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Terminal sterilization: Conventional methods versus emerging cold atmospheric pressure plasma technology for non-viable biological tissues

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Tissue products are susceptible to microbial contamination from different sources, which may cause disease transmission upon transplantation. Terminal sterilization using gamma radiation, electron-beam and ethylene oxide protocols are well-established and accepted, however, such methods have known disadvantages associated with compromised tissue integrity, functionality, safety, complex logistics, availability and cost. Non-thermal (cold) atmospheric pressure plasma (CAP) is an emerging technology that has several biomedical applications including sterilization of tissues, and the potential to surpass current terminal sterilization techniques. This review discusses the limitations of conventional terminal sterilization technologies for biological materials, and highlights the benefits of utilizing CAP.

Introduction

Tissues and biological transplant materials (TBTM), including bone, soft tissues and other biologically-derived biomaterials should be sterilized to prevent transmission of microorganisms to the recipient. TBTM typically possess a natural microbial load/bioburden[a] or acquire one during collection and processing. If not eliminated, this has the potential to cause an infection leading to transplant failure, and further harm to the patient. Prevention of disease transmission is of primary concern to TBTM manufacturers and the regulatory authorities.

Sterilization is the confident and reproducible elimination of all forms of life. Terminal sterilization is considered the final step in the manufacturing process of a sterile product in its final packaging.[1] Terminal sterilization is used in the processing of TBTM to provide sterility assurance and is generally mandated by national regulatory authorities.[2] Terminal sterilization is required when: TBTM are processed in non-aseptic conditions; are not treated by any other form of decontamination; use non-sterile consumables; or are stored in conditions that do not prevent microbial growth. There is no ideal sterilization process for all biological materials. Each strategy faces advantages and disadvantages, requiring numerous considerations when choosing sterilization methods, including:

- Material compatibility
- Reliability against bacterial spores, endotoxins and viruses
- Effect on production process
- Expense

[a] The term "bioburden" is used to define the number and type of bacteria and fungi present on/in a non-sterile product.

Whilst terminal sterilization destroys contaminating microbial agents, the process also devitalizes the cells of the tissue. As a result, terminal sterilization is unsuitable for TBTM that are intended to include viable and healthy donor cells. Non-viable TBTM are routinely used for diverse applications ranging from homotopic, long-term transplants such as bone grafts;[3, 4] to short-term heterotopic transplants, such as amniotic membrane (AM) which can be used as an ocular surface bandage.[5, 6] In these situations, terminal sterilization is appropriate. Preservation of the original structure and function of a tissue, both mechanically and biochemically is essential. However, most sterilization methods can substantially damage biological materials.

Classical terminal sterilization methods such as ionizing radiation and ethylene oxide (EtO) have limitations including TBTM degradation, prolonged sterilization cycles, cost, logistical difficulties and inefficiency against viruses, prions and endotoxins. Thus, an effective sterilization procedure capable of maintaining a balance between sterilization efficacy, cost and retention of TBTM structural and functional properties is required for elimination of such resistant contaminants.

Trials of non-thermal (cold) atmospheric pressure plasma (CAP) sterilization methods on viable cells and tissues are promising.[7-11] Whilst CAP is potentially restricted to the sterilization of thinner TBTM, further optimization could deliver reduced cost, time, logistics and damage. In contrast with conventional sterilization methods, literature has reported that CAP possesses the ability to eradicate all resistant contaminants such as viruses, prions, endotoxins and biofilm from the treated surfaces.[12, 13]

This review compares the limitations of classic terminal sterilization of TBTM including gamma (γ), electron beam (e-beam) and EtO, and addresses the possibility of implementing CAP technologies with a particular focus on the sterilization of non-viable AM.

Challenges of TBTM manufacturing and risk of contamination

Tissue processing protocols vary, depending upon tissue structure and composition and the intended application, which determines how well the structural and functional properties of the native tissue need to be maintained. Processing of TBTM may include dissection, intensive and prolonged washing steps to remove blood and cellular components to diminish the presence of antigens and disease, defatting, demineralization and decellularization. TBTM may be preserved dry, or in hydrated form, at ambient or cold storage conditions; they may be aseptically prepared, high level decontaminated, or terminally sterilized.[14] Optimized manufacturing procedures are crucial for the quality and safety of the final product. There are specified international regulatory directives set to ensure prevention of risk from communicable disease transmission agents during procurement, processing, preservation, storage and use of TBTM (Directive 2004/23/EC).[15]

In the UK, under Human Tissue Authority (HTA) regulations, viable and non-viable tissues can be prepared without terminal sterilization. However, such procedures require stringent preparation and in-process monitoring compared with terminally sterilized tissues. Long-term storage of cryopreserved tissue requires specialist storage at -135 °C and with specific cryoprotectants such as dimethyl sulfoxide or glycerol; whereas non-viable, dehydrated (freeze-, heat- or vacuum-dried) tissue can be vacuum-packed and stored at ambient temperature for up to 5 years.[16]

In the US, transplantable materials such as bone, tendon, ligament, dura mater, heart valves, skin, cornea and AM are designated as “human cell, tissue, cellular or tissue-based products” (HCT/P) by the US Food and Drug Administration (FDA), with the stipulation that it is minimally manipulated as regulated under section 361 of the Public Health Service Act (PHSA) and the Code of Federal Regulations (CFR no. 21, Parts 1270 and 1271 of the FDA cGTP. Minimal manipulation is defined as negligible alteration to the tissues’ original

biological structure including physical integrity, tensile strength and elasticity; whilst allowing for preservation *via* drying, cryopreservation and denuding (removal of epithelium).[17] FDA regulation does not mandate terminal sterilization for HCT/Ps, however, aseptic processing must be used during tissue manufacturing to prevent contamination.[18]

Amniotic membrane as an example of a TBTM

AM is a TBTM that has a long history of surgical use, but has varied processing techniques and associated challenges. AM can be used for membrane repair on wound surfaces, or to fill spaces left by non-healing ulcers.[19-21] AM is commonly used in ophthalmic applications to treat a variety of corneal conditions.[5] Alongside acting as a transparent, physical shield, AM reportedly possesses protective, antimicrobial, analgesic and anti-inflammatory properties, that promote wound healing.[5, 6] Due to its healing properties and low immunogenicity, AM is often applied directly to open wounds.

AM undergoes stringent and tightly regulated processing procedures for clinical application. AM is typically collected from women undergoing preplanned, full-term elective cesarean-sections.[22] Therefore, donor screening, which involves assessment of information on health and social history, is the first line in disease transmission prevention. Donors must comply with set inclusion criteria, for example test-negative for human immune-deficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and *Treponema pallidum* (syphilis), amongst other criteria.[23, 24]

Under normal health, AM is naturally free of bioburden whilst in the womb. However, normal flora of the lower genital tract (e.g. gram-positive and gram-negative aerobic and anaerobic organisms such as genital mycoplasmas and *Streptococcus agalactiae*), are capable of crossing intact membranes to invade the amniotic cavity during labor[25]. However, AM

obtained from healthy full-term cesarean sections should have no bioburden. The UK advisory committee on Safety of Blood, Tissues and Organs (SaBTO) guidelines state that:

"Estimation of bioburden of skin and amnion is not recommended as the former carries a substantial bioburden and the latter is surgically recovered under aseptic conditions. However, a heavy growth of bacteria from pre-process samples may signify gross contamination and the tissue should not be released unless able to be terminally sterilized by irradiation or other techniques. The potential damage to the integrity of the tissue by the high numbers of bacteria should also be considered before it is used for transplantation".[26]

Cesarean deliveries do have the potential to introduce some contamination, likely originating from the donor's skin microbial flora, transmitted during AM removal.[27, 28] Other contamination may arise from the operating room environment. Further processing of AM usually takes place in GMP environments subjected to high-level decontamination procedures. AM is generally rendered non-viable during preservation, either by drying or freezing.[29] As a non-viable tissue, currently used surgically, AM is an ideal candidate for terminal sterilization.

Sterilization of abnormal biological contaminants

Processing and sterilization protocols are effective for TBTM with low to normal bioburden. Tissues infected with a high bioburden of endotoxin producing, gram-negative bacteria or virulent, antibiotic-resistant bacteria, for example pseudomonas, methicillin-resistant *Staphylococcus aureus* (MRSA) or *Clostridium difficile,* represent a greater hazard and are unsuitable for transplantation unless a terminal sterilization procedure is used, that is validated for effective removal or complete inactivation of microbial loads.[30] TBTM can also be contaminated by non-living biological agents such as endotoxins, prions and viruses, which

are known to resist most conventional sterilization methods.[31] Most ISO standards exclude viruses and prions from their sterilization validation documents. For example, ISO 14160:2011[32] for liquid chemical sterilization controls risks associated with only bacteria and fungi contamination; however, it is not applicable to TBTM. Also, ISO 11137-1:2006, for validation of radiation sterilization dose does not cover viral contamination and ISO 11135:2014, for validation and control of EtO sterilization methods excludes prion contamination from its scope. Categorically, these standards indirectly recognize the downsides of industrial sterilization technologies towards particular contaminations.

The risk of harvesting and transplanting infected tissue can be reduced, but not eliminated, by specific donor exclusion criteria in the form of detailed medical/social history questionnaires and advanced serological testing. However, terminal sterilization remains the most effective method for eliminating biological contamination hazards associated with donor source, procurement and manufacturing. Selection of the appropriate sterilization protocol for TBTMs must be considered on a case-by-case basis; considering the possibility of sterilization resistant contaminants.

Endotoxins

Endotoxins are the lipopolysaccharides (LPS) that make up the outer membranes of the pathogenic and non-pathogenic gram-negative bacteria.[33] Endotoxins present in the blood stream are life-threatening in high doses,[34] due to activation of the immune system causing "endotoxin shock". Current sterilization technologies only deactivate endotoxin producing bacterial species and are incapable of removing endotoxins themselves. Therefore, there is potential endotoxin existence on sterile products. Endotoxin contamination is of great concern in pharmaceutical and TBTM manufacturing industries, as they are thermostable, resistant to pH change, vary widely in size, and have heterogeneous molecular structures.[35, 36]

Aggressive endotoxin removal methods such as high temperatures (250 °C for 30 min, or 180 °C for 3 hours)[37] are unsuitable for TBTM. The terminal sterilization radiation doses required to degrade endotoxins are effective only at very high levels[38], which can be destructive to TBTM. As product removal of endotoxins is more problematic than sterilization; avoiding contamination by employing aseptic technique and current good tissue practice (cGTP) are currently the favored approach.

