Biomaterials Journal
http://www.biomatj.com
Online ISSN: 2812-5045

Type of the Paper (Research Article)

Efficacy of nanoparticles in plant disease control and their phytotoxicity

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Citation: ***Yashwant Sompura , Vanshika Sharma , Chayadevi H , Maruthi G R , Jeenat Banu , Tansukh Barupal , and Shyam Sunder Meena . Efficacy of nanoparticles in plant disease control and their phytotoxicity .*** *Biomat. J., 2 (2),1 – 15 (2023).*

https://doi.org/10.5281/znodo.5829408

Received: 15 February 2023
Accepted: 25 February 2023
Published: 28 February 2023

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Abstract: Every year approximately 20 to 40 percent crop yield lost due to disease which is caused by pathogenic fungi, bacteria, virus etc. the plant disease management totally dependent on chemical pesticides, herbicides which are harmful for human life and may be cause severe diseases. Nanotechnology play a significant role in agriculture, especially in disease resistance crop production, enhancement of quality of crop, high yield production, resistance from biotic as well as abiotic stresses. In this review, we have not included all the plant diseases but we have tried to include all the newest information related to role of nanotechnology in disease resistance in plants.

Keywords: : *Nano-sensors, Nano-emulsion, Carbon-nanotubes, Nanocomposites, Biosensor.*

In 1974, 'Nanotechnology' term was coined by Taniguchi. The science that deals with particles of nano size (10^{-9}). When we reduce material at nano scale, their physical and chemical properties are also change. Nanoparticles exhibit smaller size than bacterial cell. It may be less than size of influenza virus or tobacco mosaic virus. In future, Nanomaterials will reduce the use of chemical pesticides, herbicides etc. Today, loss of crop yield due to diseases is major concern of agriculture scientist and farmers. Most of the cultivars suffer from this problem. Nanocomposites (Bioengineered chitosan-iron nanocomposite, BNCs) plays significant role in crop yield and production of disease free crop plants. E.g. Inhibit rice crop against bacterial leaf blight (BLB) disease which is caused by *Xanthomonas oryzae* and improve crop nutrition. Nano-enabled agrochemicals are good alternatives of pest control methods. Ni-chitosan nanoconjugate play an important role as an antifungal agent for combating fusarium rot of wheat (Chouhan et al., 2022). Copper-based nanopestisides have efficiency for Solanum lycopersicum disease control (Liu et al., 2022). TiO_2 nanoparticles obtained by shell extract of *Caricaceae* used as antifungal (Saka et al., 2022). Sulfur nanoparticles (SNPs) enhance disease resistance in Tomatoes (Cao et al., 2021). Fluorescent silica nanoprobes used for diagnosis of plant disease (Banik and Sharma 2011). Another way, nanosensor paly significant role in disease management, crop production (Banerjee et al., 2021). However, unregulated use of nanoparticles causes several severe problems such as lack of soil fertility. In this review article, recent scenario of nanotechnology in plant disease management, application, synthesis of nanoparticles and phytotoxic effect of nanoparticles have been discussed. We try to include the use of various type of nanoparticles in plant disease control.

Synthesis of nanoparticles

Nanoparticles can be synthesized by various types of methods such as chemical, biological and physical method. Nanoparticles are eco-friendly, biodegradable and reproducible, have less toxicity, more effective, and have antimicrobial, antifungal, antibacterial as well as antiviral properties. These properties depend on method of synthesis (Fig.1). The green synthesis of nanoparticles from plants extract is a good approach. It is pollutant less, eco-friendly and production of harmful waste in low quantity (Banerjee et al., 2021). Recently, TiO_2 NPs synthesized by using *Carica papaya* (Saka et al., 2022). Microorganism such as fungi, bacteria known as "Bio factories" for nanoparticle production (Banik and Sharma 2011).

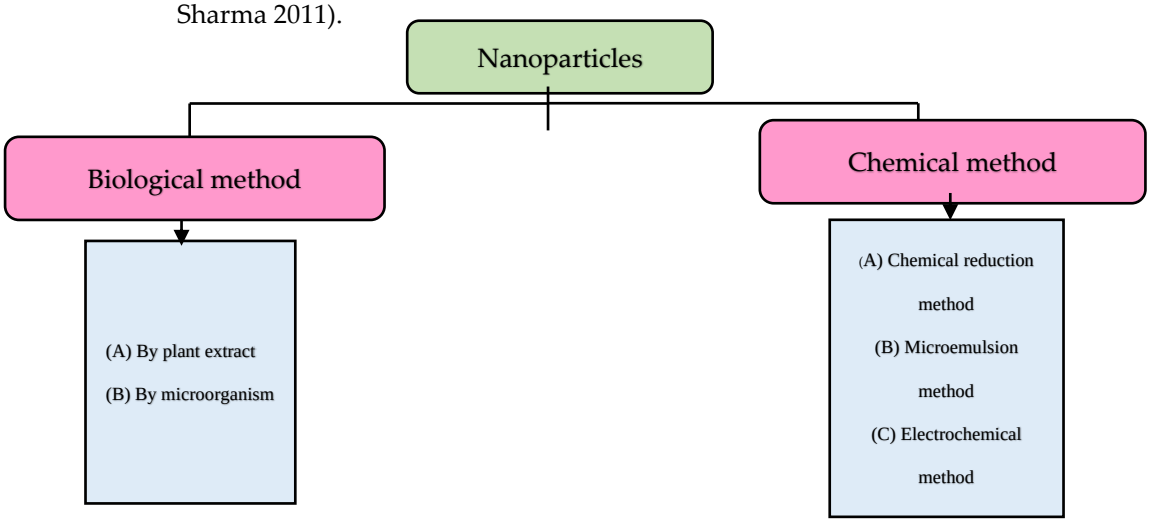

Figure 1. Different types of methods using for synthesis of nanoparticles (Khan and Rizvi, 2014**).**

Biological method

A. By plant extract

Recently, different types of approaches have been developed to synthesize nanoparticles from extract of plants (Makarov et al., 2014). Nanoparticles extracted from plants play more significant role in biological application. Copper nanoparticles (Cu NPs) can be biosynthesized by using *Magnolia , Syzygium aromaticum* and *Zinziber officinale* plants extract (Lee et al., 2011; Subhankari and Nayak, 2013). *Azadirachta indica* and *Citrus lemon* has been used in the synthesis of gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs) (Table 1)..

B. By microorganism:

Some fungi are used in biosynthesis of silver nanoparticles (Ag NPs) such as *Verticillium sp, Phoma sp., Fusarium oxysporum, Phaenerochaete chrysosporium, Aspergilus flavus* (Sastry et al., 2003; Chen et al., 2003; Duran et al., 2005; Vigneshwaran et al., 2006). On the otherhand, some bacteria also used for silver nanoparticles (Ag NPs) biosynthesis e.g. *Clostridium versicolor, Bacillus subtilis* (Sanghi and Preetiverma, 2009; Saifuddin et al., 2009). Plant virus capsids are also used as bio-templates for nanoparticles synthesis e.g. Tobacco mosaic virus (TMV) used in the biosynthesis of Ag and Ni nanoparticles (Dujardin et al., 2003).

Table 1 Biosynthesized Nanparticles by using plant extract.

Bio-Synthesized Nanoparticles	Plant extract	Activity	Reference
Cu NPs	*Magnolia*, *Syzygium aromaticum* and *Zinziber officinale*	Antibacterial	(Lee et al., 2011; Subhankari and Nayak, 2013).
Au NPs	*Eclipta alba*, *Nepenthes khasiana* leaf	Antibacterial	(Ahmed et al., 2016; Ibrahim, 2015)
Ag NPs	*Azadirachta indica*, *Musa acuminate peel*	Antibacterial	(Vijayakumar et al., 2020; Bhau et al., 2015)
TiO_2 NPs	*Caricaceae*	Antifungal	(Saka et al., 2022)
Fe_2O_3 NPs	*Mentha spicata*	Antifungal	(Khan et al., 2022)

Chemical method:

It is a commercial method of synthesis of nanoparticles. There are many types of chemical methods which can be used in the synthesis of nanoparticles such as chemical reduction method, microemulsion method, electrochemical method etc (Khan and Rizvi, 2014). The chemical reduction method was firstly discovered by Michael Faraday in 1857. This method is useful for the synthesis of nanosized copper nanoparticles (Cu NPs) (Song et al., 2004). On the otherhand, the electrochemical method used as a metal nenoparticles. It is done by passing electric current between electrodes (Raja et al., 2008).

Nanotechnology in plant disease control

There are different types of pesticides and herbicides have been used for controlling disease for many years (Talibi et al., 2011). Recently, use of nanoparticles in controlling disease in plants, is very effective in future aspect. Nanotechnology have different ways of controlling plant disease. Nanotechnology have major advances in plant disease management. In future prospects, it will be used as a tool of diagnosis of disease caused by bacteria, virus, fungi, insects etc. It will be used as a tool of diagnosis of disease caused by bacteria, virus, fungi, insects etc. Biosensor as a nanoanalytical device (Kumar et al., 2022). Nanoparticles have potential to provide protection against bacteria, virus, insects and fungi. Ag, Cu, ZnO and TiO_2 have antibacterial and antifungal properties. Various types of nanoparticles are used in the management of disease in plants (Fig.2).

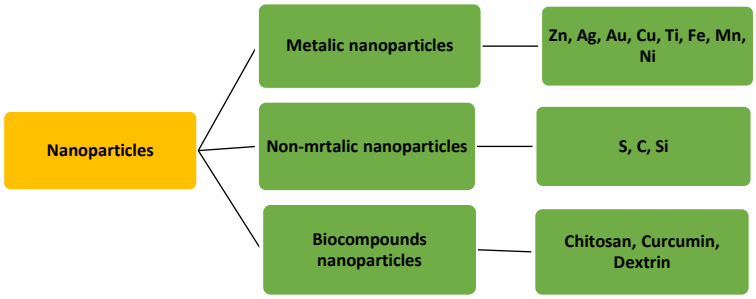

Figure 2. Types of nanoparticles synthesized for disease management.

Metallic NPs have more efficiency than non-metallic and bio compounds nanoparticles.

Zinc NPs

Zinc nanoparticles (Zn NPs) have low toxicity so it can be used in various disease management. It has viricidal, antibacterial properties (Abdelkhalek et al., 2020). It had been reported that Zinc nanoparticles (Zn NPs) effective against several pathogenic fungi *Mucor plumbeus, Botrytis cinerea, Penicillium expansum* (Banerjee et al., 2021).

