use of living cells instead of reporters, or the combination [44, 45, 52, 105, 106] of both. This addition increases the model complexity and makes it less tunable, but brings it closer to real tissue and enables to study the mechanism of plasma action on cells in a tissue-like matrix and assess the effect of plasma distribution and penetration on this mechanism. Reporters could however have an effect on cells and thus bias the results and should thus be selected carefully.

3.3.5. Influence of time The last critical parameter to take into account when performing an experiment, and especially for its analysis, is the delay between the plasma treatment and the analysis of the gel. Indeed, due to the slow diffusion of the RONS in the gel, the plasma action also shows a delayed effect. It has been shown in several studies, mainly from Szili group [34, 69–72], that the gel acts both as a buffer and a reservoir for RONS. Consequently, the reactions with the dye resulting in a color change are delayed as well and the color/fluorescence change of the gel continue to spread and intensify for minutes to hours after treatment. It is thus paramount to establish a clear approach for the experiment to analyze each sample after a designated duration following plasma treatment. This duration should preferably be the same for all samples to be able to compare plasma treatment, except for experiments investigating this diffusion time lag. This duration can be chosen to be minimum (analysis right after treatment) or a fixed value, for example to assess the plasma effect 1 hour after treatment, which could represent real in vivo conditions with a delayed effect as mentioned in Szili et al. [72].

3.4. Limitations

Similarly to the hydrogel matrix, reporters do not fully represent the *in vivo* effect of RONS, although they represent a simple and reliable measurement method of their concentration. It is hence relevant to keep in mind several limitations of to their use when performing experiments:

- Limited specificity, as discussed earlier.
- Sensitivity, as discussed earlier
- Detection of short-lived species: most reporters summarized in this review are used for the detection of long-lived species (O_3, H_2O_2, NO_2^- and NO_3^-). Short-lived species are not much investigated whereas they have been shown to have an effect on tissue [1,3]. However, many of these species usually produce the aforementioned long-lived species and can be thus taken into account to some extent, with broad spectrum reporters such as KI starch, which will react with all ROS (including short-lived, long-lived and long-lived produced by short-lived species)
- Intracellular generation: chemical reporters only report the presence of exogenous RONS, but this model do not take into account the in vivo intracellular RONS generation that can be triggered by exogenous RONS delivery [142].
- Cell-related mechanisms: in addition to intracellular generation, reporters do not render the other cellular mechanisms including signaling pathways (cell-to-cell communication, cascade effect [143])
- Compatibility reporters-gel: Pogue et al. reports the compatibility of fluorophores with gelatin and agarose [16], and other studies mentioned in this review showed other combinations, but it does not insure the compatibility of all hydrogels with all reporters.

4. Discussion and future work

4.1. Achievements

Hydrogels have proven to be a relatively simple yet powerful tool in plasma medicine allowing to study the distribution, intensity and penetration of CAP treatment, as summarized previously in Table 2. The results allow to better understand the mechanism of action of CAP on in various situations (different tissues, presence of skin barrier, etc.) and consequently adapt either plasma (power, gas mix, configuration, flow) or treatment (duration, angle, distance) parameters to improve the future therapy. Main achievements of these models are noted below :

Distribution

- The distribution pattern of RONS significantly varies (pointlike, circular, ring-shaped or even star-shaped [57]) depending on multiple factors (gas mix [11,47,58], distance [43,47,64,65]) or flow [42,47]. This distribution also varies along the depth of the tissue [47,64,65]. This shows that tuning gas mix (e.g. adding O_2), distance (typically $< 10mm$) or using shielding gas [46] allows to maximize the treatment and control its distribution, for example for localized tumors.
- The angle of treatment (i.e. inclination) changes the distribution of RONS depending on the main and added gas [58].
- There is an optimal flow to achieve maximum ROS delivery due to a balance between larger delivery of gas and turbulent flow [49].
- Gas flow influences distribution and increases treatment depth [37,47,57]. Low flow produces a circular treatment while high flow produces a ring-shaped distribution [47].
- Composition of the treated tissue (i.e. nature but also concentration) may drastically influence the RONS delivery, such as proteins and O_2 which increase or decrease it depending on concentration [34].

Depth and transport

- The effect of CAP treatment is observed millimeters deep in hydrogels [45, 57, 72], suggesting that treatment can be achieved in real tissues beyond superficial layers.
- Deep treatment is mainly due to RONS [10], and long-lived species (O_3, H_2O_2, NO_2^- and NO_3^-) in particular, which are also the main species transported through barriers.
- While delivery of RONS by CAP is an on/off phenomenon, diffusion still occurs for minutes to hours inside the tissue model [59, 67, 71, 71] due to a slow transport phenomenon. This time-lag allows RONS to accumulate in the treated tissue (e.g. skin) and subsequently be released in its surroundings.

- RONS delivery through a barrier (i.e. skin) decreases as function of barrier thickness [43].
- Electric fields greatly improves the transport of RONS through barriers [12, 37].
- There are plasma-induced forces influencing fluid dynamics during CAP treatment [51], potentially due to electric fields and charges [72].
- CAP can propagate and act into complex structure [28], particularly promising for bronchoscopy for example.
- CAP can induce apoptosis in cancer cells up to 1mm in a 3D matrix [45]
- An appropriate irradiation (relatively short) distance is critical to provide RONS in depth [61, 65], important in the treatment of tumors for example. The ROS/RNS ratio is also influenced by this distance [50].