Prions

Prions are another challenging form of non-living contamination. They are a pathogenic form of misfolded protein responsible for introducing neurodegenerative disorders such as transmissible spongiform encephalopathies. These potentially fatal prion diseases can be transmitted to healthy patients through contaminated tissue allografts such dura mater and ocular tissue transplants.[39] Prions exhibit a resistance to most known physical and chemical sterilization methods. Even methods combining autoclaving with chemical treatment of concentrated sodium hydroxide are incapable of completely abolishing prion contamination from reusable hospital devices.[40]

Biofilm

A biofilm is a complex stratified mass of single or varied genotypes of microbes (bacteria or fungi), compacted to each other and strongly attached to surfaces. Biofilms have high protection capacity against disinfectants and antimicrobials compared to normal microbial form. Thickness and compaction determines microbial biofilm resistance to penetrating oxidative agents. Biofilms are formed on synthetic transplant materials[41], but are not known to be present on TBTM. However, biofilm presence in water pipes, channels and surfaces of TBTM manufacturing equipment can be a source of frequent contamination, representing a

considerable safety concern. Once these biofilm making microorganisms reach the manufacturing equipment and materials, a reliable sterilization technique is required to eradicate it. Examples of biofilm forming bacteria include: MRSA, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Escherichia coli*.[41]

Viruses

Non-viable TBTM that have been exposed to chemical treatment, drying or terminally sterilized are less virally infectious than viable tissue preserved at low temperatures.[42] Therefore, transmission of viruses through non-viable TBTM is rare. However, viruses with small, and/or double stranded genomic material have more ability to repair damage to genetic material than viruses with complex genomes[43], and can resist high radiation doses of 100 kGy.[44]

Established sterilization techniques for TBTM

Challenges of TBTM manufacturing include standardization, maintenance of tissue quality and sterility, which may ultimately affect clinical outcome. The predominant issue with contemporary terminal sterilization techniques such as γ, e-beam and EtO, is maintenance of tissue structure and biological properties.[45] However, failure to provide sterile TBTM can result in significant health complications, and cost implications to health care providers.

Standardization and regulation of sterilization techniques

High temperature sterilization techniques, such as high-pressure steam methods, are unsuitable due to their denaturing and destructive effects on tissue extracellular matrix; they can also coagulate soft tissues[46] and are insufficient for endotoxin inactivation. Other non-

thermal, conventional sterilization methods such as EtO, γ and e-beam are recognized techniques, which are effective when validated correctly.[40] Radiation dosage for sterilization is validated using ISO 11137 (2006)[47], EtO and pressured steam sterilization are also standardized by ISO 11135 (2007)[48] and ISO 17665-1 (2006)[49] respectively.

The achievement of absolute sterility is difficult to determine without destructively testing all batch materials.[50] Therefore, sterile production standards of medical supplies, drugs and TBTMs refer to the sterility assurance level (SAL). SALs are based on the theoretical probability of overkill and demonstration of non-sterility of a particular item following sterilization. SAL 10^{-6} is adopted for medical devices and biological products and is endorsed by most major tissue banking societies and specialized organizations.[51, 52] Using SAL 10^{-6} implies that no more than one item in one million may be non-sterile, or that there is a one in a million chance that an individual microbe will survive sterilization. Nevertheless, the FDA does not specify a particular method of terminal sterilization or a specific SAL for TBTMs. SAL selection is the tissue manufacturer's responsibility and appropriate SALs lower than 10^{-6} can be used if the product is unable to withstand the sterilization process (AAMI ST67 standard). SAL 10^{-3} is used for inanimate medical supplies and have not been reported to be less assured than SAL 10^{-6} products in terms of preventing nosocomial infections[b]. Whilst current terminal sterilization techniques have revolutionized many industries including medicinal products and tissue supply, higher SALs may come at the cost of damaging TBTM.[53]

Limitations of current low temperature sterilizations

[b] Nosocomial infections refer to infections acquired in healthcare facilities such as hospitals that were not present upon administration.

Current terminal sterilization techniques along with their advantages and disadvantages with respect to TBTM are summarized in **Table 1.**

Ionizing radiation sterilization at moderate doses is used at large-scale for medical supplies and synthetic prostheses, but there are concerns regarding their destructive effects on TBTMs at higher doses. γ and e-beam radiation are widely applied for tissue sterilization including AM.[54-57] 25 kilo gray (kGy) from both e-beam and γ sources has been internationally accepted as a guaranteed dose, however, the International Atomic Energy Agency (IAEA) standards[58] recommend a dose of below 25 kGy.[30] Conversely, concerns regarding viral transmission *via* allografts from deceased donors potentially infected with HIV/HCV/HBV justify higher doses (e.g. 35 kGy).[54] Debates regarding optimal sterilization dose reflect concerns regarding unacceptable structural damage or physicochemical changes caused by high radiation doses[59, 60] and irradiation cost, especially for small tissue manufacturers, is an additional issue.

Chemical sterilization, such as EtO, has toxicity concerns due to high oxidative stresses of free radicals and residues, time-consuming processes, expense and difficult operational requirements. EtO sterilization unavoidably leaves residuals such as ethylene chlorohydrin and ethylene glycol on treated products,[61] limiting the functionality of biological tissues.[62] Other chemical sterilization methods, occasionally used for TBTM; are rarely regarded as terminal sterilization due to technical, safety or biocompatibility barriers. Super critical carbon dioxide ($SCCO_2$)[63], characterized by its efficacy against spores, penetration and biomaterial compatibility,[63-66] is reported to cause damage to materials, leaves residues and is ineffective against endotoxins. [53] [67] Vaporized peracetic acid (PAA) gas plasma, has been proven to have broad germicidal action over short time periods,[68] and is compatible with most collagenous materials and tissues.[69-71] However, PAA has time and cost constraints[72] and additionally, PAA sterilized ophthalmic instruments have been documented to cause

serious ophthalmic and skin damage with contact, leading to withdrawal from the market place [65, 73, 74]. Similarly, vaporized hydrogen peroxide (H_2O_2) gas plasma is used efficiently for TBTM sterilization and is FDA approved.[75, 76] However, it has problems regarding adverse reactions with the functional properties of the tissue and medical materials. [77, 78] Glutaraldehyde sterilization protocols are relatively inexpensive, effective against most bacteria and viruses, but limited by long process cycles and toxicity to recipient tissues and potentially crosslinking effects.[65]

Cold atmospheric pressure gas plasma for terminal sterilization

Plasma[c] has been widely investigated for medical applications in recent years. Progressive findings regarding the role of plasma in the deactivation and elimination of biological contamination demonstrates the potential for this technology to overcome the drawbacks of conventional sterilization methods of heat sensitive materials including TBTM.

Thermal and non-thermal plasmas

Plasmas at atmospheric pressure can be classified in terms of temperature into two categories, thermal[80, 81] and non-thermal.[82-84] In thermal plasma, the charged particles, neutral electrons and heavy particles all have the same high temperatures (in thermodynamic equilibrium with the surrounding temperature) and are almost fully ionized; whilst, in the non-thermal plasma the temperature of gas, atoms and molecules remains low[85] because of the slight ionization

[c] Plasma in physics is the fourth state of matter basically constituted of partly or entirely ionized gas contains free radicals, charged ions and electrons, neutral atoms and other radiation.[79]

of the used gas[80], with only electrons at high temperature. Due to this variation of the constituent's temperature, non-thermal plasma is also termed non-equilibrium plasma.[86, 87]

Low and atmospheric pressure plasmas

Given that thermal plasma produces high gas temperature, non-thermal "cold" plasma is much more suitable as an emerging sterilization method[81, 86], for inactivation of microbial loads on human tissues and heat sensitive surfaces.[88] Furthermore, cold plasma can be produced at low pressures, under vacuum, but for practicality and economic reasons, it is more convenient and cost-effective when generated at atmospheric pressure and therefore called cold atmospheric-pressure plasma (CAP).[87]

CAP is obtained by exposing gas flow (either air, or noble gases such as oxygen, nitrogen, argon, or helium) to a high electric field which partially ionizes the gas atoms producing a sustained plasma. This plasma contains a collection of excited electrons, negative and positive ions, excited gas species e.g. O, $O_2^{\bullet}$, O_3, $OH^{\bullet}$, reactive oxygen species (ROS), NO and NO_2 reactive nitrogen species (RNS), free radicals and UV-photons at different wavelengths.[89] These nontoxic gases become germicidal only after the plasma is ignited, since they are not biocidal on their own.[90] Concentration of produced plasma agents (i.e. ROS, RNS, UV, free radicals, charged particles) depends on the operation parameters of the plasma source; namely loaded gas, gas pressure, flow rate, electric voltage, *etc*.

CAP systems

CAP can be provided by three different systems (**Figure 1**): (i) Direct plasma, known as the dielectric barrier discharge (DBD) system. By design, the DBD system safely uses the material to be sterilized as a grounded electrode, which the current flows through, whilst the

other electrode is connected to a high voltage; air is utilized as the operating gas, and the distance between plasma source tip and the object is a few millimeters.[80] (ii) 'Indirect plasma', also known as atmospheric pressure plasma jet, which is deployed by a plasma needle/pen (narrow focused jet) or plasma torch systems which have broader streams and cover larger surface areas.[91] These devices generate plasma between two electrodes, transferred through a gas flow to the object to be treated, the distance between the object and the device ranges from millimeters to centimeters. The reactive species are generated by igniting plasma in an operating gas such as air, or by helium or argon plasmas in an admixture of oxygen or nitrogen.[92] (iii) Hybrid systems or coronal discharge, have characteristics of both direct and indirect plasmas.[80, 93]

Mechanisms of microbial inactivation by CAP

CAP technologies have been used effectively to eliminate microorganisms on living tissues in several clinical applications[8, 80, 94-101], including inactivation and eradication of fungi, and vegetative and spore-forming bacteria.[86, 89, 102] The microbial inactivation effect of CAP can be attained by direct exposure to both the ignited plasma discharge products such as ROS, RNS, UV radiation, or indirectly through the long–lived species that are capable to reach the treated substrate without the electric field effect[91]. Generally, decontamination through plasma treatment is accomplished *via* oxidative stress arisen from synergetic actions of plasma discharge products.[88, 103]

The specific plasma agent responsible for the mechanism of CAP killing is not clearly corroborated in literature[7, 89]; although CAP is proven to be damaging to bacterial cell walls, primarily due to ROS and RNS.[104] These charged particles have sufficient ability to induce high oxidative stress which cause damage in microbial cells via fast direct interactions.[81] It has been postulated that the accumulation of plasma charged particles over the bacterial cell

membrane causes membrane rupture through electrostatic disruption[105]; or that the reactive species permeabilize and penetrate the cell walls before reacting with DNA via complex mechanisms.[106] Membrane damage through lipid peroxidation is another major cause of bacterial death by CAP. The effect by heat and UV is argued to be indirect or negligible.[107, 108] However, CAP's microbicidal action produced by various plasma sources are reported to target viable cellular components such cell membrane, protein and DNA. The effect of CAP is believed overwhelming the DNA repair mechanisms of the bacterial cells.[7]