Silver NPs

It is a most effective NP. It has antibacterial, antiviral, nematocidal and antifungal activity. It has been resulted that Silver nanoparticles (Ag NPs) have viricidal activity, it protects faba bean plant from BYMV (Rajani et al., 2022). Foliar application of Silver nanoparticles (Ag NPs) spray has efficacy to provide resistance against Tomato mosaic virus (ToMV) and Potato mosaic virus (PMV) (Noha et al., 2018). It has been also reported that AgNPs have viricidal activity against banana bunchy top virus (BBTV) (Mahfouze et al., 2020) when banana plants treated with AgNPs it reduces viral infection. It has antibacterial activity against *Staphylococcus aureus, E. coli, P. aeruginosa* and *Bacillus subtilis* (Brayskova et al., 2011). It shows antibactericidal and antimicrobial activity against *Staphylococcus aureus, E. coli,* and *P. aeruginosa* (Guzman et al., 2009).

Iron NPs

Iron nanoparticles (Fe_2O_3 NPs) have less toxic effect so it can be used in ordinary use (Abbaszadeh and Hejazi et al., 2019). It is a highly reactive and shows antiviral activity against tobacco mosaic virus (TMV) (Rajani et al., 2022). Khan et al., 2022 have been reported that Fe_2O_3 NPs have potential to inhibit the growth of *Phytophthora infestance*.

Nickel NPs

When cucumber plant treated with Ni NPs it shows antiviral activity and after the treatment leaf number and dry weight are also increases (Derbalah et al., 2019)**.** It shows antimicrobial activity against Methicillin-resistant *Staphylococcus aureus* infection (Zarenezhad et al., 2022). Ni NPs have antimicrobial activity against *E. coli*, *Bacillus subtilis* which is synthesized by using plant extract of *Ocimum sanctum* (Pandian et al., 2016).

Titanium NPs

It has ability to oxidize biomolecules due to this it has high antiviral activity. It has been resulted that when *Viccia faba* L. treated with TiO2 NPs it shows reduction in viral infection caused by broad bean stain virus (BBSV) (Elsharkawy et al., 2019).

Gold NPs

Due to their physiochemical properties it shows great antimicrobial activity. Au NPs have been used as biosensor components for diagnosis of plant disease (Biju, 2014). Au NPs play a significant role for detection of pathogen of karnal bunt disease of wheat by surface plasmone resonance (SPR) method and late blight of potato and tomato (caused by *Phytophthora infestance*) by using Au NPs based lateral flow biosensor (Singh et al., 2010; Zhan et al., 2018). Au NPs have been used as the detection label (Lee et al., 2021).

Copper NPs

It has excellence potential of plant disease control and antimicrobial activity. Fungicides developed by Cu NPs has potential to inhibit growth of *Phytophthora infestance* in

tomato plant (Giannousi et al., 2013). Bordeax mixture produced by Cu NPs suppress the *Xiphinema index* (Elmer et al., 2018). (Varympopi et al., 2022) reported that Cu NPs shows antibacterial activity against *Xanthomonas compestris pv. vesicatoria,* in Tomato. CuO NPs have most effective antifungal activity against root rot disease in cucumber which is caused by *Phomopsis sclerotioides* (Kamel et al., 2022).

Mg NPs

It shows effective antibacterial activity against Gram-positive and Gram-negative bacteria. It had been demonstrated that MgO NPs shows antibacterial activity against *Ralstonia solanacearum* (Imada et al., 2016). Recently, some studies hypothesized that MgO NPs has antibacterial activity against bacterial wilt in tomato caused by *Ralstonia solanacearum.*

Table 2 Nanoparticles in bacterial disease management.

Nanoparticles	Activity	Effect against	Reference
ZnO NPs	Antibacterial	*P. aeruginosa*	(Jayaseelan et al., 2012).
Ag NPs	Antibacterial	*Staphylococcus aureus*, *E. coli*, *P. aeruginosa* *Bacillus subtilis*	(Brayskova et al., 2011).
		Fusarium oxysporum	(Birla et al., 2013)
CuO NPs	Antibacterial	*Staphylococcus aureus*, *E. coli*, *P. aeruginosa* *Bacillus subtilis*	(Azam et al., 2012).
Ag NPs	Antimicrobial, Antibactericidal	*Staphylococcus aureus*, *E. coli*, *P. aeruginosa*	(Guzman et al., 2009).

Table 3. Nanoparticles against pathogenic fungi.

Nanoparticles	Pathogenic fungi	Disease	Reference
$CuSO_4$, $Na_2B_4O_7$	*Uromyces viciae-fabae*	Rust disease of field peas	(Singh et al., 2013).
Mn and Zn NPs	*Pythium spp., Fusarium spp.*	Damping off and charcoal rot diseases in sunflower	(Abd El-Hai et al., 2009).
ZnO NPs	*Botrytis cinerea*,	Grey mould of strawberry	(Khan, A.R and Rizvi T.F., 2014)
	Penicillium expansum	Blue mold disease in fruits and vegetables	

Phytotoxic effect of nanoparticles

There are many studies on interaction between nanoparticles and plant which reported their negative as well as positive impacts on plant. It has been reported that nanoparticles show toxic effect on plants (Table 3). Phytotoxicity depends upon physicochemical property of plant. Konotop et al., 2014 observed that colloidal solution of nanoparticles supress the growth of root of *Allium cepa* (L.). However, some metal-nanoparticles show more toxic than other such as Cu NPs more toxicity than Fe NPs (Cu>Zn>Ag>Fe). CuO NPs inhibit root and shoot elongation of *Hordeum sativum* (Rajput et al., 2018). Nano Zn and ZnO NPs have inhibitory effect on seed germination in ryegrass and corn, respectively. It has been reported that Ag NPs damage the cells of root tip of Allium cepa (Parthasarathi, 2011).

Table 4 Phytotoxic effect of various type of nanoparticles on plants

Nanoparticles	Plant species	Toxic effect	Reference
CuO NPs	*Hordeum sativum*,	Inhibition of shoot and root elongation	(Rajput et al., 2018)
Ag NPs	Tobacco	Retardation of plant growth and crop yield	(Liu et al., 2016)
Fe_2O_3 NPs	*Lactuca sativa*	Retardness in root elongation	(Liu et al., 2016)
ZnO NPs	*Allium cepa*	Effect on cell-cycle progresion	(Sun et al., 2019)
TiO_2 NPs	*Lactuca sativa*	Decrease in CO_2 fixation	(Madanayake and Adassooria, 2020)

Future aspects of nanotechnology in plant disease control

To provide necessary amount of food for this fast-growing world, we are Using so many chemicals as fungicides, pesticides and insecticides which cause damage to living organisms. There is a need to adapt Chemical less farming. The best way for chemical less disease management is Nanotechnology. Which plays crucial role in current agriculture practices like high yield crops, hormone delivery, seed germination, transfer of desired gene nanobarrcoding, decrease the usage of chemical fertilizer. Some of the researches on metal nanoparticles shows antiviral, antifungal and antibacterial properties. Metal nanoparticles like Silver (Ag), Copper (Cu), Zinc oxide (ZnO) and Titanium oxide (TiO) Are used to supress the Activities of paint pathogens like *Alternaria aleternata*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina* etc (Lamsal et al., 2011). In the Silver, Copper, Zinc and Titanium, Silver nanoparticles showed hopefully results against Powdery mildew, late blight in tomato by it's host defense mechanism. Silver nanoparticles shows Activities like disturbing cell membrane of pathogen, prevent H+ ATPase activity and blockage of nutrient flow. Silver nanoparticles (AgNPs) are considered as an effective tool for crop disease management. Powdery Mildew is one of such disease which is found in the plant family of cucurbits by fungi. As the studies proved that silver ions are highly reactive in nature. They can decrease the metabolic activities of the bacteria by damaging the bacterial

DNA. The activity of silver nanoparticles was tested against the fungi Ascomycetous which resulted in the 75% inhibition of late blight disease in plants and also experiments concluded that Ag nanoparticles with silica can damage many bacteria like *Pseudomonas syringae* and showed 25% disease resistance. Chitosan is the another widely used nanoparticles which showed antimicrobial activity and prevent viral infection like tobacco mosaic virus, bean mild mosaic virus, tobacco necrosis virus. Chitosan nanoparticles inhibit microbial activities by synthesizing mRNA and protein and distorting the cell membrane of pathogen resulting in control of root rot in tomato and bunch rot in grapes. Some experiments are going on the nanoparticles like TiO2, MnO, CuO and so on which are estimated to show hopeful results to prevent disease caused by many bacteria, fungi and viruses. By the successful application of these methods we can prevent many diseases like powdery milder, root rot, late blight, bunch rot and so on (Elmer and White 2018).

Conclusion

From this review this is concluded that nanoparticles (NP) play a significant role in control of plant disease. Nanoparticles (NP) have antibacterial, antiviral and antifungal properties against plant pathogens which causes various types of disease in plants. These nanoparticles are synthesized from plants extract and by microorganisms. These have potential tool of detect the plant disease. Nanoparticles (NPs) act as nano-herbicides, nanopesticides, nano-fungicides and play a significant role in control of plant disease. Nanoparticles provide unprecedented advantage in the field of plant disease management. In future it will be alternative of fungicides, pesticides and herbicides and become a sustainable tool in agriculture field to control management of plant disease. In another way, nanoparticles also play a significant role in improving crop yields and delivery of plant hormones etc. however, due to inappropriate use of nanoparticles have some phytotoxic effects which retards the growth of plant and also effects physiology of plants.

Funding source

None

Conflict of interest

No conflict of interest.

Authors contribution

Yashwant Sompura provide general concept of manuscript. Yashwant Sompura, Vanshika Sharma, Chayadevi H, Maruthi G R, Jeenat Banu, Tansukh Barupal and Shyam Sunder Meena wrote the manuscript. All authors read and approved it for publication.

Acknowledgement

The author would like to thank Dr. Tansukh Barupal and Dr. Shyam Sunder Meena S.B.K. Govt. College, Jaisalmer, Rajasthan and Chayadevi H and Maruthi G R Department of Biotechnology, Government Science College, Chitradurga, Karnataka , Jeenat Banu Department of Botany, JNV University, Jodhpur, Rajasthan. Vanshika Sharma Department of Botany, MLS University, Udaipur, Rajasthan.