These examples have strong technical and clinical implications and show the interest of the hydrogel model. Hopefully more work will be achieved to better understand and optimize CAP treatment. Based on current challenges in plasma medicine, the following leads are proposed :

- Help to understand the biological mechanisms by which CAP reaches deep into tissues by discriminating the contribution of diffusion (happening both in gel and tissues) and the contribution of cell-to-cell communication (as mentioned notably in Graves work [3]). To this end it will probably be necessary to sophisticate the model by incorporating complex reporters as done by Szili group [10, 14, 103] or even live cells.
- Further study the parameters influencing penetration to better control it, from plasma source configuration to treatment settings. This will be critical to treat either deep lesions (e.g. tumors) or superficial tissues (and preserving deeper layers).
- Similarly, continue to explore the parameters influencing distribution to enhance superficial treatment of large surfaces or constrain the CAP effect to localized zones.
- Investigate the treatment of closed structures as initiated by Park et al. [28] to open new possibilities in minimally invasive treatments such as endoscopy.
- Study how electrical parameters of tissues affect distribution, intensity and penetration of CAP treatment, notably with electrical models simulating human impedance as developed by [15]. This parameter has been too often neglected by the plasma medicine community but seems critical to tailor an effective treatment.
- Better understand how the chemical composition of the target can influence CAP treatment.
- More practically, tune the new plasma sources and treatments settings developed by each group, with a method arguably closer to reality (i.e. not in free jet) than more conventional ones (Optical Emission Spectroscopy, X-ray Photoelectron Spectrometry, EPR, mass spectrometry, etc.).

Obviously, many other research questions could be explored by this young method in the frame of new plasma medicine projects. To that end, an experimental protocol is proposed based on the matters discussed in this article.

4.2. Proposition of experimental protocol for the manufacturing of hydrogel-reporter model

For the interested reader aspiring to implement his own hydrogel model for plasma medicine, we suggest the following steps :

(i) Establish the **goal of the experiment** and the questions to be answered to infer the needed model.

(ii) Select the best suited **model configuration**, gel nature and **mass fraction** depending on the targeted tissue.

(iii) **Adapt the gel** and medium in terms of conductivity and permittivity, thickness and temperature. Examine the need for other potential improvements.

(iv) Identify the **needed reporter(s)** depending on the RONS to be measured, keeping in mind specificity.

(v) Determine the reporter **protocol in terms of concentration and sensitivity**.

(vi) Establish the **analysis protocol** to measure CAP effect and taking into account the time between CAP treatment(s) and measure(s).

4.3. Conclusion

This work gives a thorough view of the use of hydrogels models in plasma medicine. Regarding the tissue model, several aspects have been discussed, including material, mass fraction, configuration, manufacturing, diffusion and electrical parameters (conductivity and dielectric constant), which are critical to tailor the tissue model. Their use in literature was summarized to guide the development of such models. Other parameters to consider, such as medium, thickness, aging and temperature were also discussed. The chemical counterpart of the model, i.e. the reporters, and their use in the literature were also listed as it is not so straightforward to choose the species to study and the corresponding reporters. The use of these reporters in gel model and their measurement were then described and challenged. Finally, crucial aspects of these reporters such as specificity, sensitivity and medium were also discussed. This will hopefully establish a strong basis for the development of such models and give an overview of the relevant literature.

The association of a simple yet representative model of living tissue in terms of composition, conductivity and penetration (i.e. agarose and gelatin) with incorporated reporters (colorimetric and fluorescent dyes) is a recent development encountering growing popularity. Indeed, it constitutes a useful tool, either in fundamental studies investigating distribution and penetration of various types of species, or as a step in plasma

medicine studies possibly combined with other techniques. Indeed, its intermediary position between rigorous, highly repeatable lab experiment on one side and assessment of plasma effect *in vivo* on the other side make it particularly valuable. This method is relatively young and the number of articles on the subject so far is still limited. However, in just 10 years, a lot of significant information has been learned and will guide the development of medical devices using CAP. Future research in the field promises further important advances and will help the emerging field of plasma medicine to mature.

However, several aspects remain either not or poorly investigated, such as the correlation between several critical parameters (gel mass fraction, electrical parameters, diffusion) and the type of tissue it models. For the reporters, the range of chemicals is still limited and other types of dyes, or even other types of reporters should be investigated. In addition, the measurement of RONS using this method presents several bias to keep in mind when interpreting results, both regarding the detection (no direct relationship between RONS and reacting reporters) and the analysis technique (spectroscopy).

In order to refine the model, the gel should be improved by studying and correlating its parameters to real tissues for a better adequacy. Concerning reporters, specificity, sensitivity and the influence of many parameters such as the medium or other interference should be explored. Finally, it must be reminded that this model has its limitations, with its quite simple structure and the reporters that merely detect RONS amid the many actors in cold plasma. It will never fully account for the penetration and reactions taking place in real living tissues. This simplicity constitutes nevertheless its strength and it proves highly valuable in the fastly growing field of plasma medicine, especially as treatment on humans is becoming more of a reality.

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