Effects of CAP on vegetative and spore-forming bacteria

Plasma agents destroy different microbial entities irrespective of their molecular defense mechanisms. Examples reported of CAP capability for decontaminating multidrug resistant bacteria including the highly resistant MRSA[109-111] and *Pseudomonas aeruginosa*.[110] Notably, CAP is also effective on the sterilization resistant *Deinococcus radiodurans* bacteria.[112] Less resistant vegetative bacteria have also been tested against the antimicrobial activity of CAP, and were eliminated with different degrees of survival, including gram-negative: *Escherichia coli*[111, 113, 114], *Salmonella typhimuriu* [115], and gram-positive *Staphylocuccus epidermidis*[90, 113, 116], *Staphylococcus aureus*[90, 114], *Micrococcus luteus*[95], *Streptococcus pyogenes*[90], *Enterococcus faecalis*[117] and *Enterococcus faecium*.[90] Gram-negative bacteria are more susceptible to gas plasma treatments than gram-positive[104, 118] and differences in susceptibility are thought to be due to variation in the thickness of the peptidoglycan murein layer in the bacterial cell wall.[86, 104]

Skin floral bacteria *Staphylococcus epidermidis* is of particular importance in sterilization of TBTM given its frequent association with contamination from procurement and processing sites. It is normally found in the hair follicle where it is protected from antiseptics. CAP

penetration has the capacity to reach deep into hair follicles[119] and disinfect bacteria by 94%.[120] Gram-positive skin flora *Propionibacterium acnes*, reported to cause post-transplantation and implantation infection, are also effectively sterilized by CAP even in protected aggregated forms.[121]

Spore-forming bacteria are typically resistant to many established sterilization protocols, and are often used as a model organism for sterilization verification. Studies have demonstrated CAP efficacy against bacterial spores, including: *Bacillus subtilis*, *Clostridium difficile, Bacillus atrophaeus*, *Bacillus safensis*, *Bacillus megaterium*, *Bacillus megaterium* 2c1 and *Bacillus thuringiensis* E24.[122, 123] Studies have shown CAP has effective sporicidal action even in humid conditions[124, 125], when spores are stacked together or covered by debris.

Effect of CAP on abnormal contamination

TBTM manufactured in a sterile environment using cGTP protocols are unlikely to have resistant contamination such as endotoxins, prions, viruses and biofilms unless a pre-processing infection has occurred. Efficient sterilization should be able to inactivate all forms of disease causing contamination. The advantage of CAP sterilization compared to traditional methods is its powerful efficacy to eliminate resistant contamination and removal through its etching action, which is highly dependent upon applied plasma source. For instance, plasma jets have more etching capabilities than DBD systems. The plasma density, gas mixture and radical content are important parameters in etching of protein residues from treated surface by chemical degradation and volatilization mechanisms.[126] CAP has been demonstrated to be effective for inactivation of LPS endotoxin removal from treated surfaces within minutes.[127, 128] Similarly, it has been shown that CAP is efficient in removing amyloid fibril aggregates (protein structure mimics prion) from a surfaces, and that aggregates outside the plasma diameter were degraded, whilst those within the plasma focus were consistently removed.[129]

The use of a CAP system with a negative corona discharge has been shown to have a significant effect on prions and may reduce the infectivity of prion particles by several orders of magnitude.[130] This was believed to be mediated by the generated reactive particles. Additionally in this study, the source of CAP had different effects on the viability of brain cells; a positive streamer discharge killed the cells, whereas point-to-point, cometary and negative corona discharges did not result in a loss of cell viability. This indicates that the discharge configuration is an important parameter to consider when sterilizing viable tissues with CAP.

TBTM contaminants such as the anaerobic gram-positive *Propionibacterium acnes* and *Staphylococcus* have been proven to form *in vivo* and *in vitro* biofilms, and develop resistance to gentamicin-loaded implants for orthopedic surgery[41], causing biomaterial/prosthesis transplant infection and failure.[131] Biofilms of *Propionibacterium acnes* bacteria have been found to be considerably inactivated by two types of plasma jet over short time periods.[121] Biofilms of MRSA, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Escherichia coli* are also removed by CAP treatment.[12] Similarly, CAP have efficiently removed biofilms of antifungal resistant *Candida albicans.*[132] This effect can potentially be enhanced by adding oxygen to the plasma discharge.[133] Studies have shown that viruses can be eliminated by CAP, due to the destruction of the protein coat, rather than a direct effect on viral DNA.[134]

Several factors may affect the dose or the exposure time required to achieve sterilization. These factors are related to the plasma device operational conditions or the microorganism conditions before and after exposure, e.g. bacterial species, bacterial culture age (growth phase), bacterial medium during and post-treatment.[135] The eradicating effect of CAP on various microorganisms is well-established in literature [91, 130], however, studies on sterilization and removal of biological non-living disease causing agents would have a promising medical applications.[13, 130] However, CAP system variation, the flexibility of

utilizing variable operational conditions along with the different applications have resulted in complication when comparing the literature findings. **Table 2** shows examples of the sensitivity of microorganisms of medical importance and other biological contaminants resistant to sterilization, treated with different CAP devices under various experimental conditions, with differences in exposure times and contamination reductions. CAP device specifications and the applied dose were omitted due to the aforementioned plasma delivery complexities. Often, the time required to inactivate or sterilize the microbial/biological contaminations is much shorter than those applied in other established sterilization methods regardless of the CAP system used. To achieve SAL 10^{-6} sterilization, exposure duration (plasma dose) may be extended. Therefore, it is important to have information on the level of initial material contamination if CAP is chosen as a sterilization protocol. It can be difficult to contrast and compare CAP efficacy on biological contamination due to the variability in CAP parameters and systems; however, this does not indicate that CAP is ineffective.

Use of CAP for TBTM sterilization

CAP has been evaluated as a safe and efficient procedure for decontamination of skin wounds, ulcers and infected tissues in patients, and there are commercially available products such as the kINPen.[136] and the DBD plasma generator (PlasmaDerm® VU-2010). [137] Reports often emphasize the biocompatibility of plasma treatment on viable cells and tissues, including ocular surface tissues. [101, 138-140] These effects are deleterious to microbial contamination, but have thus far not been proved to induce necrotic, apoptotic or morphological changes in surrounding tissues. Furthermore, no significant effects on living cell viability were reported.[111] In other studies, a slight reduction in viability through necrosis of *in vitro* culture of mucosal cells was observed, however, no mutagenic effects were observed.[136, 141] The reason CAP mechanisms are generally harmless to human cells

and tissue is attributed to CAP's superficial penetration[142]{Shintani, 2016 #459}, and potentially that multicellular eukaryotic tissues have more complicated mechanisms to enable survival of oxidative stresses introduced by plasma agents, than prokaryotic cells.[143] Limited penetration could be an obstacle to CAP sterilization of TBTM with thick, heterogeneous size and architecture such as bone graft materials.[142] However, dried, thin, soft tissues such as skin, facia, dura mater, AM, tendons and laminate of cartilage and bone are all candidates for CAP sterilization[144]. The defined advantages and disadvantages of CAP sterilization of TBTM are listed in **Table 3.**

CAP as a potential technique for thin tissue sterilization

At the time of this review there were no studies available evaluating CAP for sterilization of non-viable TBTM. However, if we consider non-viable AM as an example, a number of factors should be considered when optimizing CAP specifically, tissue thickness, treatment area, surface morphology, probable type and count of contamination, and whether sterilization will occur before or after packaging.

A reliable sterilization method must have sufficient penetration capability. Studies have demonstrated that CAP delivers ROS in the range of 150 μm - 1,500 μm[145] when employed on a tissue-equivalent material and at over 5,000 μm for agarose gels.[101] For intact viable skin, reactive species can penetrate up to 10 μm depth.[136] The penetration of H_2O_2 produced by CAP into euthanized rat skin and chicken breasts was shown to go up to 4,000 μm.[146] Furthermore, experiments on polyester fabric materials assert that the distance from plasma nozzle to substrate, exposure time, and thickness of substrate, have an effect on plasma penetration, yet suggests longer exposure time renders a sufficient amount of reactive species accumulating on one side to diffuse to the other side of the porous material.[147, 148] Moreover, it has been demonstrated that nitric oxide (NO) generated by topical application of DBD over

a human skin and reconstructed epidermiswere safe on living tissue and able to penetrate and cause changes in microcirculation up to 6000-8000 µm on treated skin areas.[149]Dried AM with a thickness of approximately 20 µm[150] will therefore lie within the penetration range of CAP and should not require lengthy exposure times.

Surface topography of treated tissue present challenges in sterilization, as changes can occur during chemical or mechanical processing, creating rough surfaces and microgrooves where microorganisms can reside and proliferate. Tissue surface irregularity enables contaminant bacteria to migrate from the surface into the tissue, protecting it from the biocidal effect of sterilization.[151] There are natural intercellular microholes on AM epithelial surface (**Figure 2A, B**), which have been proven to allow gas transmission through dried and cryopreserved AM upon transplantation.[152, 153] These microholes are impermeable to bacteria such as *Escherichia coli*, *Pseudomonas*, *Klebsiella* and *Staphylococcus*.[154] CAP can easily reach narrow, confined spaces and microgrooves on surfaces.[82] and mobility of plasma gas can allow for treatment of microspaces[123, 155]. Recent studies reported CAP efficacy in reducing microbial contamination adhered to rough surfaces of chicken skin[151], titanium implant surfaces[156] and root canals, especially in dry state conditions.[157] This supports the appropriate use of CAP for sterilization of dry AM with a comparably soft homogenous surface (**Figure 2A, C**).

Additionally, CAP treatment of large tissues requires a plasma source capable of delivering plasma components over wider surface areas. Single CAP jets are limited to a few millimeters, e.g. 25 mm^2[158], thus are unsuitable, but micro-discharge plasma devices have been used effectively for biofilm treatment on a 177 cm^2 surface area.[132] Plasma torches are able to treat areas of approximately 25 cm^2 and DBD and jet torches appear to be favorable for large size membranous grafts[82] and have potential to be developed to operate as a

systemic scanning system for tissue materials with large surface area. The area of AM grafts are generally 4-100 cm^2, thus CAP systems could be developed to accommodate this.