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Biomaterials Journal
http://www.biomatj.com
Online ISSN: 2812-5045

Type of the Paper (Review Article)

Pulp capping materials and effect of biomaterials on angiogenesis

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Abstract: Regenerative endodontics has gained great interest recently in an attempt to fill the root canal space with living tissues instead of artificial materials. The most important goal is to provide a suitable environment for the regeneration of healthy tissue and restore the lost biological tissues. Based on the treatment site, this field is divided into two categories of dentin-pulp complex regeneration and dental pulp regeneration. The dentin-pulp complex regenerative procedures or vital pulp therapies include direct pulp capping, indirect pulp capping, and pulpotomy

Keywords: *pulp capping,pulp regenerations,pulp capping materials,calcium hydroxide,MTA,angiogenesis,angiogenic growth factors.*

Citation: ***Menna S. Gaber . Pulp capping materials and effect of biomaterials on angiogenesis*** *. Biomat. J., 2 (2),16 – 28 (2023).*

https://doi.org/10.5281/znodo.5829408

Received: 10 February 2023
Accepted: 20 February 2023
Published: 28 February 2023

I.Vital pulp therapies

Direct pulp capping is defined as covering an exposed dental pulp with a protective agent and indirect pulp capping is referred to the application of a protective agent, on a thin layer of dentin over the nearly exposed dental pulp. The other treatment in vital pulp therapies is the pulpotomy which is the surgical removal of inflamed coronal part of the dental pulp in the exposed pulpal tissue to save the remaining healthy tissue. In dentin-pulp complex regeneration, clinicians attempt to provide an effective pulp capping with appropriate sealing ability, and maintain the vitality of irritated pulp tissues and promote the formation of a dentinal bridge and other tissues including neural cells.[1]

In these procedures, the progenitor dental pulp stem cells (DPSCs) are migrated, recruited, and differentiated into odontoblast-like cells, which have the ability to produce reparative dentin. The homeostasis of pulp should be restored by reestablishment of the vascular network, through up- or down regulation of pro- or anti-angiogenic growth factors, which guides the regenerative procedure toward survival or necrosis of pulp tissue. [1]

II.Non-vital pulp therapies

The second field of regenerative endodontics deals with regeneration of dental pulp tissue in necrotic teeth. The treatment involves complete removal of necrotic dental pulp tissue, which is referred to as pulpectomy. After canal preparation, the regenerative treatment, the revascularization process, begins with instrumentation of periapical tissue to cause bleeding into the canal space. The blood clot formed inside the canal provides a provisional matrix scaffold for the recruited stem cells from apical papilla. [1]

Beside the stem cells derived from apical papilla, other investigators have used tissue engineered DPSCs for transplantation into the empty canal. In this treatment protocol, the establishment of a functional vascular network in transplanted tissue is the challenging goal for a successful result. The formation of this vascular structure is possible through angiogenesis, which is defined as the formation of new blood vessels from pre-existing vasculature. [1]

Pulp tissue regeneration approach

A. Cell transplantation approach

For the cell transplantation method, the transplanted cells can be either collected from the host (autologous) and/or from other individuals (allogenic) or can be processed and grown in cultures to increase the numbers.

- In this method, exogenous stem cells are either directly injected to the needed anatomic site (cell injecting) of the host or applied by being loaded onto scaffolds: (cells seeded scaffolds) either incorporated with or without signaling molecules.

For cell injecting method, main problems at directly injection were the difficulty for adequate localization and the direct contact with the immune system which prevents efficacy of the therapy.

It has been reported that cells encapsulated into a delivery vehicle were able to proliferate and differentiate more precisely. Therefore, using a delivery vehicle to carry and deliver the material thought as a solution to increase the efficacy of the therapy. The cells seeded into scaffolds can allow superior control for stem cell delivery, saturation with time-release signaling molecules and control of stiffness, pore size and cell-substrate interaction. [32]

On the other hand, cell transplantation method can be complex according to the procedure patterns as tooth extraction, pulp extirpation, in vitro cell culture, selection of stem cell populations, storage and shipping. In addition, there is a risk for contamination, development of tumorigenesis during ex vivo cell manipulation, immunological rejection and the ability of injected cells to maintain their phenotype. [32]

B.Cell homing/Cell-free approach

Cell homing/Cell-free approach is developed to overcome the drawback of cell transplantation approach and it is considered as a better alternative method.

In cell homing method the main purpose is to induce regeneration by the chemotaxis, proliferation and differentiation of host endogenous stem cells to injured tissue via biological signaling molecules loaded onto scaffolds. Furthermore, signaling molecules used for this method should promote angiogenesis, migration of endogenous stem cells and mineralization. Cell types responding to the signaling molecules during cell homing are found as DPSCs, SCAP and BMMSCs. [32]

There are two ways to attract the host's stem cells:

1.To apply signaling molecules and scaffolds into the root canals. Therefore, platelet-rich plasma (PRP) application can be thought of as a cell-free approach which can be considered as a combination of signaling molecules. However, it has disadvantages like drawing blood from the patient and additional centrifuge and purification processes.

2.To induce transport of both signaling molecules and stem cells into the root canals. This method is consisting of the disinfection of the root canals without mechanical preparation, application of antibiotics and blood clotting which is a simple technique of revascularization.

Blood clot acts as a scaffold, bleeding of the apical area can directly transport the signaling molecules and stem cells or inflammation caused by this trauma can induce mi-

gration of the stem cells through the root canal. However, this method is used for dentin-pulp complex regeneration of immature permanent teeth in the clinic, the outcome of the treatment is inconsistent.[32]

In addition to regeneration of dental pulp tissue, apexogenesis and apexification are other endodontic procedures that are performed in immature permanent teeth. Apexogenesis is the procedure that enables the immature permanent teeth to continue root end development, while the apexification provides a calcified barrier at the end of immature root by biocompatible material next to periapical tissue. It has been reported that the revascularization process occurs through the angiogenesis events derived from the periapical tissues that grow into the engineered pulp tissue. Furthermore, the immature teeth with open apices are the best candidates for these regenerative procedures.[2]

III. Pro-angiogenic and Anti-angiogenic Factors

- Vascular endothelial growth factor(VEGF) :is one of the major proangiogenic growth factor secreted by many cells as dental pulp stem cells, macrophages and tumor cells.

  Actions

  1. Proliferation and migration of endothelial cells and maturation of sprouted capillary vessels

  2. Activation of intrinsic tyrosine kinases

  3. Differentiation of DPSCs into endothelial cells and odontoblasts. [3]

- FGF: Fibroblast growth factor is proangiogenic growth factor inducing angiogenesis by interacting with various endothelial cell surface receptors causing proliferation and migration of endothelial cells.[4]

- PDGF: platelet derived growth factor is proangiogenic factor induces angiogenesis by up-regulating VEGF production and modulating the proliferation and recruitment of perivascular cells. The hDPSCs express this growth factor as well. It was demonstrated that the dentin matrix contains a higher amount of PDGF than other growth factors [5]

- Angiopoietins:

  Angiopoietins have 2 major forms including Ang-1 and Ang-2, which interact with specific receptors on endothelial cells. Dental fibroblasts express Ang-2 along with other growth factors such as VEGF, FGF-2, PDGF. These factors play a critical role in initiation and stabilization of angiogenesis through competitive interactions with the receptor.[6]

  - Matrix metalloprotienase (MMPs):

    Are a group of important enzymes in angiogenesis. These biological molecules have a key role in degradation of the ECM of vessel walls, allowing the migration of endothelial cells. [6]

  - Bone morphogenetic proteins (BMPs):

    Bone morphogenetic proteins (BMPs) belongs to transforming growth factor-β family. BMPs play a critical role in embryonic and postnatal development, and also in maintaining homeostasis in different organs and tissues by regulating cell differentiation, proliferation, survival and motility. Also, BMPs have important role in angiogenesis either di-

rectly regulate the functions of vascular endothelial cells or indirectly via regulation of angiogenic factors, such as vascular endothelial growth factor (VEGF).[7]

- Transforming growth factor beta (TGF-β)

The TGF-β regulates various cell activities inside the cell, including the growth and division (proliferation) of cells, the maturation of cells to carry out specific functions (differentiation), cell movement (motility), and controlled cell death (apoptosis).[8]

- Biochemical Inhibitors:

It was reported that DPSCs can produce antiangiogenic factors including endostatin, thrombospondins (TSP) and angiostatin. It was found that the dentin matrix components in low concentrations have proangiogenic impact, whereas in high concentrations they have inhibitory effects on angiogenesis events of dental pulp. Hyperglycemia also has negative influence on immune system function and angiogenesis. There is now a large family of endogenous inhibitors of angiogenesis whose function in tooth development and regeneration needs evaluation. These include thrombospondin-1(TSP-1) and thrombospondin-2(TSP-2), whose expression plays a significant role in vascular homeostasis and avoid pathologic neovascularization. Another important physiologic antiangiogenic agent is platelet factor 4 is effective inhibitor of angiogenesis process and are useful agents for therapeutic purposes. [9]

Stimuli of angiogenesis:

❖ Mechanical Stimulation: Higher capillary shear stress increases the expression of VEGF and angiogenesis. An increase in the number of microvessels in pulp tissue was detected in orthodontically moved teeth that was due to the increased expression of the angiogenic growth factors. Thus, mechanical alteration in tooth has a significant impact on local angiogenesis and tissue regeneration. [3]

❖ Biochemical Stimulation: The biochemical stimulation of angiogenesis is due to the production of proangiogenic and antiangiogenic factors including growth factors such as bone morphogenetic proteins (BMPs). The DPSCs can secrete proangiogenic factors such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [3]

IV. Pulp capping materials and the effect on Angiogenesis in Dentin-Pulp Complex Regeneration

- 1. Calcium hydroxide: The introduction of vital pulp therapies including direct or indirect pulp capping date back to 1939. An ideal pulp capping material should provide easy handling, antibacterial effect, good sealing ability, and induce dentinal bridge formation. Among several materials, calcium hydroxide was one of the most common material used in pulp capping. Due to its alkalinity, $Ca(OH)_2$ has antibacterial activity and stimulates dentin formation. Calcium hydroxide has been considered the "gold standard" for the capping of exposed pulp.[10]

- Composition:

✓ Calcium hydroxide cements are paste/paste systems. One paste contains calcium hydroxide and the other contains salicylate. Salicylate is a weak acid that is reacts with the calcium hydroxide by acid–base reaction which is responsible for setting, the reaction forms an amorphous calcium disalicylate, for example, Dycal [11]. A visible light cured calcium hydroxide was introduced to overcome the limitations of the chemical cure calcium hydroxide; that is, they set on command, improved strength and minimal solubility in water. A visible light-cured (VLC) calcium hydroxide liner consists of calcium hydroxide and barium sulfate dispersed in a urethane dimethacrylate resin containing initiators and accelerators activated by visible light VLC calcium

hydroxide liners are mainly indicated for indirect pulp capping and as a cavity liner under all types of restoratives, for example, Calcimol (Voco GmbH, Cuxhaven, Germany) and Lime-lite (Pulpdent Corporation, Watertown, MA, USA).[11]

- The effect of $Ca(OH)_2$ on dentin-pulp complex regeneration and angiogenesis:

✓ It was reported that $Ca(OH)_2$ increases the recruitment, migration, proliferation of DPSCs, and periodontal ligament stem cells (PDLSCs) through the expression of STRO-1 and CD146 markers. [12]

✓ It was also found that the regenerative effects of $Ca(OH)_2$ are due to calcium ion release and the high pH value. Calcium ions promote the migration of pulp progenitor cells, increase the synthesis of biomolecules such as fibronectin and bone morphogenic proteins (BMPs), and participate in mineralization. The alkaline pH can present antibacterial and anti-inflammatory effects, activate transforming growth factor β (TGF-β), increase the activity of alkaline phosphatase (ALP), and enhance the dissolution of dentine extracellular matrix (ECM).[1, 13]

✓ The pro-angiogenic effects of $Ca(OH)_2$ is mainly attributed to the release of growth factors preserved in the dentin matrix including TGF-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF). It was also found that chelating agents as (EDTA) can dissolve the dentin matrix and release pro- angiogenic factors.[13]

✓ Dentine matrix also contains vascular endothelial growth factor (VEGF), which is one of the most important factors in angiogenesis events. It has been suggested that $Ca(OH)_2$ can activate notch signaling pathway. In dental pulp tissue, notch signaling pathway is activated due to injury, and participates in angiogenesis through the proliferation and migration of endothelial cells, smooth muscle, and arterial-venous differentiation.

❖ During healing 3 zones are formed: -

Zone of obliteration: The pulp tissue immediately in contact with the calcium hydroxide is usually completely distorted because of the caustic effect of the drug. This zone consists of debris, dentinal fragments, hemorrhage, blood clot, and particles of calcium hydroxide.

Zone of coagulation necrosis: A weaker chemical effect reaches the subjacent, more apical tissues and results in a zone of coagulation necrosis and thrombosis, also called layer of 'firm necrosis'.

Zone of demarcation: A line of demarcation develops between the deepest level of the zone of coagulation necrosis and the subjacent vital pulp tissue. Exposed human dental pulp will heal with hard tissue bridging.

❖ Disadvantages:

• Highly soluble in oral fluids.

• Lack of adhesion.

• Presence of porosities known as "*tunnel defects*" in reparative dentin bridge. Therefore fail to provide barriers, which act as pathways for microleakage and bacterial reinfection.[14]

2. Bonding Agents:

• Dental adhesive systems were suggested for use as an indirect pulp capping material to overcome the disadvantages of calcium hydroxide as they produce superior adhesion.

• However, they have poor outcome due to its cytotoxic effect and absence of calcific bridge formation.[15]

- Effect on angiogenesis:

- By comparing the effect of dentine adhesive resin and $Ca(OH)_2$ on the pulp cells, it was found that the adhesive resin causes more inflammation of pulp tissue due to its missing antibacterial efficacy and foreign body reactions.

- Decrease the release of FGF and has no effect on PDGF therefore it cannot induce the formation of an acceptable tertiary dentine bridge. [16]

3.Laser:

- Pulp capping therapy using lasers results in good prognosis for the tooth however, the sealing of exposed pulp with one of the dental materials after laser treatment is still required.

- For Example: CO_2, Diode and Nd: YAG lasers.

Mechanism of action:

- Sterile field is provided by the bactericidal effect of the laser. Area of coagulation is created by a superficial necrosis, with an underneath area of reversible damage, this stimulates odontoblasts to produce reparative dentin.[17]

Effect of laser on angiogenesis:

It was reported that low-level laser therapy caused higher proliferation of DPSC and higher gene expression of VEGF with the lower energy densities (0.7, 1.5, and 3 J/cm^2) but not with higher ones (9 J/cm^2), so it could be concluded that low-level laser therapy could be a useful tool to promote angiogenesis and dentinogenesis of the dentin-pulp complex when parameters are optimized.[18]

4.Calcium silicate based materials:

4.a. MTA:

- Composition:

The MTA is composed of tricalcium silicate, dicalcium silicate, tricalcium aluminate and bismuth oxide.

❖ Two types of MTA were available: White and Gray MTA.

The grey version is due to the addition of iron. Tooth discolouration has been reported with the use of grey MTA. Therefore, the use of white MTA has generally been recommended. [14]

- Mechanism of action:

The primary reaction product of MTA with water is calcium hydroxide. Therefore, the formation of calcium hydroxide explains a similar action with the calcium hydroxide paste.

The second reaction is depending on MTA is calcium silicate-based materials. Calcium silicate-based materials are bioactive materials capable of forming apatite by using calcium silicates or calcium aluminates. these formed deposits can stimulate dentin bridge formation and remineralization by promoting the release of cytokines and interleukins from white blood cells.[14]

The differences between the MTA and Ca (OH) 2:

MTA has a higher sealing ability, lower solubility, higher-strength and stability, MTA can set in a moist environment, prevent bacterial infiltration, produce thicker dentin bridge formation with a lesser inflammatory response, less hyperemia, and less necrosis of pulp tissue compared to calcium hydroxide. Therefore, many clinical reports demonstrated that the success rate of direct pulp capping was higher with MTA than with calcium hydroxide. [14]

Disadvantages of MTA:

It has shown high solubility, demonstrating a 24% loss after 78 days of storage in water, the presence of iron in the grey MTA formulation may darken the tooth, prolonged setting time of approximately 2 hours and 45 minutes, the handling is difficult and high cost.

Effect on angiogenesis:

By comparing $Ca(OH)_2$ and MTA, it was found that MTA had positive effects on angiogenesis and differentiation of dental pulp cells when it was placed in direct contact with dental pulp. It was also reported that MTA, as a direct pulp capping agent, can induce the expression of VEGF, osteocalcin and dentin sialoprotein. The in vitro culture of hDPSCs with MTA facilitated their differentiation, and also increased the expression of angiogenic factors as angiopoietin-1 (Ang-1).[19]

4.b. Biodentine:

Biodentine is a new class of dentin material which has been recently introduced as a pulp capping material. It was developed as a silicate-based restorative material from the addition of calcium chloride to MTA. It is represented in a capsule form.

•Composition:

The powder: Mainly contains tricalcium and dicalcium silicate ($3CaO\ SiO_2$ and $2CaO\ SiO_2$), the principal component of Portland cement, calcium carbonate ($CaCO_3$). In addition, zirconium dioxide (ZrO_2) serves as a contrast medium.

The liquid: Consists of calcium chloride ($CaCl_2.H2O$), which is used as a setting accelerator and water-reducing agent in aqueous solution with an admixture of polycarboxylate (a super plasticizing agent). [13]

Effect on angiogenesis:

It was reported that the induction effect of Biodentine cement on differentiation of DPSCs is through the mitogen-activated protein kinase (MAPK) and calcium/calmodulin-dependent protein kinase II (CaMKII) pathways. The angiogenic effect of this cement was evaluated and it was suggested that Biodentine can induce early miner-

alization in dental pulp due to an increase in release of TGF-β1, a pro-angiogenic factor produced by pulp cells.[13]

4.c. TheraCal

Theracal is a Light-curable resin-modified calcium silicate-based materials. It is used as a pulp capping agent and as a protective liner for use with restorative materials, cement, or other base materials. This is material has been classified as a 4th generation calcium silicate material.

- Composition:

The material is single paste light cured calcium silicate based cement and composed of Portland cement (30-50%), resin (10-30%), and barium sulfate (1-10%). TheraCal is opaque and "whitish" in colour, so it should be kept in thin layers so as not to show through composite materials that are very translucent affecting final restoration shade.[20]

- Mechanism of action:

TheraCal may act as a scaffold for reparative dentine formation. Dentinal fluids are absorbed within it, resulting in the release of calcium and hydroxyl ions and the tooth responds to form apatite deposits. Moreover, the formed apatite deposits have a natural sealing ability that plays a crucial role in pulpal protection by the formation of an interfacial layer rich in minerals thus promoting remineralization of dentin bridge.

- Compared to conventional MTA materials, the resin-modified light-curable cement is superior as it polymerizes immediately after light-curing, prevention of materials washing out and superior physical properties.

- Disadvantages: TheraCal displayed lower calcium ion release and the presences of unpolymerized resin can exert a toxic effect on the pulp. [20]

- Effect on angiogenesis:

TheraCal showed toxic effects after 24, 48, and 72 hr. Moreover, TheraCal decreased secretion of TNF-α and IL-8 in hDPSCs.[21]

4.d. Endosequence Root Repair Material:

It is a recent nano-bioceramic material in both low and high viscosity form recommended for pulp capping, perforation repair, apical surgery, and apical plug and can be able to penetrate dentinal tubules and set using their moisture.

- Composition:

Calcium silicates, Monobasic calcium phosphate, Zirconium oxide, Tantalum oxide, fillers, thickening agents. No mixing is required due to its supply as a paste in a preloaded syringe or a moldable putty form.[22]

- Advantages:

Excellent sealing ability, highly biocompatible, dentin bridge formation, hydrophilic, improved handling characteristics over MTA and antibacterial effect.

Effect on angiogenesis:

ERRM promoted the vascularization, migration and differentiation of DPSCs through the release of VEGF and BMP growth factors from dentin matrix. [22]

5. Enamel matrix derivative (EMD)

EMD (Emdogain) is an extract derived from porcine foetal tooth material.

Composition:It is mainly consisting of amelogenins, a class of protein known to induce proliferation of PDL cells, acellular cementum and alveolar bone during tooth development.[23]

- Mechanism of action:

Enamel matrix proteins have been reported to increase the levels of mineralization markers (including bone sialoprotein and osteopontin) in odontoblasts. Based on this concept, the enamel matrix derivative (EMD) was introduced. The regenerative process of EMD consists of differentiation of odontoblasts with consequent dentine formation and pulpal wound healing without affecting the vitality of the remaining pulp in a manner similar to normal dentinogenesis. [23]

- Disadvantage:

It has poor sealing qualities and no effective hard tissue barrier formation.