There are parameters related to the plasma system operational conditions that will affect use, such as: the voltage, loaded gas, gas pressure, flow rate, exposure time and sample distance from plasma nozzle.[159, 160] The interplay of these parameters could yield sufficient plasma density and composition, or conversely might change the treatment outcome; causing inconsistency in sterilization cycles.[161] For instance, operational parameters such as ion and UV flux, electric field and gas flow might not have direct influence on the antibacterial efficiency, while other factors related to the method and conditions of application such as distance from the plasma nozzle and exposure time have a distinct influence on treatment efficiency.[162]

An effective treatment that causes no damage to tissues requires a highly standardized, balanced and homogenous current discharge,[163] which can be attained by adjusting the circuit, gas flow, and power supply parameters.[88] Since varied modalities of CAP systems have been used as decontamination tools on different biological substrates, such as human skin and food products, it is possible to develop a CAP device that copes with thin non-viable membranous tissue sealed in sterile packaging.

To maintain sterility of a manufactured TBTM, CAP should be applied after packaging or in-package and sealed in a sterile environment. Use of CAP for in-package sterilization has been tested effective against spores, used to decontaminate foods, and is compatible with some packaging materials.[125, 164] DBD plasma configurations developed with efficient sporicidal actions were found to safely fit several shapes of Tyvek packaging materials used for sterile wrapping of medical instruments.[165] Different packaging materials including polyethylene, polypropylene and nylon loaded with microbial pathogens have shown no change in the physical properties after treatment with low pressure plasma.[166] Recent studies have shown

that generating plasma inside a closed environment can prevent post-packaging contamination of sterilized products.[167]

Another attractive advantage of CAP over conventional sterilization is cost-efficiency. Cold plasma generators can be scaled-down as portable devices in a regular laboratory instrument size, for use on the bench, which suits application in small-scale tissue production.[8] Close proximity to tissue preparation site can potentially minimize cost of logistics and are not as complex as performing terminal sterilization away from the production site.

Direct or indirect CAP systems may be appropriate for AM sterilization, as they can be operated at atmospheric pressure and at nearly ambient temperature[168], can deliver large quantities of active species with sufficient penetration power of plasma agents[81], and can treat reasonably large areas inside the final product packaging. Small mobile handled CAP devices could be an economical choice for AM sterilization inside the sterile area or alternately, an on-bench contained chamber design for in-package sterilization may be more applicable.

Conclusion

Current terminal sterilization methods such as EtO, ionizing radiation and chemical sterilization methods have several disadvantages including toxicity, high running-costs and inefficacy against abnormal contamination. CAP is a terminal sterilization technique that is applicable to living tissues without known harm or side effects, that has been proven to destroy both normal and abnormal contamination. CAP is inexpensive but limited by tissue thickness, post-packaging penetration and reproducibility. Choice of CAP for TBTM sterilization should be done on individual basis, dependent on tissue characteristics,

manufacturing protocol and intended use. However, if CAP can be adapted for TBTM, it holds a vast amount of promise.

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References

1. B. J. Lambert, T. A. Mendelson, M. D. Craven, *AAPS PharmSciTech*, **2011,** *12,* 1116.
2. D. Hussong, *AAPS PharmSciTech*, **2010,** *11,* 1482.
3. S. Stevenson, *Orthop Clin North Am*, **1999,** *30,* 543.
4. T. Boyce, J. Edwards, N. Scarborough, *Orthop Clin North Am*, **1999,** *30,* 571.
5. H. S. Dua, J. A. Gomes, A. J. King, V. S. Maharajan, *Surv Ophthalmol* **2004,** *49,* 51.
6. F. Gindraux, R. Laurent, L. Nicod, B. de Billy, C. Meyer, N. Zwetyenga, L. Wajszczak, P. Garbuio, L. Obert, *Recent Pat Regen Med*, **2013,** *3,* 193.
7. J.-W. Lackmann, J. E. Bandow, *Applied Microbiology and Biotechnology*, **2014,** *98*.
8. P. Brun, M. Vono, P. Venier, E. Tarricone, V. Deligianni, E. Martines, M. Zuin, S. Spagnolo, R. Cavazzana, R. Cardin, I. Castagliuolo, A. L. Valerio, A. Leonardi, *PLoS One*, **2012,** *7,* 14.
9. C. Hoffmann, C. Berganza, J. Zhang, *Med Gas Res*, **2013,** *3*.

10. C. Ulrich, F. Kluschke, A. Patzelt, S. Vandersee, V. A. Czaika, H. Richter, A. Bob, J. Hutten, C. Painsi, R. Huge, A. Kramer, O. Assadian, J. Lademann, B. Lange-Asschenfeldt, *J Wound Care*, **2015,** *24*.
11. R. Laurita, F. Alviano, C. Marchionni, P. M. Abruzzo, A. Bolotta, L. Bonsi, V. Colombo, M. Gherardi, A. Liguori, F. Ricci, M. Rossi, A. Stancampiano, P. L. Tazzari, M. Marini, *Journal of Physics D: Applied Physics*, **2016,** *49*.
12. M. Hee Lee, B. Joo Park, S. Chang Jin, D. Kim, I. Han, J. Kim, S. O. Hyun, K.-H. Chung, J.-C. Park, *New J Phys*, **2009,** *11,* 115022.
13. J. L. Zimmermann, K. Dumler, T. Shimizu, G. Morfill, A. Wolf, V. Boxhammer, J. Schlegel, B. Gansbacher, M. Anton, *J Phys D: Appl Phys*, **2011,** *44,* 505201.
14. J. Komender, *Ann transplant*, **2004,** *9,* 88.
15. *Guide to safety and quality assurance for organs, tissues and cells, 2nd edition,* C.o.E. Publishing, Council of Europe Publishing, Strasbourg, Belgium **2004**
16. H. Gillan, K. Bennett, P. Yates, *Review of long-term storage of tissue products*, N.B.a. Trnasplant, Editor. 2010, NHS Blood and Transplant: UK.
17. F. D. A. U.S. Department of Health and Human Services, *Minimal manipulation of human cells, tissues, and cellular and tissue-based products -draft guidance for industry and food and drug administartion staff*. 2014, FDA: New Hampshire Avenue, Silver Spring, USA.
18. S. Kuroda, A. S. Virdi, Y. Dai, S. Shott, D. R. Sumner, *Calcif Tissue Int*, **2005,** *77,* 212.
19. I. Mermet, N. Pottier, J. M. Sainthillier, C. Malugani, S. Cairey-Remonnay, S. Maddens, D. Riethmuller, P. Tiberghien, P. Humbert, F. Aubin, *Wound Repair Regen*, **2007,** *15,* 459.
20. F. E. Kruse, K. Rohrschneider, H. E. Völcker, *Ophthalmology*, **1999,** *106,* 1504.
21. M. T. Rodríguez-Ares, R. Touriño, M. J. López-Valladares, F. Gude, *Cornea*, **2004,** *23,* 577.
22. P. J. Adds, C. Hunt, S. Hartley, *Br J Ophthalmol*, **2001,** *85,* 228.
23. *Current Good Tissue Practice,* American Association of Tissue Banks, McLean, Virginia. **2006**
24. *International Standards for Tissue Banks,* World Scientific, Singapore **2003**
25. C. A. Combs, T. J. Garite, J. A. Lapidus, J. P. Lapointe, M. Gravett, J. Rael, E. Amon, J. K. Baxter, K. Brady, W. Clewell, *Am J Obstet Gynecol*, **2015,** *212,* 482.
26. SaBTO, *Guidance on the microbiological safety of human organs, tissues and cells used in transplantation*, in *Retrieval of material for donation testing*. 2011, Infectious Diseases and Blood Policy: UK.
27. S. Hill, *AORN J*, **2008,** *88,* 731.
28. H. R. Aghayan, P. Goodarzi, A. Baradaran-Rafii, B. Larijani, L. Moradabadi, F. Rahim, B. Arjmand, *Cell Tissue Bank*, **2013,** *14,* 401.
29. A. Hopkinson, R. McIntosh, P. Tighe, D. James, H. S. Dua, *Invest Ophthalmol Vis Sci*, **2006,** *47,* 4316.
30. J. Morales Pedraza, A. Lobo Gajiwala, M. E. Martinez Pardo, *Cell Tissue Bank*, **2012,** *13,* 15.
31. Norimah Yusof, Abdul Rani Shamsudin, Hasim Mohamad, Asnah Hassan, A. C. Yong, M. N. F. A. Rahman., *Bioburden estimation in relation to tissue product quality and radiation dose validation*, in *Sterilisation of Tissues Using Ionising Radiations* J.F. Kennedy, G.O. Phillips, and P.A. Williams, Editors. 2005, Woodhead Publishing Limited,: Abington, Cambridge, England p. 319-329.
32. ISO-14160, *Sterilization of health care products - Liquid chemical sterilizing agents for single-use medical devices utilizing animal tissues and their derivatives -*

Requirements for characterization, development, validation and routine control of a sterilization process for medical devices, in *The International Organization for Standardization*. 2011 Geneva, Switzerland.
33. G. Ramachandran, *Virulence* **2014,** *5,* 213.
34. N. Mamalis, H. F. Edelhauser, D. G. Dawson, J. Chew, R. M. LeBoyer, L. Werner, *J Cataract Refract Surg*, **2006,** *32,* 324.
35. E. T. Palva, P. H. Makela, *Eur J Biochem*, **1980,** *107,* 137.
36. R. C. Goldman, L. Leive, *Eur J Biochem*, **1980,** *107,* 145.
37. T. Miyamoto, S. Okano, N. Kasai, *Appl Environ Microbiol*, **2009,** *75,* 5058.
38. H. Mrazova, J. Koller, G. Fujerikova, P. Babal, *Cell Tissue Bank*, **2014,** *15,* 429.
39. P S-Juan, H J T Ward, R De Silva, R S G Knight, R. G. Will, *Br J Ophthalmol*, **2004,** *88,* 446.
40. S. Lerouge, *Introduction to sterilization: definitions and challenges*, in *Sterilization of biomaterials and medical devices*, S. Lerouge and A. Simmone, Editors. 2012, Woodhead Publishing Limited. p. 10.
41. M. M. Tuney, M.M. Tunney, N. Dunne, G. Einarsson, A. McDowell, A. Kerr, S. Patrick, *J Ortho Res*, **2007,** *25,* 2.
42. P. Solves, V. Mirabet, M. Alvarez, *World J Gastroenterol*, **2014,** *20,* 7434.
43. M. Silindir, A. Yekta Ozer, *FABAD J. Pharm. Sci.*, **2009,** *34*.
44. N. Yusof, *Chapter 9, Radiation killing effects on bacteria and fungi*, in *Radiation in tissue banking - Basic science and clinical applications of irradiated tissue allografts*, Nather A, Yusof N, and Hilmy N, Editors. 2007, © World Scientific Publishing Co. Pte. Ltd.: Singapore. p. 129.
45. D. R. McAllister, M. J. Joyce, B. J. Mann, C. T. Vangsness, Jr., *Am J Sports Med*, **2007,** *35,* 2148.
46. F. von Versen-Hoynck, C. Syring, S. Bachmann, D. E. Moller, *Cell Tissue Bank*, **2004,** *5,* 45.
47. ISO-11137-1, *Sterilization of health care products, Radiation, Part 1, Requirements for development, validation and routine control of a sterilization process for medical devices*, in *International Organization for Standardization*. 2006: Geneva, Swetzerland.
48. ISO-11135, *Sterilization of health care products – Ethylene Oxide – Requirements for development, validation, and routine control of a sterilization process for medical devices*, in *International Organization for Standardization*. 2014: Geneva, Switzerland.
49. ISO-17665, *Sterilization of health care products — Moist heat — Part 1: Requirements for the development, validation and routine control of a sterilization process for medical devices* in *The International Organization for Standardization*. 2006: Geneva, Switzerland
50. V. C. Abraham, D. L. Taylor, J. R. Haskins, *Trends Biotechnol*, **2004,** *22,* 15.
51. ISO-11137-2, *Sterilization of health care products – Radiation – Part 2: Establishing the sterilization dose.*, in *International Organization for Standardization*. 2013: Geneva, Switzerland.
52. *Microbiological Surveillance Program and Process Validation* American Association of Tissue Banks, McLean, Virginia **2012**
53. Q. Q. Qiu, W. Q. Sun, J. Connor, *Sterilization of Biomaterials of Synthetic and Biological Origin*, in *Comprehensive Biomaterials*, P. Ducheyne, Editor. 2011, Elsevier. p. 127-143.
54. A. Dziedzic-Goclawska, A. Kaminski, I. Uhrynowska-Tyszkiewicz, W. Stachowicz, *Cell Tissue Bank*, **2005,** *6,* 201.