Effect on angiogenesis:

Moreover, EMD has also been reported to contain growth factors such as transforming growth factor-beta 1 and small amelogenin peptides that are actively involved in cell signalling to stimulate matrix formation and mineralization. These growth factors are recognized as mediators in processes such as tissue homeostasis, inflammation, healing and neogenesis. Enamel matrix derivative (EMD) can also be used as a pulp capping material. It was found that EMD was more capable of inducing the differentiation and proliferation of human tooth germ stem cells (hTGSCs) compared with calcium calcium hydroxide and mineral trioxide aggregate (MTA). EMD can exhibit angiogenic effects by chemotactic effect on endothelial cells and stimulation of periodontal cells to produce VEGF. [1] [23]

6. CEM cement:

Calcium-enriched mixture (CEM) cement is one of the pulp capping agents composed of calcium oxide (CaO), sulfur trioxide (SO3), phosphorous pentoxide (P2O5), and silicon dioxide (SiO2), which has osteogenic, cementogenic and dentinogenic functions.

Effect on angiogenesis:The ability of CEM and MTA as capping agents was compared. It was concluded that CEM could increase the expression of FGF and bone morphogenic protein 2 (BMP-2) when compared to MTA.[24]

7.Natural pulp capping materials

a.Propolis

Propolis is collected from trees and shrubs by honeybees. The main chemical classes present in propolis are flavonoids, phenolics and other various aromatic compounds. Propolis has potent antimicrobial and anti-inflammatory properties. Propolis has shown to inhibit the synthesis of prostaglandins and supports the immune system by promoting

phagocytic activities, stimulating cellular immunity and augmenting healing effects. Moreover, it contains some elements as zinc and iron that are important for the collagen synthesis.

- Composition: Propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other various substances, including organic debris.

- Effect on angiogenesis:In previous studies, showing the ability of Propolis to stimulate the production of transforming growth factor (TGF) Beta 1, which is important for the differentiation of odontoblasts. It also induces the synthesis of collagen by dental pulp cells. [25]

b.Eggshell powder

Eggshell has attracted research interest recently due to its bioactive effect in different biomedical applications. It is thought to have anabolic effects on human bone without side effect. This may be attributed to its unique chemical composition.

- Composition:

Contains 94 wt.% $CaCo_3$,1 wt.% $MgCo_3$, 1 wt.% calcium phosphate ($Ca_3(Po_4)_2$) and trace amounts of strontium (Sr) and fluoride (F).

- Mechanism of action:

It was reported that the natural biological origin of the eggshell makes it similar in its crystalline structure to the custom-made hydroxyapatite and human bone-like crystals. It has been reported that a significantly thicker dentin bridge formation with less pulp inflammation and fibrosis within the micro-sized eggshell powder when compared to calcium hydroxide paste. However, the material still needs further investigation to reveal it is a mode of action of the pulp tissue. [26]

c. Grapessed extract:

Grapeseed extract has been used in dentistry in multiple applications due to its anti-inflammatory, antibacterial, antioxidant and anticancer effect. Grape seed oil contains a large amount of phenolic compounds, including flavonoids, carotenoids. It has anti-angiogenic effect especially VEGF.[27]

V. The effects of canal irrigating solutions and disinfecting agents on angiogenesis:

V.1.NaOCl

The first step in dental pulp regeneration is the complete disinfection of root canal system. At first, root canal space should be irrigated with sodium hypochloride (NaOCl) which has proteolytic and antimicrobial activities which dissolves the organic debris and eliminate microorganisms inside the dental canal. It was suggested that NaOCl has toxic effects on on human bone marrow mesenchymal stem cells (MSCs).[28]

It was also reported that high concentrations of NaOCl can drastically effect the survival and differentiation of stem cells of the apical papilla (SCAPs) and significantly reduce the expression of dentin sialophosphorprotein (DSPP). It has been suggested that using lower concentrations like 1.5% and using 17% EDTA after NaOCl irrigation reduced the NaOCl negative effects, and increased the survival rate of SCAPs and expression of DSPP. [28]

V.2.Chlorhexidine gluconate

The other disinfecting endodontic solution used in regenerative procedures is 2% chlorhexidine gluconate (CHX). The effect of CHX on DPSCs was measured and no viable cell was detected in samples irrigated with 2% CHX. It was reported that 2% CHX, as well as 6% NaOCl, showed cytotoxic effects on DPSCs due to negative influence on their attachment to root canal wall surface.[29]

V.3.EDTA

EDTA is an endodontic irrigating solution with chelating activity, which is suggested to be added to canal irrigation protocol as a final rinse for smear layer removal or in combination with NaOCl and CHX solutions. Although, the addition of EDTA to other rinsing solutions can increase the viability of DPSCs, and positively affect stem cell's attachment to root canal wall, authors showed that the time of irrigation with EDTA should be 1 min, while after 3 min the microhardness of dentin can significantly reduce.

It was also noted that EDTA can induce DPSCs cell attachment and odontoblastic/osteoblastic differentiation. Also, it was indicated that EDTA can stimulate the release of pro-angiogenic growth factors in dentin matrix including TGF-β, VEGF, FGF-2, PDGF, and BMP-2.[1]

V.4.Triple antibiotic paste (TAP)

TAP is a disinfecting regimen containing three antibiotic pastes including: ciprofloxacin, metronidazole, and minocycline used for complete elimination of microorganisms inside necrotic root canal in regenerative procedures. It was indicated that the scaffolds containing 5%wt ciprofloxacin were safe for hDPSCs, and only 25%wt ciprofloxacin had cytotoxic effects on pulp stem cells. Also, it was suggested that ciprofloxacin could decrease the expression of inflammatory cytokine IL-6, and increase the expression of the IL-8.

It was indicated that metronidazole plus clindamycin had anti-agiogenic activity and could strongly interact with pro-angiogenic factors like FGF-2. It was demonstrated that minocycline has anti-angiogenic activity due to suppression of vascular endothelial growth factor (VEGF) expression. It has showed that minocycline can inhibit hypoxia-induced neovasculogenesis. [30, 31]

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Biomaterials Journal
http://www.biomatj.com
Online ISSN: 2812-5045

Type of the Paper (Review Article)

Artificial Intelligence In Dentistry

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Citation: ***Salma K.Rizk*** . ***Artificial Intelligence In Dentistry*** . *Biomat. J., 2 (2),29 – 45 (2023).*

https://doi.org/10.5281/znodo.5829408

Received: 10 February 2023
Accepted: 20 February 2023
Published: 28 February 2023

Abstract: The term "artificial intelligence" (AI) refers to the idea of machines being capable of performing human tasks. It describes how technology is used to develop a software or a machine that can easily mimic human intelligence and perform specific activities. John McCarthy, a mathematician coined the term artificial intelligence in 1955, and widely recognized as the father of artificial intelligence. He chose this term to explain the potential of machines to perform tasks that can fall in the range of "intelligent" activities. In the following review article different applications of artificial intelligence in dentistry, achievements, limitations and challenges will be discussed.

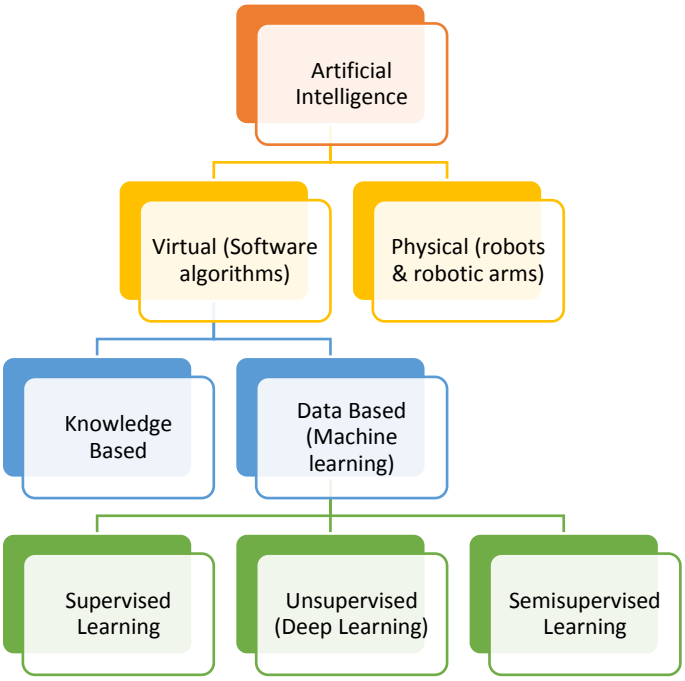
Keywords: *Artificial intelligence, applications of AI & Dental AI*

I. Introduction to artificial intelligence

The term "artificial intelligence" (AI) refers to the idea of machines being capable of performing human tasks. It describes how technology is used to develop a software or a machine that can easily mimic human intelligence and perform specific activities. (1, 2)

John McCarthy, a mathematician coined the term artificial intelligence in 1955, and widely recognized as the father of artificial intelligence. He chose this term to explain the potential of machines to perform tasks that can fall in the range of "intelligent" activities.(3)

AI is a branch of applied computer science that provides machines with the ability to mimic intelligent human behavior. Two types of AI are available for general health care delivery: **physical** and **virtual.** Physical applications are represented by sophisticated robots or automated robotic arms.Virtual components are software-type algorithms that support clinical decision making. (4)



Attempts at implementing AI were initially based on the assumption that human intelligence can be fully digitized and integrated into machines.

In dentistry, the applications of AI are mostly virtual, employing AI algorithms to distinguish between lesions and normal structures, prioritize risk factors, and simulate and evaluate prospective results. Virtual AI methodologies are divided into **knowledge-based** and **data-driven** AI according to the Barcelona Declaration for the Proper Development and Usage of Artificial Intelligence in Europe. (4)

Knowledge-based AI attempts to model human knowledge and is built in a top-down fashion from the self-reported concepts and knowledge that humans use to solve problems. However, knowledge acquisition and formalization are 2 major bottlenecks, which consume development time and require significant initial effort. (4)

Conversely, **data-driven AI**, commonly known as **machine learning** (ML), built in a bottom-up approach by training mathematical models with data derived from human activities. Because of the large amount of dental data available in electronic form, data-driven AI receives a lot of attention in dentistry. (4)

Data-driven AI or ML may be divided as supervised, unsupervised, and semisupervised learning. On **the supervised** platform, algorithms employ manually labeled training data sets to learn the correlations between data instances and labels, yielding the desired and known outcomes. It is defined by its use of labeled datasets to train algorithms to **classify data or predict outcomes** accurately. It is frequently mentioned that supervised learning is limited by the lack of annotated data. (4)

Examples:

- **Support vector machines** (SVMs) set up an imaginary high-dimensional space, place samples according to their features, and separate them by a hyperplane, resulting in data classification.
- **Decision tree (**DT) a hierarchical data structure used for classification.
- **Random forest** (RF) combines the output of multiple decision trees to reach a single result. It handles both classification and regression problems.
- **Artificial neural network** (ANN) is an attempt to simulate the network of neurons that make up a human brain so that the computer will be able to learn things and make decisions in a human like manner. ANNs are created by programming regular computers to behave as interconnected brain cells. The greatest advantage of these systems is that they have capability to solve the problems that are too complex to be solved by conventional methods. They are useful in various areas of medicinal science like diagnosis of diseases, biomedical identification, image analysis and data analysis. In dental practice also the clinical support systems are actively progressing.

A study done by Kim et al. used Artificial neural network to build a model that can predict toothache on the basis of association between toothache and daily toothbrushing frequency, toothbrushing time, use of dental floss, toothbrush replacement pattern, undergoing scaling and other factors like diet and exercise. This successful study aided in the development of a **toothache predictive model** with great accuracy. This model recognizes adequate eating habits, oral hygiene, and stress prevention as the most important factors in preventing toothaches.(5)

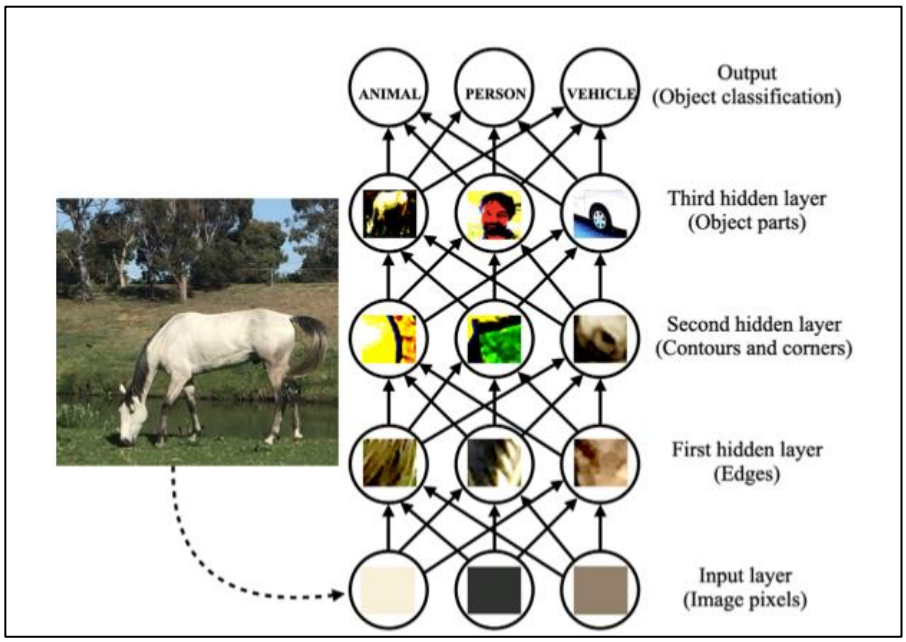


Fig 1: an artificial neural network for object identification

Unsupervised learning uses machine learning algorithms to analyze and cluster unlabeled datasets. These algorithms discover hidden patterns or data groupings without the need for human intervention, yielding unknown results.

- **Deep neural networks**, commonly known as deep learning (DL), is a subset of ML that can be operated in unsupervised scenarios. The term "deep" refers to multiple neural layers between the input and output layers. **Convolutional neural networks** (CNNs) are the most widely used DL architecture in dentistry, employing a convolutional process to learn features contained within data. The purpose of deep learning is **to construct a neural network that automatically identifies patterns to improve feature detection.** Unsupervised learning has also been criticized for failing to identify the initial pattern.

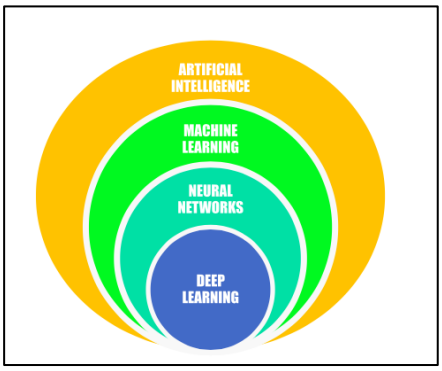


Fig 2: Key aspects of artificial intelligence(2)

Semisupervised learning is an amalgamation of supervised and unsupervised learning that analyzes a collection of data while augmenting the pattern recognition abilities with a small amount of labeled data. (4)

Smart and Intelligent Materials

Smart materials are **materials that are manipulated to respond in a controllable and reversible way by modifying some of their properties as a result of external stimuli** such as certain mechanical stress or a certain temperature.(6)

Smart materials have the ability to perceive and respond but not to self-optimize and improve, whereas intelligent materials perceive and analyze to summarize the experience and adapt to optimize the responsive performance.

Intelligent material is self-aware, and responds purposefully. intelligent materials should have the ability to be aware of external stimuli and learn from it to optimize response behaviors for achieving goals to the greatest extent. (7)

II. Applications of Artificial Intelligence in Dentistry

Two types of AI are available for dental health care delivery: **physical** and **virtual.** Physical applications are represented by sophisticated robots or automated robotic arms.Virtual components are software-type algorithms that support clinical decision making.

A) <u>Applications of Virtual AI in dentistry:</u>

i. Diagnosis:

Oral and Maxillofacial lesions. Machine learning algorithms, including SVM, ANN, RF, have been experimentally investigated for their ability to identify cysts, benign tumors, oral cancer, and lymph node metastasis.(4)

- Yilmaz et al. 2017 found that CBCT with SVM was 94% accurate in differentiating periapical cysts from keratocystic odontogenic tumors.(8)
- Sunny et al. 2019 Utilized CNN to score the malignancy of cytology images derived from a telemedicine platform, this model showed high sensitivity in detecting oral malignant (93%) and high-grade potential malignant (73%) lesions. (9)

Despite these excellent results, contemporary AI models for oral and maxillofacial surgery diagnosis focus on only 1 type of data, such as radiographic results or cytopathologic images. For highly accurate diagnosis, models that integrate more medical information about the patient are required.

Cariology and Endodontics. With the ability to perform automated lesion segmentation, Deep Learning with CNN has become the predominant AI component used in cariology and endodontic diagnostics. The segmentation process divides radiographs or images into multiple non overlapping regions using sets of rules, such as similar pixels or intrinsic features, to convert them into a meaningful form that can be conveniently analyzed. A number of studies have explored automated detec- tion of periapical radiolucencies using either panoramic radiographs or CBCTs. Computer vision and neural networks facilitate interpretation of CBCTs at a level uninterpretable to human vision. Early detection of periapical lesions might prevent complications and improve patient outcomes. (4, 10)

Deep Learning segments CBCT voxels into lesion, tooth structure, bone, restorative materials, and background, achieving results comparable to those of clinicians in diagnosing periapical lesions.

Focusing on the binary presence or absence of lesions. Volumetric measurement in CBCT, following DL-based segmentation, was reported to be comparable to the results obtained from manual segmentation of periapical lesions.(4,18)

Periodontics. Although periodontal disease is a complex inflammatory disease contributed by multiple causal factors simultaneously and interactively. Focusing on periapical radiographs, CNN achieved 81.0% and 76.7% accuracy in diagnosing periodontally compromised premolars and molars. However, due to the hysteresis of imaging characteristics and the visual field of periapical radiographs, this technique cannot distinguish incipient lesions or make a final diagnosis of periodontal disease. (4,22)

DT and SVM performed well in classifying healthy periodontium, gingivitis, chronic periodontitis, and aggressive periodontitis by integrating a patient's medical history, clinical information, and radiographs.

Temporomandibular Joint Disorder. Clinical clues from a patient's complaint and history are important for diagnosing temporomandibular joint disorders (TMDs). Natural language processing is a technology that transforms natural human language into structured computer language. A natural language processing–based model was successful in differentiating TMD-mimicking conditions from genuine TMDs, according to the frequency of word usage in the patient's chief complaint and mouth-opening size. (4,23)

ii. Treatment:

Prosthodontics. Integrating AI with CAD/CAM improves its chair-side application. ANNs based on panoramic radiographs, periapical radiographs, micro–computed tomography images, and 3-dimensional scanning of dental surfaces have been explored for tooth segmentation and classification. With >90% accuracy, such automatic classification is instrumental for bridging the gap between data acquisition and manufacturing in CAD/CAM technology. (4)

3D printing

Dental research has expanded the scope of 3D printing to a wide range of applications including surgical guides, orthodontic brackets and metal framework for removable partial dentures. Researchers are exploring methods to further improve dimensional accuracy of 3D printing, one of which is the implementation of ML algorithms to **learn** shape deviations from past printing jobs, **predict** shape distortions and **compensate** for these deviations in future printing jobs. This has the potential to reduce costs, time and the number of visits required for accurate fitting. (4)

B) Applications of physical AI in dentistry:

Dentronics is a hypernym of the range of advanced dental technologies, including medical robotics and artificial intelligence.

Robotics is an interdisciplinary field that integrates computer science and engineering. Robotics is defined as the "intelligent connection between perception and action". by artificial intelligence, robots are able to reason about current situations and new events in order to adapt to new circumstances autonomously. The inherent advantages of robots are their high accuracy and precision, high work efficiency, and stability.

1. Robots in Implant & Maxillofacial Surgeries.

- **Robots in implant surgeries:**

The development of computer-assisted implant surgery based on the concept of prosthetic-driven implantology and CT-scan analysis have been reviewed.

A surgical robotic application is an invasive robotic assistant for dental implantology. It was permitted for operative use by the FDA (Food and Drug Administration) in March 2017. The product is called ***Yomi*** and is produced by *Neocis* (Neocis Inc., Miami, USA). Based on 3D- data from a CT the dentist plans the implant position. During surgery the robotic arm drills the hole in the jawbone and places the implant according to the planning while the dentist can follow the position of the bur in real-time, owing to the software, which allows the dentist to adjust placement position of the implant intraoperatively.(3)

Robotic-Guided Dental Implant Placement in Fully Edentulous Patients was done using yomi robots. A total of 58 implants were placed in 11 arches in 8 patients. Intraoperative outcomes captured included safety, efficacy, surgical time, and a Likert scale evaluation of user experience. No adverse events were reported, and user experience was rated highly with respect to the standard of care. First cases using Yomi robotic guidance in fully edentulous patients were notable for brief procedural times, compati- bility with minimally invasive soft tissue management and access to immediate loading of restorations for candidate patients. (11)

Other industrial robot systems (MELFA RV-3S, Mitsubishi Electric Corporation, Tokyo, Japan). Those robots have six degrees of freedom (DOF), a position repeatability of ±0.02 mm and showed an error of 1.42 ± 0.70 mm were also used in studies describing robot-assisted implant placement.(12)

- **Robots in Maxillofacial Surgeries.**

A lot of systems have been proposed comprising surgical robots with optical surgical navigation systems and some kinds of hard tissue lasers that are able to automatically perform an osteotomy operation according to a preformed surgical plan. During the operation, the robot is proposed to register patient movements by real-time tracking. Robotic surgical techniques are being used for milling of bone surfaces, drilling of holes, deep sawing osteotomy cuts, selecting osteosynthesis plates, bending and intraoperative positioning in a defined position, and orthognathic surgery planning.(13)

Advantages:

- Minimize human-related factors such as reduced concentration, trembling, distraction or reduced vision that affect the accuracy and safety in maxillofacial surgery.
- Enabled drilling of complex forms of dental implants thus enhancing flexibility in prosthetic rehabilitation in patients with reduced bone supply.