55. C. R. Balsly, A. T. Cotter, L. A. Williams, B. D. Gaskins, M. A. Moore, L. Wolfinbarger, Jr., *Cell Tissue Bank*, **2008,** *9,* 289.
56. R. Singh, P. Gupta, P. Kumar, A. Kumar, M. P. Chacharkar, *Cell Tissue Bank*, **2003,** *4,* 95.
57. N. Marsit, S. Dwejen, I. Saad, S. Abdalla, A. Shaab, S. Salem, E. Khanfas, A. Hasan, M. Mansur, M. Abdul Sammad, *Cell Tissue Bank*, **2014,** *15,* 603.
58. *Radiation sterilization of tissue allografts:requirements for validation and routine control: a code of practice,* International Atomic Energy Agency, Vienna **2007**
59. S. Endres, M. Kratz, *J Musculoskelet Neuronal Interact*, **2009,** *9*.
60. M. Silindir, A. Y. Ozer, *FABAD J Pharm Sci*, **2009,** *34,* 43.
61. B. E. Butterworth, J. R. Chapman, *Regul Toxicol Pharmacol*, **2007,** *49,* 149.
62. D. W. Jackson, G. E. Windler, T. M. Simon, *Am J Sports Med*, **1990,** *18,* 1.
63. A. White, D. Burns, T. W. Christensen, *J Biotechnol*, **2006,** *123,* 504.
64. E. Shieh, A. Paszczynski, C. M. Wai, Q. Lang, R. L. Crawford, *J Microbiol Methods*, **2009,** *76,* 247.
65. W. A. Rutala, D. J. Weber, *Am J Infect Control*, **2013,** *41,* S2.
66. A. Simmons, *Future trends for the sterilisation of biomaterials and medical devices*, in *Sterilization of biomaterials and medical devices*, S. Lerouge and A. Simmone, Editors. 2012, Woodhead Publishing Limited,. p. 310-319.
67. M. Perrut, *J Supercrit Fluids*, **2012,** *66,* 359.
68. M. S. a. H. P. P. Wutzler, *J Clin Microbiol*, **1975,** *1,* 246.
69. C. S. Frauke von Versen-H¨oynck, S. Bachmann and D.E. M¨oller, *Cell and Tissue Banking*, **2004 a,** *5*.
70. P. D. Kemp, *Peracetic acid sterilization of collagen or collagenous tissue* U.S. Patent, Editor. 1995, Organogenesis Inc., Canton, Mass: USA.
71. A. Pruss, C. Perka, P. Degenhardt, U. Maronna, K. Buttner-Janz, B. Paul, K. Muller, C. Klumpp, J. C. Bruck, R. Von Versen, *Cell Tissue Bank*, **2002,** *3,* 235.
72. W. A. Rutala, D. J. Weber, *Am J Infect Control*, **2013,** *41*.
73. S. Killeen, M. McCourt, *Surgery*, **2012,** *30,* 687.
74. W. A. Rutala, D. J. Weber, *J Hosp Infect*, **1999,** *43,* S43.
75. K. Shimizu, H. Yano, E. Nakamura, N. Kaku, *Ann Transplant*, **2001,** *6,* 26.
76. M. N. Bathina, S. Mickelsen, C. Brooks, J. Jaramillo, T. Hepton, F. M. Kusumoto, *J Am Coll Cardiol*, **1998,** *32,* 1384.
77. S. D. Ferreira, W. S. Dernell, B. E. Powers, R. A. Schochet, C. A. Kuntz, S. J. Withrow, R. M. Wilkins, *Clin Orthop Relat Res*, **2001,** *388,* 233.
78. Y. Ikarashi, T. Tsuchiya, A. Nakamura, *Biomaterials*, **1995,** *16,* 177.
79. G. Fridman, G. Friedman, A. Gutsol, A. B. Shekhter, V. N. Vasilets, A. Fridman, *Plasma Processes and Polymers*, **2008,** *5*.
80. J. Heinlin, G. Morfill, M. Landthaler, W. Stolz, G. Isbary, J. L. Zimmermann, T. Shimizu, S. Karrer, *J Dtsch Dermatol Ges*, **2010,** *8,* 968.
81. M. Moreau, N. Orange, M. G. Feuilloley, *Biotechnol Adv*, **2008,** *26,* 610.
82. M. G. Kong, G. Kroesen, G. Morfill, T. Nosenko, T. Shimizu, J. van Dijk, J. L. Zimmermann, *New J Phys*, **2009,** *11,* 115012.
83. C. Cheng, L. Peng, X. Lei, Z. Li-Ye, Z. Ru-J., *Chin Phys*, **2006,** *15,* 1544.
84. O. Schlüter, J. Ehlbeck, C. Hertel, M. Habermeyer, A. Roth, K. H. Engel, T. Holzhauser, D. Knorr, G. Eisenbrand, *Mol Nutr Food Res*, **2013,** *57,* 920.
85. B. Haertel, T. von Woedtke, K.-D. Weltmann, U. Lindequist, *Biomol Ther*, **2014,** *22,* 477.

86. R. Morent, N. De Geyter, *Inactivation of Bacteria by Non Thermal Plasmas*, in *Chapter 2, Biomedical Engineering – Frontiers and Challenges*, R. Fazel, Editor. 2011, In Tech. p. 25-54.
87. L. Bárdos, H. Baránková, *Vacuum*, **2008,** *83,* 522.
88. K. P. Arjunan, V. K. Sharma, S. Ptasinska, *Int J Mol Sci*, **2015,** *16,* 2971.
89. M. Y. Alkawareek, S. P. Gorman, W. G. Graham, B. F. Gilmore, *Int J Antimicrob Agents*, **2014,** *43,* 154.
90. S. A. Ermolaeva, A. F. Varfolomeev, M. Y. Chernukha, D. S. Yurov, M. M. Vasiliev, A. A. Kaminskaya, M. M. Moisenovich, J. M. Romanova, A. N. Murashev, Selezneva, II, T. Shimizu, E. V. Sysolyatina, I. A. Shaginyan, O. F. Petrov, E. I. Mayevsky, V. E. Fortov, G. E. Morfill, B. S. Naroditsky, A. L. Gintsburg, *J Med Microbiol*, **2011,** *60,* 75.
91. G. Fridman, G. Friedman, A. Gutsol, A. B. Shekhter, V. N. Vasilets, A. Fridman, *Plasma Process Polym*, **2008,** *5,* 503.
92. Xu Han, William A. Cantrell, Erika E. Escobara, S. Ptasinskab, *Eur Phys J D*, **2014,** *68,* 46.
93. C. Sadiqali, M. Černák, M. Pavel, H. Josef, *J Appl Biomed*, **2010,** *8,* 55.
94. S. Emmert, F. Brehmer, H. Hänßle, A. Helmke, N. Mertens, R. Ahmed, D. Simon, D. Wandke, W. Maus-Friedrichs, G. Däschlein, M. P. Schön, W. Viöl, *Clinical Plasma Medicine*, **2013,** *1*.
95. G. Daeschlein, S. Scholz, R. Ahmed, T. von Woedtke, H. Haase, M. Niggemeier, E. Kindel, R. Brandenburg, K. D. Weltmann, M. Juenger, *J Hosp Infect*, **2012,** *81,* 177.
96. A. S. Wu, S. Kalghatgi, D. Dobrynin, R. Sensenig, E. Cerchar, E. Podolsky, E. Dulaimi, M. Paff, K. Wasko, K. P. Arjunan, K. Garcia, G. Fridman, M. Balasubramanian, R. Ownbey, K. A. Barbee, A. Fridman, G. Friedman, S. G. Joshi, A. D. Brooks, *J Surg Res*, **2013,** *179,* e1.
97. B. Yang, J. Chen, Q. Yu, H. Li, M. Lin, A. Mustapha, L. Hong, Y. Wang, *J Dent*, **2011,** *39,* 48.
98. X. Zhou, Z. Xiong, Y. Cao, X. Lu, D. Liu, *IEEE Transactions on Plasma Science*, **2010,** *38,* 3370.
99. N. O'Connor, O. Cahill, S. Daniels, S. Galvin, H. Humphreys, *J Hosp Infect*, **2014,** *88,* 59.
100. M. R. Boscariol, A. J. Moreira, R. D. Mansano, I. S. Kikuchi, T. J. Pinto, *Int J Pharm*, **2008,** *353,* 170.
101. E. Curran, R. Duffy, D. Peretz, S. Park, B. Seiber, R. Smalley, A. Raghavan, M. Gurjar, D. Dobrynin, K. Wasko, A. Fridman, G. Fridman, D. Paog, *Plasma Med*, **2013,** *3,* 153.
102. G. Avramidis, B. Stüwe, R. Wascher, M. Bellmann, S. Wieneke, A. von Tiedemann, W. Viöl, *Surf Coat Technol*, **2010,** *205,* S405.
103. S. Ptasinska, B. Bahnev, A. Stypczynska, M. Bowden, N. J. Mason, N. S. Braithwaite, *Phys Chem Chem Phys*, **2010,** *12,* 7779.
104. E. Stoffels, Y. Sakiyama, D. B. Graves, *Plasma Science, IEEE Transactions*, **2008,** *36,* 1441.
105. Mendis D.A., Rosenberg M., A. F., *IEEE Trans Plas Sci*, **2000,** *28,* 1304.
106. J. L. Zimmermann, T. Shimizu, H. U. Schmidt, Y. F. Li, G. E. Morfill, G. Isbary, *New J Phys*, **2012,** *14,* 073037.
107. M. Laroussi, F. Leipold, *Int J Mass Spectrom*, **2004,** *233,* 81.
108. M. Laroussi, *Plasma Processes and Polymers*, **2005,** *2*.
109. M. L. Burts, I. Alexeff, E. T. Meek, J. A. McCullers, *Am J Infect Control*, **2009,** *37,* 729.

110. E. Kvam, B. Davis, F. Mondello, A. L. Garner, *Antimicrob Agents Chemother*, **2012,** *56,* 2028.
111. T. Maisch, T. Shimizu, Y. F. Li, J. Heinlin, S. Karrer, G. Morfill, J. L. Zimmermann, *PLoS One*, **2012,** *7,* e34610.
112. T. Maisch, T. Shimizu, A. Mitra, J. Heinlin, S. Karrer, Y. F. Li, G. Morfill, J. L. Zimmermann, *J Ind Microbiol Biotechnol*, **2012,** *39,* 1367.
113. C. M. Edelblute, M. A. Malik, L. C. Heller, *Bioelectrochemistry*, **2015,** *103,* 22.
114. M. Korachi, C. Gurol, N. Aslan, *J Electrostat*, **2010,** *68,* 508.
115. A. Fernandez, N. Shearer, D. R. Wilson, A. Thompson, *Int J Food Microbiol*, **2012,** *152,* 175.
116. J. J. Cotter, P. Maguire, F. Soberon, S. Daniels, J. P. O'Gara, E. Casey, *J Hosp Infect*, **2011,** *78,* 204.
117. C. Wei, H. Jun, D. Ning, L. Xiao-Di, L. Guo-Hua, W. Xing-Quan, Z. Guo-Ping, G. Li-Hong, Y. Si-Ze, *Chin Phys Lett*, **2012,** *29,* 075203.
118. R Matthes, C Bender, R Schluter, I Koban, R Bussiahn, S Reuter, J Lademann, K D Weltmann, A Kramer, *PlosONE*, **2013,** *8,* e70462.
119. O. Lademann, A. Kramer, H. Richter, A. Patzelt, M. Meinke, J. Roewert-Huber, V. Czaika, K. Weltmann, B. Hartmann, S. Koch, *Laser Phys Lett*, **2011,** *8,* 313.
120. O. Lademann, A. Kramer, H. Richter, A. Patzelt, M. Meinke, V. Czaika, K.-D. Weltmann, B. Hartmann, S. Koch, *Skin Pharmacol Physiol*, **2011,** *24,* 284.
121. A. Ali, Y. H. Kim, J. Y. Lee, S. Lee, H. S. Uhm, G. Cho, B. J. Park, E. H. Choi, *Curr Appl Phys*, **2014,** *14,* S142.
122. Y. F. Hong, J. G. Kang, H. Y. Lee, H. S. Uhm, E. Moon, Y. H. Park, *Lett Appl Microbiol*, **2009,** *48,* 33.
123. S. Shimizu, S. Barczyk, P. Rettberg, T. Shimizu, T. Klaempfl, J. L. Zimmermann, T. Hoeschen, C. Linsmeier, P. Weber, G. E. Morfill, H. M. Thomas, *Plant Space Sci*, **2014,** *90,* 60.
124. J. Jeon, T. Klaempfl, J. Zimmermann, G. Morfill, T. Shimizu, *New J Phys*, **2014,** *16,* 103007.
125. S. Patil, T. Moiseev, N. N. Misra, P. J. Cullen, J. P. Mosnier, K. M. Keener, P. Bourke, *J Hosp Infect*, **2014,** *88,* 162.
126. F. Rossi, O. Kylián, *Sterilization and decontamination of surfaces by plasma discharges*, in *Sterilisation of biomaterials and medical devices*, S. Lerouge and A. Simmons, Editors. 2012, Woodhead Publishing Series in Biomaterials: Philadelphia, USA. p. 117-150.
127. H. Shintani, *Biocontrol Sci*, **2016,** *21*.
128. B. J. Park, K. Takatori, M. H. Lee, D.-W. Han, Y. I. Woo, H. J. Son, J. K. Kim, K.-H. Chung, S. O. Hyun, J.-C. Park, *Surf Coat Technol*, **2007,** *201,* 5738.
129. D. L. Bayliss, J. L. Walsh, G. Shama, F. Iza, M. G. Kong, *New J Phys*, **2009,** *11,* 115024.
130. J. Julák, O. Janoušková, V. Scholtz, K. Holada, *Plasma Process Polym*, **2011,** *8,* 316.
131. Holmberg A, Lood R, Mo¨rgelin M, So¨derquist B, Holst E, Collin M, Christensson B, Rasmussen M, *Clinical Microbiology and Infection*, **2009,** *15*.
132. T. Maisch, T. Shimizu, G. Isbary, J. Heinlin, S. Karrer, T. G. Klampfl, Y. F. Li, G. Morfill, J. L. Zimmermann, *Appl Environ Microbiol*, **2012,** *78,* 4242.
133. K. Fricke, I. Koban, H. Tresp, L. Jablonowski, K. Schroder, A. Kramer, K. D. Weltmann, T. von Woedtke, T. Kocher, *PLoS One*, **2012,** *7,* e42539.
134. H. Yasuda, T. Miura, H. Kurita, K. Takashima, A. Mizuno, *Plasma Process Polym*, **2010,** *7,* 301.

135. A. Mai-Prochnow, A. B. Murphy, K. M. McLean, M. G. Kong, K. K. Ostrikov, *Int J Antimicrob Agents*, **2014,** *43,* 508.
136. S. Bekeschus, A. Schmidt, K.-D. Weltmann, T. von Woedtke, *Clin Plas Med*, **2016,** *4,* 19.
137. F. Brehmer, H. A. Haenssle, G. Daeschlein, R. Ahmed, S. Pfeiffer, A. Gorlitz, D. Simon, M. P. Schon, D. Wandke, S. Emmert, *J Eur Acad Dermatol Venereol*, **2015,** *29*.
138. E. Stoffels, I. E. Kieft, R. E. J. Sladek, *J Phys D Appl Phys*, **2003,** *36,* 2908.
139. T Jiang, Huina Zhao, Chao Wang, Guozhou Rao, Ang Li, Harm Tolner, B Li, *Plasma Med*, **2012,** *2,* 179.
140. E. Stoffels, A. J. Flikweert, W. W. Stoffels, G. M. W. Kroesen, *Plasma Sources Sci Technol*, **2002,** *11,* 383.
141. C. Welz, S. Becker, Y.-F. Li, T. Shimizu, J. Jeon, S. Schwenk-Zieger, H. M. Thomas, G. Isbary, G. E. Morfill, U. Harréus, *J Phys D Appl Phys*, **2013,** *46,* 045401.
142. X. Pei, X. Lu, J. Liu, D. Liu, Y. Yang, K. Ostrikov, K. C. Paul, Y. Pan, *J Phys D Appl Phys*, **2012,** *45,* 165205.
143. D. Dobrynin, G. Fridman, G. Friedman, A. Fridman, *New J Phys*, **2009,** *11,* 115020.
144. Ashkan Heidarkhan Tehrani, Pooya Davari, Sanjleena Singh, Adekunle Oloyede, *J Mater Sci Mater Med*, **2014,** *25,* 953.
145. E. J. Szili, J. W. Bradley, R. D. Short, *Journal of Physics D: Applied Physics*, **2014,** *47*.
146. D. Dobrynin, G. Fridman, G. Friedman, A. A. Fridman, *Plasma Med* **2012,** *2,* 71.
147. C. X. Wang, Y. Liu, H. L. Xu, Y. Ren, Y. P. Qiu, *Appl Surf Sci*, **2008,** *254,* 2499.
148. C. X. Wang, Y. P. Qiu, *Surf Coat Technol*, **2007,** *201,* 6273.
149. K. Heuer, M. A. Hoffmanns, E. Demir, S. Baldus, C. M. Volkmar, M. Rohle, P. C. Fuchs, P. Awakowicz, C. V. Suschek, C. Oplander, *Nitric Oxide*, **2015,** *44*.
150. F. von Versen-Hoeynck, A. P. Steinfeld, J. Becker, M. Hermel, W. Rath, U. Hesselbarth, *Biologicals*, **2008,** *36,* 248.
151. E. Noriega, G. Shama, A. Laca, M. Díaz, M. G. Kong, *Food Microbiol*, **2011,** *28*.
152. S. S. Ab Hamid, N. K. Zahari, N. Yusof, A. Hassan, *Cell Tissue Bank*, **2014,** *15,* 15.
153. Nor Kamilia Z, Suzina SAH, Y. N, *Regenerative Research*, **2014,** *3,* 64.
154. R. Singh, M. P. Chacharkar, *J Tissue Viability*, **2011,** *20,* 49.
155. S. Kitazaki, Akimasa Tanaka, N. Hayashi, *Vacuum*, **2014,** *110,* 217.
156. M. Annunziata, L. Canullo, G. Donnarumma, P. Caputo, L. Nastri, L. Guida, *Med Oral Patol Oral Cir Bucal*, **2016,** *21,* e118.
157. E. Simoncelli, D. Barbieri, R. Laurita, A. Liguori, A. Stancampiano, L. Viola, R. Tonini, M. Gherardi, V. Colombo, *Clin Plasma Med*, **2015,** *3,* 77.
158. C.-W. Kan, C.-F. Lam, C.-K. Chan, S.-P. Ng, *Carbohydr Polym*, **2014,** *102,* 167.
159. L. Bárdos, H. Baránková, *Thin Solid Films*, **2010,** *518,* 6705.
160. C. Wang, Y. Qiu, *Surf Coat Technol*, **2007,** *201,* 6273.
161. S. Lerouge, M. R. Wertheimer, L. Yahia, *Plasmas Polym*, **2001,** *6*.
162. E. Stoffels, I. E. Kieft, R. E. J. Sladek, L. J. M. v. d. Bedem, E. P. v. d. Laan, M. Steinbu, *Plasma Sources Sci Technol*, **2006,** *15,* S169.
163. N. De Geyter, R. Morent, *Annu Rev Biomed Eng*, **2012,** *14,* 255.
164. S. Pankaj, N. Misra, P. Cullen, *Innov Food Sci Emerg Technol*, **2013,** *19,* 153.
165. H. Eto, Y. Ono, A. Ogino, M. Nagatsu, *Appl Phys Lett*, **2008,** *93,* 221502.
166. T. Lee, P. Puligundla, C. Mok, *Food Control*, **2015,** *51,* 149.
167. H. I. Yong, H.-J. Kim, S. Park, A. U. Alahakoon, K. Kim, W. Choe, C. Jo, *Food Microbiol*, **2015,** *46,* 46.