2. Robots in orthodontics:

- **Wire Bending and Customized CAD/CAM Appliance Robotics.**

Accurate arch wire bending is a key technology for fixed orthodontic treatment. Compared with the traditional manual bending system, the accuracy and efficiency of arch wire bending can be improved by using the robot with its precise posture control ability.

Different types of arch wire bending robots have been proposed in the last decade including the **Motoman** UP6 robot, optimizing bending process and properties, **LAMDA system** (Lingual Arch wire Manufacturing and Design Aid), bending only 1st-order bends in the XY plane, **Cartesian type** arch wire bending robot using the third-order and **arch wire bending robot** that could change the pincer automatically as needed . (13)

Moving on to the customized CAD/CAM full appliances including customized brackets and wires manufactured by robots, clinical outcomes were assessed in terms of effectiveness and efficiency in different CAD/CAM systems in comparison to conventional approaches, showing premise in improving or at least achieving similar outcomes to conventional appliances, it also **reduced overall treatment duration**. (14, 15)

- **Robotics in Automated Aligner Production.**

In 2011, Hilliard patented a robotic system for forming features in orthodontic aligners, including a control system, a platen for three-dimensional positioning of the aligner, a heating station for selectively heating a small region of the aligner, and a thermoforming station for manipulating the heated region to form a desired feature in the aligner. The control system can include a processor with CAD software to enable a user to design features for aligners. The present invention enables an automated process for installing activation features and other types of features needed for polymeric shell orthodontic aligners to receive auxiliary devices that serve to expand their usefulness, range, and duration of application. (13)

3. Rehabilitative Robots in Management of TMD.

Massaging robots and mouth training robots have been proposed for the implementation of safe and effective maxillofacial massage and exercises to treat patients with myofascial pain and limited mouth opening by decreasing muscle stiffness significantly. Suitable treatment regimens have been discussed and evaluated, reaching an efficacy of 70.3%. (13)

Different designs were suggested including shoulder- mounted robotic exoskeleton for better esthetics and portability, incorporating visual feedback into therapy routines to promote active participation with safety design considerations. Assisted motion of the jaw using EMG- based feedback systems accurately tracking the progress of a patient over time. (16)

4. Nano-/Microrobots

o Nanorobotic Dentifrice (Dentifrobots).

Subocclusal dwelling nanorobotic dentifrice delivered by mouthwash or toothpaste could patrol all supragingival and subgingival surfaces, performing continuous calculus debridement. with catalytic-ability to destroy biofilm so it is used for prevention of tooth decay or peri-implant infection.

These invisibly small dentifrobots would be inexpensive, safely deactivating themselves if swallowed, and would be programmed for better cleaning of the teeth.

o Nano-sensors for Remote Monitoring of Removable Appliance Wear.

Monitoring of Obstructive Sleep Apnea Oral Appliance Compliance. Sleep apnea monitoring devices are being developed for diagnostic and treatment applications. These can be a safe, reliable, effective, feasible, and affordable option to monitor a person's sleeping patterns and to objectively measure compliance in wearing the OSA oral appliances.

Monitoring of Compliance of Active and Passive Removable Appliance Wear. Compliance in removable appliance wear is a highly variable, multifactorial issue that requires objective measures to be safely addressed in research designs and in clinical practice. Electronic microsensors, such as the **Smart Retainer** and the **TheraMon** proved to be reliable and accurate enough to measure wear time of removable orthodontic appliances by identifying temperature changes, which are then transformed to wear time information. Moreover, they provide the basis for more individualized wear time recommendations for patients with removable appliances, resulting in a more efficient, shorter, and less painful orthodontic therapy. (17)

5. *Robots for Tooth preparation*

Tooth preparation for crowns and bridges is a routine task for dentists, even though it is still challenging. The challenge is to reduce the tooth sufficiently to create space for the prosthetic rehabilitation with a minimum of damage to sound tooth structure.

A mechatronic system to support the dentist in drilling has been tested in vitro and showed good results. The dentist‘s position accuracy was 53% better with the mechatronic system than without it. Yet, it has not been validated in a clinical setting.(18)

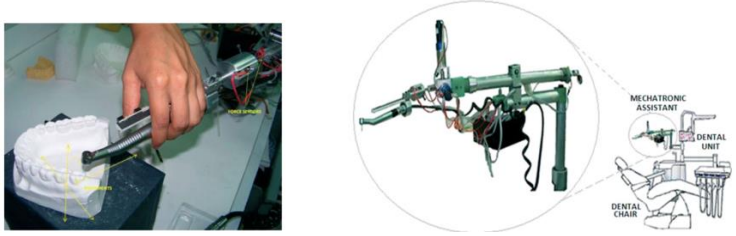


Fig 3: Mechatronic system attached to drill & Mechatronic system attached to dental unit

Yuan et al. described a robotic tooth preparation system with the following hardware components: (19)

- An intraoral 3D scanner to obtain the 3D data of the patient's target tooth, adjacent teeth, opposing teeth and the teeth fixture.

- Computer-aided design (CAD)/computer-aided manufacturing (CAM) software for designing the target preparation shape and generating a 3D motion path of the laser
- An effective low-heat laser suitable for hard tissue preparation.
- 6 DoF robot arm
- A tooth fixture connecting the robotic device with the target tooth and protecting the adjacent teeth from laser cutting.

The developed robotic device achieved precise 3D motion control of a laser focal point and is small enough to be used in the narrow workspace of the oral cavity. which can meet the requirement of typical dental operations.

Another tooth preparation system for veneers with a rotating diamond instrument mounted on a robotic arm was compared to human hand crown prep in an invitro study and showed better results than the tooth preparation carried out by the dentist.(19)

6. Robots for Root Canal Treatment.

Root canal treatment is a procedure which is based on high accuracy. Usually, a dentist specialized in endodontics works using magnification to assure adequate view of the root canal.

Nelson et al. published the idea of a **robotic system for assistance** during root canal treatment. The so-called "**vending machine**" was supposed to supply the dentist with the necessary root canal instruments during treatment in order to reduce deflection from the operating site. (21)

7. Educational robotics

A humanoid full-body patient simulation system **(SIMROID**) is standing 165 cm tall. It comes with a metal skeleton and vinyl chloride-based gum pattern of skin.It was tested in a study among dental students to find out whether a robotic patient was more realistic for the students to familiarize with real patients than the usually used dummies.

The "**Hanako**" is an interesting contribution to education in dentistry as it imitates human in its actions and expressions. It can verbally express pain, roll its eyes, blink, shake its head in pain, perform movements of

jaw, tongue, elbow and wrist. It can even simulate a vomiting reflex with a uvula sensor, and also simulate functions to induce bleeding and saliva flow.

A haptic-based tooth drilling simulator was introduced for dental education with an implemented collision detection system to give force sensation to the user and make the virtual reality (VR) experience more realistic. Moreover, haptic devices for training of dental implant placement or oral anesthesia

It was found that best learning of dental basic motor skills in trainees receiving a combination of VR training with haptic feedback and human instructor verbal feedback.

The **ROBOTUTOR** is robotic educational equipment. That was was developed as an alternative to a clinician to demonstrate tooth-cleaning techniques to patients. It is a robotic device to train and show brushing techniques. A study among patients showed that the ROBOTUTOR was the most attractive method according to patient evaluation or dental health care education compared to other methods (clinician or video audio tutorial).

8. X-ray imaging robots

Positioning of the film/sensor and the X-ray source was proposed to be executed by a 6 Degree of Freedom robotic arm and was found to have no adverse effects. Results showed that the robotic system was superior to the mechanical alignment approach, due to its excellent accuracy and repeatability.(4)

9. Robotics in tissue engineering

In an attempt to improve conventional tissue culture methods and expand them to large-scale manufacturing, advanced robotics are used in manufacturing. Not only could robotic manufacturing produce a larger scale of cells without the need for training personnel, but fabrication could be performed in a closed system, reducing the risk of contamination and thus further saving costs by eliminating the need to discard contaminated cells.

Moreover, using machine learning algorithms and imaging techniques, manufacturing robots could be trained to identify cells in a culture that have successfully undergone genetic modification or differentiation and then isolate them, thus improving the efficiency of stem cell-derived cells for tissue engineering. (24)

III. Achievements of AI in dentistry

- Assists the clinicians so they can offer **high-quality dental care** to their patients.
- Dentists can use AI systems as an **ancillary tool for increasing the accuracy** of diagnosis, treatment planning, and predicting the treatment outcomes.
- Non-specialty dentists can receive **diagnostic support** via the deep-learning systems.
- Automated systems can save a lot of **time** and increase the **efficiency** of the clinicians
- The use of these systems for **secondary opinions** can improve the accuracy of diagnosis. (2)

IV. Limitations and Challenges

- **Data Acquisition**
  - Insufficient data
  - The lack of information on data processing & measuring
  - Sample size used for training and testing as well as the information for reference and comparative tests are deficient & sometimes unclear.

It is necessary to improve data quantity, quality, and readability by standardizing methodology in data reporting. Establishing an open-access standard data base, which contains comprehensive demographic, clinical, experimental, and treatment data, would be a crucial task in the next stage of AI development to facilitate evaluation and comparison of different algorithms. (4)

- **Interpretability**

Data-driven AI calculates output in a purely computational manner; however, it fails to illustrate the decision-making process in a medically acknowledgeable format. The lack of interpretability and transparency reflects the black-box nature of many ML approaches.

Interpretability matters for 2 reasons. First, ensuring that the algorithm is a reasonable interpretation of medical incidents is important for the **rapport between technology and humans**. Failure to explain the inner working will inevitably disrupt practitioners' trust in the clinical value of AI.