168. O. V. Penkov, M. Khadem, W.-S. Lim, D.-E. Kim, *J Coat Technol Res*, **2015,** *12,* 225.
169. C. Wiegand, M. Abel, P. Ruth, T. Wilhelms, D. Schulze, J. Norgauer, U.-C. Hipler, *J Biomed Mater Res B Appl Biomater*, **2009,** *90,* 710.
170. E. Y. Elenes, S. A. Hunter, *J Bone Joint Surg Am*, **2014,** *96,* 1321.
171. C. M. Deeley, *Rad Phys Chem*, **2004,** *71,* 505.
172. D. Cheung, N. Perelman, D. Tong, M. Nimni, *J Biomed Mater Res*, **1990,** *24,* 581.
173. S. Baloda, J. Martin, J. Carter, E. Jenness, B. Judd, K. Smeltz, I. Uettwiller, M. Hockstad, *The irradiation and sterilization subcommittee of the bioprocess systems allience*, **2008,** *2,* 10.
174. A. Hoburg, S. Keshlaf, T. Schmidt, M. Smith, U. Gohs, C. Perka, A. Pruss, S. Scheffler, *Cell Tissue Bank*, **2014,** *16,* 219.
175. M. Smith, R. Galloway, *radiat Phys Chem*, **2004,** *71,* 531.
176. G. da Cunha Mendez, T. da Silva Brandao, C. Miranda Silva, *Expert Rev Med Devices*, **2008,** *5,* 323.
177. G. C. Mendes, T. R. Brandao, C. L. Silva, *Am J Infect Control*, **2007,** *35,* 574.
178. T. M. Moore, E. Gendler, *J Orthop Res*, **2004,** *22,* 1358.
179. A. Nather, J. L. L. Chew, Z. Aziz, *Types of terminal sterilization of tissue grafts*, in *Radiation in tissue banking - Basic Science and Clinical Applications of Irradiated Tissue Allografts*, A. Nather, N. Yusof, and N. Hilmy, Editors. 2007, © World Scientific Publishing Co. Pte. Ltd.: Singapore.
180. ISO-10993-7, *Biological evaluation of medical devices – Part 7: Ethylene oxide sterilization residuals.*, in *The International Organization for Standardization*. 2008: Geneva, Swetzerland.
181. T. S. Roberts, D. Drez, Jr., W. McCarthy, R. Paine, *Am J Sports Med*, **1991,** *19,* 35.
182. *Ethylene Oxide (EtO): Evidence of Carcinogenicity,* U.S. Department of Health and Human Services, National Institute for Occupational Safety and Health, USA **1981**
183. E. W. Kyle Steenland, James Deddens, Leslie Stayner & Elizabeth Ward, *Cancer Causes Control*, **2003,** *14,* 531.
184. V. M. Steelman, *AORN J*, **1992,** *55,* 773.
185. *Preventing worker injuries and deaths from explosions in industrial ethylene oxide sterilization facilities,* Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health,, Atlanta, GA **2007**
186. C. T. Vangsness, Jr., P. P. Wagner, T. M. Moore, M. R. Roberts, *Arthroscopy*, **2006,** *22,* 1351.
187. Y. C. Chien, H. H. Liu, Y. C. Lin, P. C. Su, L. H. Li, C. P. Chang, D. T. Tang, C. Y. Chen, *J Biomed Mater Res B Appl Biomater*, **2007,** *83,* 527.
188. Bong Joo Park, Kosuke Takatori, Mi Hee Lee, Dong-Wook Han, Yeon I. Woo, Hyun Joo Son, Jeong Koo Kim, Kie-Hyung Chung, Soon O. Hyun, J.-C. Park, *Surf Coat Technol*, **2007,** *201,* 5738.
189. B. A. Niemira, *J Food Sci*, **2012,** *77,* M171.
190. A. Blumhagen, P. Singh, A. Mustapha, M. Chen, Y. Wang, Q. Yu, *Am J Dent*, **2014,** *27,* 84.
191. P. Sun, Yi Sun, Haiyan Wu, Weidong Zhu, Jose L. Lopez, Wei Liu, Jue Zhang, Ruoyu Li, J. Fang, *Appl Phys Lett*, **2011,** *98*.
192. B. Dasan, M. Mutlu, I. Boyaci, *Int J food Microbiol*, **2016,** *216,* 50.
193. M. Selcuk, L. Oksuz, P. Basaran, *Bioresour Technol*, **2008,** *99,* 5104.
194. N. S. Panikov, S. Paduraru, R. Crowe, P. J. Ricatto, C. Christodoulatos, K. Becker, *IEEE Transactions Plasma Science*, **2002,** *30,* 1424.

195. H. Shintani, N. Shimizu, Y. Imanishi, T. Sekiya, K. Tamazawa, A. Taniguchi, N. Kido, *Biocontrol Sci*, **2007,** *12,* 131.
196. R. A. Venezia, M. Orrico, E. Houston, S. M. Yin, Y. Y. Naumova, *Infect Contol Hosp Epidemiol*, **2008,** *29,* 430.
197. N. Alshraiedeh, M. Alkawareek, S. Gorman, W. Graham, B. Gilmore, *J Appl Microbiol*, **2013,** *115,* 1420.
198. O. Terrier, B. Essere, M. Yver, M. Barthélémy, M. Bouscambert-Duchamp, P. Kurtz, D. VanMechelen, F. Morfin, G. Billaud, O. Ferraris, *J Clin Virol*, **2009,** *45,* 119.
199. D. Ziuzina, L. Han, P. J. Cullen, P. Bourke, *Int J Food Microbiol*, **2015,** *210,* 53.
200. M. Moisan, P. Levif, J. Séguin, J. Barbeau, *J Phys D Appl Phys*, **2014,** *47,* 285404.
201. Q. Y. Nie, Z. Cao, C. S. Ren, D. Z. Wang, M. G. Kong, *New J Phys*, **2009,** *11,* 115015.
202. A. N. Aleynik, A. N. Baykov, M. B. Baskakov, G. T. Dambaev, O. I. Deneko, E. P. Krasnozhenov, E. V. Semichev, O. S. Zhdanova. *Application of non-equilibrium atmospheric pressure plasma for coagulation and sterilization*. in *Strategic Technology (IFOST), 2012 7th International Forum*. 2012. Tomsk Polytechnic University, Russia

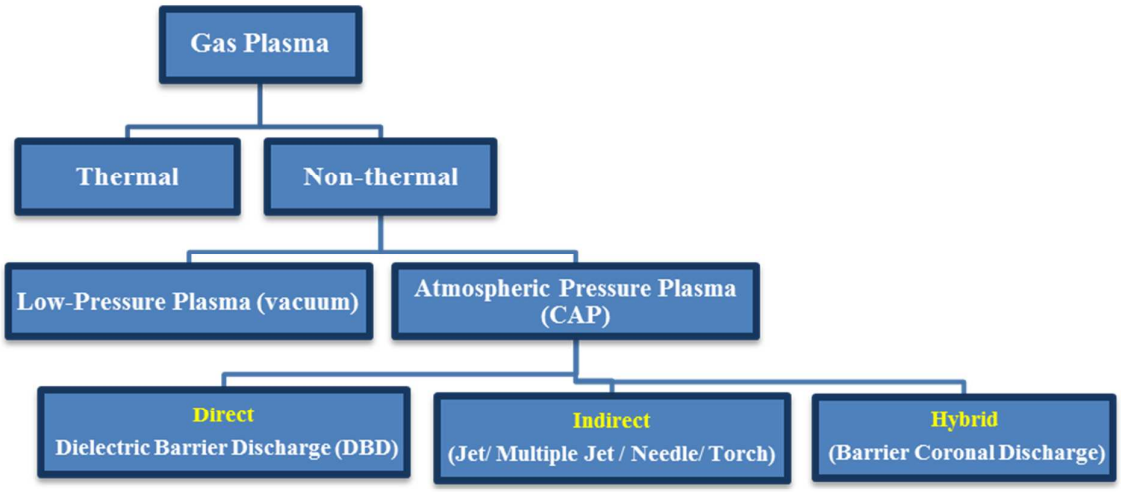

Figure 1. Schematic diagram showing non-thermal, cold atmospheric pressure plasma system types

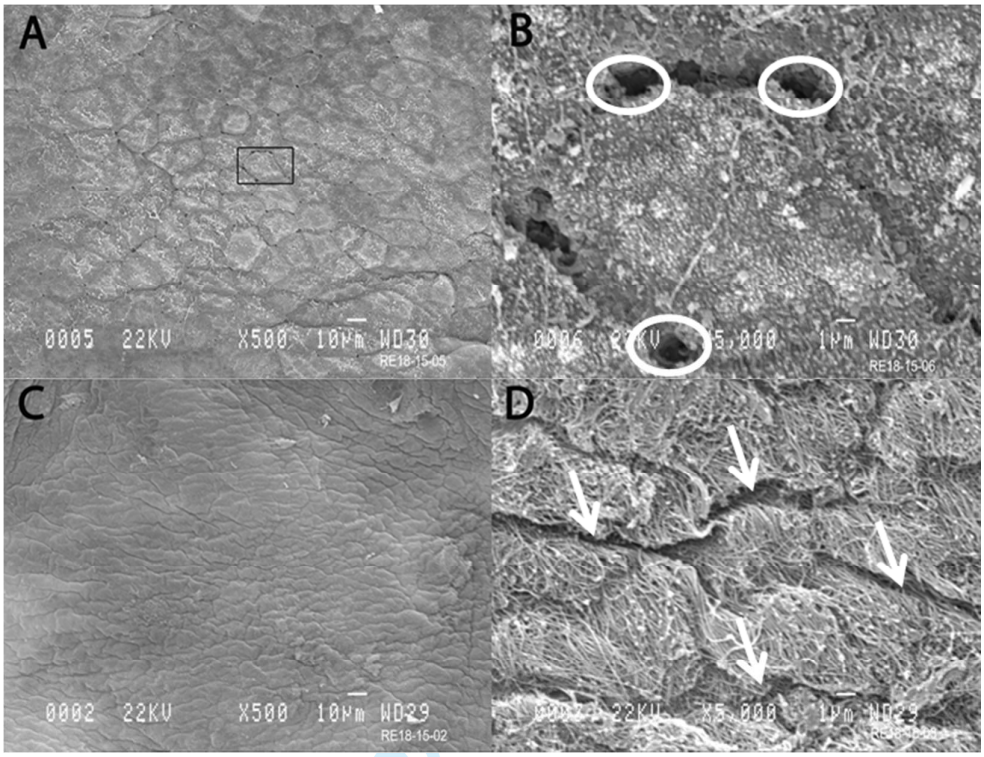

Figure 2: Scanning electron microscopy image of dried AM (A,B – epithelial side; C,D – stromal side). (A) Intact polygonal epithelial surface cells with occasional intracellular spaces (scale bar: 10 µm). (B) Box in (A) magnified to show distinct natural intracellular microholes (white circles) around the cell (scale bar: 1 µm). (C) Smooth regular stromal surface of AM (scale bar: 10 µm). (D) Collagen fibrils on the stromal side with tiny microgrooves (white arrows), scale bar: 1 µm)

Table 1. Physical and chemical terminal sterilization techniques and their advantages and disadvantages.

Technique	Advantages	Disadvantages
Physical:		
Pressurized steam (autoclaving)	Inexpensive simple, safe, rapid and efficient, leaves no toxic residues.[74] Easy to monitor. [40] Can sterilize liquids.[40] More effective than dry heat against prions.[40] Used in both industry and clinical settings.	Incompatible with tissues and polymeric biomaterials due to high temperature and moisture that disrupts biochemical structures and causes coagulation of proteins.[40, 169] Materials require special permeable packages to ensure steam reaches all surfaces.[40]
Ionizing radiation:		
Gamma (γ) Radiation. from ^{60}Co	Effective against spore forming bacteria. Radiation doses ≤ 25kGy do not alter the mechanical performance of soft tissues.[170] Suitable for heat and moisture sensitive materials. Higher penetration (50-80 cm).[60, 171] Dose rate approximately 10 kGy/h. Immediate release after processing. No need for permeable packaging. Leaves no chemical residues or radioactive substances.[169, 172, 173] Cost-effective compared to EtO.[171]	Predominantly only available in industrial and research centers.[40] High capital cost for installation and maintenance. Expensive ^{60}Co source replenishment.[171] Unsuitable for small tissue batch producers. Requires high doses to deactivate viruses (89 kGy). Higher doses than 35 kGy cause tissue damage.[53] Causes crosslinking and/or peptide chain breakage.[53, 169] Low dose rate (slow dose delivery).[60] Not appropriate for viral or prion infected tissue.[47] High doses required to eliminate endotoxins.[38]
Electron (e-beam) Radiation	Can sterilize heat and moisture sensitive materials, and generally more compatible than γ.[40] Doses up to 34 kGy are less harmful on soft tissues.[174] High dose rate (quick sterilization dose delivery within minutes).[60] No need for permeable packaging. Immediate release after processing.	High equipment and operational cost.[175] Higher doses than 35 kGy damaging to tissues. High electric consumption.[47] Not appropriate virally infected human donated tissue grafts.[47] Low penetration depth (5-8 cm).[60, 171] Not recommended for high density

	No chemical residues or radioactive substances.[169, 172, 173] Cost effective compared to EtO.[171]	materials. Acceptable sterilization doses are not effective against prions. Ineffective against endotoxins.
Chemical:		
Ethylene Oxide Gas (EtO)	Reliable and effective sterilization.[176] Efficient bactericidal, sporicidal and virucidal activity.[177] Rapidly deactivates hepatitis B and HIV-1 viruses.[178] Causes less damage to extracellular matrix than γ, e-beam and glutaraldehyde methods.[53] Suitable for bulk sterilization.	Only available and suitable for large-scale usage. High costs for small scale use. Lengthy sterilization process[169, 179]. Requires special gas-permeable packaging.[48] Recognized as a toxic waste by the Environmental Protection Agency (EPA).[61, 169, 179-181] Potentially carcinogenic.[61, 182, 183] Process monitoring is more complex than radiation, needs to control several parameters.[40, 184] Flammable and explosive.[185] Leaves toxic residues on sterilized materials.[169] Causes potential damage to tissues.[61, 62, 184, 186] Not recommended for sterilization of tissue allografts.[181, 186] Danger to facility workers.[187] Ineffective against prions.[48]

Table 2. Examples of susceptibility of vegetative bacteria, fungi, bacterial spores, endotoxins, prions and biofilm to CAP treatment.

Microorganism	Importance	Exposure Substrate	Log reduction or % sterilization	Exposure time (s)	Ref.
Vegetative Bacteria (*gram-negative*)					
Escherichia coli	Clinical pathogen	Agar medium plates	5 log	600	[123]
Escherichia coli	Clinical pathogen	Glass slides	100 %	1	[188]
Escherichia coli KCTC[a] 1039	Clinical pathogen	Thin glass covers	10 log 100 %	7 60	[122]
Escherichia coli O157:H7 (C9490) *Escherichia coli* O157:H7 (ATCC[b] 35150) *Escherichia coli* O157:H7 (ATCC 43894) *Salmonella anatum* F4317 *Salmonella stanley* H0558 *Salmonella enteritidis* PT30	Foodborne pathogens	Dry almonds	1.34 log	20	[189]
Vegetative Bacteria (*gram-positive*)					
Propionibacterium acnes KCTC 3314	Skin flora	Coated glass slides	7 log	600	[121]
Deinococcus radiodurans	Radiation, drying and oxidizing agent resistant	Aluminium discs	3 log	1,800	[123]
Lactobacillus acidophilus *Streptococcus mutans*	Oral caries producing bacteria	Hydroxya-patite	1.5 - 2 log	6	[190]
Listeria innocua (ATCC 33090)	Non-pathogenic surrogate for	Chicken meat and skin	3.3 log	480	[151]

	the foodborne pathogen *monocytogenes*				
Fungi					
Candida albicans (thickness of 10 - 20 mm)	Pathogenic fungus	Polystyrene wafers	9 5%	300	[133]
Candida albicans *Candida krusei* *Candida glabrata*	Antifungal resistant (fluconazole-resistance)	Air Water	> 90 % > 90 %	600 60	[191]
Aspergillus flavus (ATCC 327) *Aspergillus parasiticus* (ATCC 1041)	Mycotoxin producing food contaminants	Hazelnuts	4.50 log 4.19 log	300	[192]
Aspergillus paraciticus 798 TUBITAK-MAM *Penicillum* MS1982	Bio-indicator fungus	Vegetable and legumes seed surfaces	3 log	900	[193]
Spore-forming bacteria					
Bacillus subtilis	Sterilization resistant	Thin glass covers	10 log 100 %	24 120	[122]
Bacillus safensis, DSM[c] 19292T *Bacillus megaterium*, ATCC 14581T *Bacillus megaterium* DSM 30587 (2c1)	Sterilization resistant	Aluminium discs	> 6 log 100%	3,600 5,400	[123]
Bacillus atrophaeus DSM675 *Bacillus thuringiensis* DSM 30879 (E24)	Sterilization resistant	Aluminium discs	> 3 log	5,400	[123]
Bacillus subtilis	Sterilization resistant	Glass slide & aluminium substrates	4 log (He plasma) 8 log (air plasma)	< 600	[194]
Bacillus atrophaeus	Biological indicator for thermal sterilization	Commercial spore strip	≥ 6 log10	60	[125]

	(Heat resistant)				
Endotoxins					
LPS from *Escherichia Coli* ATCC 8739	Pyrogen	Glass slides	100 %	10	[188]
Viruses					
Adenovirus, non-enveloped double stranded DNA virus	Human infectious disease causing	PBS Solution	6 log	240	[13]
Bacteriophage lambda (λ phage)	Virus that infects bacteria	Buffer	6 log	20	[134]
Bacteriophage lambda (λ phage) C-17 (ATCC 23724-B1) Lytic bacteriophage (Rambo; Microphage)	Virus that infects bacteria	NA[d]	4–6 log	600	[196]
MS2 bacteriophage ATTC 15597-B1	Surrogate of human enteric viruses such as norovirus that causes infectious gastroenteritis	Phage suspension	4.98 -7.06 log	450	[197]
Influenza virus type A (H5N2) Human parainfluenza virus type 3 strain C-243 ATCC VR-93 Respiratory syncytial virus-A ATCC VR-26	Airborne respiratory pathogen	Viral aerosol suspension	4 log (99.20 %) 6.5 log (99.22 %) 3.8 log (99.00 %)	180	[198]
Prions					
Scrapie (Rocky Mountain Laboratory strain, RML5)	Surrogate of human prion	1% mouse brain in PBS solution	100 %	600-1,200	[130]
Biofilm					
Pseudomonas aeruginosa SG81,	Clinical pathogen	Polycarbonate discs.	4.83 log 7.11 log	300 600	[118]
Staphylococchus	Skin flora	Polycarbonate	2.77 log	300	[118]

epidermidis RP62A		discs	3.38 log	600	
Salmonella enterica serovar typhimurium *Listeria monocytogenes* *Escherichia coli* *Enterococcus faecalis*	Clinical pathogens	Lettuce	5 log	300	[199]
Candida albicans	Clinical pathogen	Inanimate surface	6 log	240	[132]

[a)]KCTC= Korean Collection for Type Cultures, Korea; [b)]ATCC= American Type Culture Collection, USA; [c)]DSM= Leibnitz Institute DSMZ-German Collection of Microorganism and Cell Cultures, Germany; [d)]NA= not available

Table 3. Advantages and disadvantages of CAP sterilization of TBTM

Advantages	Disadvantages
Biocompatible with viable and non-viable tissues.	Methods not yet validated.
No toxic chemical residue left on treated object.[136]	Low penetration power so may be not suitable for thick TBTM materials.[142]
Shorter exposure time than established sterilization methods (seconds to minutes).	Difficult to apply to packaged materials.
No toxic effects to operators and the environment.[200]	Not validated as terminal sterilization that offers SAL of 10^{-6}.
Can remove bacterial cells and viruses from sterilized surfaces.	Many parameters (e.g. loaded gas, gas pressure, flow rate, voltage, exposure time and sample distance) need to be controlled to get the desired capacity of charged species.
Effective on endotoxins, prions and bacterial and fungal biofilms.[132]	
Delivers uniform treatment to uneven surfaces of biological materials.[201]	
CAP gas capable of reaching confined and narrow spaces.[82]	
Microorganism resistance to CAP is unlikely to occur.[106]	
Inexpensive.[202]	
Can be used portably or in smaller scale (such as in laminar flow hoods and isolators).[202]	

Table of Contents Entry

This review discusses the challenges surrounding terminal sterilization of tissues and biological transplant materials. The limitations of conventional terminal sterilization techniques of biological materials are identified, particularly the damage caused to tissue structure, and the benefits of using non-thermal (cold) atmospheric pressure plasma in terminal sterilization of soft thin tissues and biomaterial constructs discussed.

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Terminal sterilization: Conventional methods versus emerging cold atmospheric pressure plasma technology for non-viable biological tissues

Table of Contents Figure (50 mm x 55 mm)