Second, the lack of transparency and interpretability makes it difficult to predict failures and generalize specific algorithms for similar contexts. (4)

Computing Power

Extracting information from constantly updating medical and dental databases for the application of AI requires continuous upgrading of processing power. Because the computational power of classical computers has been largely saturated, the insufficient computational resources in data processing have become one of the obstacles that constrain the efficacy of AI. (4)

Ethical Considerations

Development of AI should ensure that such intelligent technologies do no harm to humans. Incorporating AI into health care would inevitably replace some established services and potentially exacerbate current health inequalities. These ethical paradoxes emphasize the need to establish clear guidelines for the manner in which AI is applied clinically.

Judgment of legal responsibility is another ethical dilemma. At present, AI is not accountable. The physician takes total responsibility for each patient and for how information is used. Applying the same social and ethical norms acceptable to humans is inappropriate when the border of human responsibility is increasingly blurred by the advent of chatbot-based, unsupervised AI–based diagnosis. (4)

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Biomaterials Journal
http://www.biomatj.com
Online ISSN: 2812-5045

Type of the Paper (Review Article)

Characterization of Dental Materials: Time-of-Flight Secondary Ion Mass Spectroscopy, Dynamic Mechanical Analysis, and-Focused Ion Beam

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Abstract: Characterization of dental materials is necessary for understanding and identifying the materials composition, structure, and properties. Many characterization techniques and methods are used for surface characterization analysis, evaluation of dynamic moduli, and investigation of the materials surface and subsurface through electrons and ions. Time-of-flight secondary ion mass spectroscopy technique is used for materials surface characterization, analysis of surface molecules and surface-mediated reactions. Dynamic mechanical analysis is a technique that measures the complex moduli and study the viscoelastic properties of solids. Focused Ion Beam technique is used in imaginging of materials' surfaces using ion beams, deposition of materials such as platinum and carbon onto the materials surface, and milling of materials.

Keywords: *ToF SIMS; Dynamic mechanical analysis; complex modulus; surface characterization; Focused Ion Beam.*

Citation: ***Yasmeen S. Mahmoud . Characterization of Dental Materials: Time-of-Flight Secondary Ion Mass Spectroscopy, Dynamic Mechanical Analysis, and-Focused Ion Beam.*** *Biomat. J., 2 (2),46 – 52 (2023).*

https://doi.org/10.5281/znodo.5829408

Received: 10 February 2023
Accepted: 20 February 2023
Published: 28 February 2023

1. Time-of-flight secondary ion mass spectroscopy (ToF SIMS)

ToF SIMS is a qualitative technique used for the surface characterization analysis in materials science as it provides information about entire or fragments of molecules from the outermost surface of the sample. It is applicable to any surface-mediated reactions such as sorption, redox, dissolution, precipitation, coprecipitation, catalysis, etc [1].

ToF SIMS is based on measuring the mass to electric charge ratio of a given ion (m/z). From the weight to charge ratio of an ion, it is possible to determine the molecular weight of the entire or fragments of the molecule. The ionization methods are chosen such that the charge (z) is 1 for most ions, so when interpreting the spectrum, it can be assumed that m/z corresponds simply to the molecular weight of the ion [2].

1.1. Basic principle of ToF SIMS:

ToF SIMs uses a pulsed ion beam (commonly Cs, Bi, or Ga) that penetrates 1–2 nm under the surface, to remove molecules. The molecules removed from the atomic monolayers of the surface (dislocated secondary

ions with positive or negative charge), are accelerated into a "flight tube" until reaching the detector which detected the molecular mass by measuring the time of flight [1].

1.2. Types of Secondary ion mass spectroscopy (SIMS) analysis:

1.2.1. Static (SSIMS):

The incident ion on the surface (about 10-10 A) generates the ejection of secondary ions coming from the first or two layers of the material surface and the damages on the surface can be ignored because they are minimal. When insulating materials like polymers are studied under SSIMS, some charging problems on its surfaces can be observed, and this problem affects the analysis, decreasing the sensitivity.

1.2.2. Dynamic SIMS:

The incident ion on the surface (about 10-7 A) causes a rapidly eroded surface and the information obtained is not exactly from the first layers (1–2 nm). For that reason, dynamic SIMS is ideal to get information from depth profiling.

1.3. Disadvantages of ToF SIMS:

- Due to the high sensitivity of this technique, only high purity solvents should be used.
- It is a destructive technique due to the impact of the ion beam that destroys the first monolayers on the surface's sample.
- This equipment has limited optical capabilities; sometimes has difficulties in collecting positive or negative ion data.
- Depending on the nature on the sample, the analysis can vary between 30 min and 5 h
- ToF SIMS is a qualitative technique able to determine fragments of the material exposed to analysis. The presence of a peak of specific element does not represent a quantitative information [1, 2].

1.4. Advantages of ToF SIMS:

- It can determine elemental and chemical mapping on a sub macroscale by surveys (of positive and negative ions (positive and negative spectra), individual isotopes, and molecular compounds) of all masses on materials science.
- Samples such as metals, ceramics, organic and biological materials, polymers, biomaterials, composites can be analyzed distinguishing species of similar nominal mass.

- The traces of elements are detected in the order of ppm (parts-per-million (10−6)) for most chemical species[1].

2. Dynamic mechanical analysis (DMA)

Dynamic mechanical analysis (DMA) is an important materials characterization tool which measures the complex elastic moduli of solids. Static force-assisted mechanical studies cannot resolve the complex moduli of a material. There are different moduli based on the type of deformation, such as Tensile modulus, Compressive modulus, and Shear modulus [3].

The complex modulus of a material contains both storage (recoverable energy, elastic) and loss (thermally dissipated, viscous) parts. The study of the solids response to dynamic stimulus is important in different fields where the frequency-dependent modulus variations is an important measure such as paint industry, adhesive development, plastic hip joints and dental fillings, contact lenses, heart valves, mechanical dampers, and airbags [3].

Evaluation of complex modulus is important to study the viscoelastic properties of a solid, where no material is ideally elastic (ideal solid) or viscous (ideal liquid) in nature. As the applied frequency becomes higher, the material becomes more like a solid (higher storage modulus) and at lower frequencies liquid-like (lower storage modulus) behavior will dominate. Moreover, the modulus depends on temperature, at higher temperatures the material behaves like a liquid [4].

2.1. Uses of DMA:

- It can give information about phase transitions in materials such as polymers. Phase transitions occur due to inter-molecular rearrangements as a response to the applied frequency or temperature[4].

- It can be used to study composites and mixtures, and the complex interactions among their constituents.

- DMA is an efficient method to resolve stress or strain hardening or softening due to cyclic loading.

- For example: Certain composites matrices such as poly(dimethylsiloxane) impregnated with vertically aligned carbon nanotubes show such self-strengthening under cyclic loading because of complex interactions between matrix materials and nanostructures [5, 6].

- DMA is helpful in the assessment of materials properties in terms of operational temperature, load, frequency, other external parameters, and inter-material interactions.

The present DMA based experiments can simulate the real-time performance of materials. They measure the strain of as small as 1 nm with a load (force) range of 0.0001N– 18 N in a

wide temperature range (150 C–600 C), in different humid conditions, and can mimic the mechanical relaxations of solids in different environments. An advanced tool in DMA can conduct dynamic mechanical studies in liquid environment. They can be used to study or simulate the performance of biological membranes in actual physiological conditions [4].

2.2. Basic working principles of DMA:

The viscoelastic behavior of a material is evaluated as a function of time, temperature, and frequency. A sinusoidal oscillatory force is applied to the material and the resulting deformation or strain is measured in response to the applied stress in the linear viscoelastic region of the material. It is important to evaluate the linear response of the material before the dynamic force (strain) based experiments. The amplitude of the dynamic perturbation should be so small that it should not go beyond the linear Hookean region of the material [4, 7].

The response of the viscoelastic material to the dynamic stimulus will not be in-phase with the stimulus. The phase difference is 90° for a pure viscous material**,** 0° for a perfectly elastic material, and intermediate for a viscoelastic material. By evaluating the phase lag (δ), the material's properties such as the ability to flow (viscosity) and the stiffness (modulus) from recovery could be calculated. The loss modulus which measures the energy dissipated as heat and represents the viscous portion. The loss factor tan δ determines whether a material presents a predominantly elastic or viscous response when subjected to load while in service. For a material with tan δ greater than 1, the viscous component predominates [8].

The sample is placed in a holder and the dynamic force is applied using a force motor. The displacements are measured by linear variable differential transformer or optical encoders with high-precision for measuring linear displacements over a wide range. The temperature is an important parameter which affects the mechanical properties of viscoelastic materials such as polymers, therefore the sample chamber should be kept at a constant temperature [4, 7].

2.3. Different DMA modes:

a) Multi-frequency:

The multi-frequency mode evaluate stress/strain variations as a function of frequency. It is used to determine the glass transition (Tg) and melting temperatures (Tm) of polymers. At higher frequencies, the material has solid-like behaviour whie it becomes liquid-like at lower frequencies.

b) Multi-stress/strain:

Varying stress/strain is assessed while frequency is held constant.

c) Controlled force/strain rate:

Stress or strain is changed at a constant rate. The Young's modulus is calculated from the linear portion of the curve. Then, it enters the plastic region until failure. The area under the curve represents the energy required to break the material which is the toughness of the material [9].

d) Iso-strain mode:

The strain is held constant during a temperature ramp. It can be used to measure the shrinkage force in films and fibers.

e) Creep–recovery:

Creep testing is performed through application of a constant stress to the sample and the deformation is measured as a function of time. After the removal of stress, the material is allowed to relax, which is called a recovery test [7].

f) Stress relaxation:

Stress relaxation is the reverse of creep recovery test. The strain is held constant, and the stress is recorded as a function of time [3].

3. Focused Ion Beam (FIB)

Biological 3D imaging is made by Serial-Section TEM, in which sequential sections of resin-embedded samples are imaged. This technique allows high-resolution imaging in x- and y-planes, while the z-resolution is limited by the slice thickness. This 3D reconstruction suffers from poor resolution, as well as from distortion and shrinkage of the tissue due to the larger electron dose. Another method for obtaining 3D images is the use of Serial block face SEMs. This method destroys the sample, and it is prone to charging artifacts and slicing artifacts such as knife marks, holes, folds, compression and/or stretching[10].

Poor control over the thickness of each slice can also generate artifacts in the 3D volume, resulting in inaccuracies in the high-resolution 3D reconstruction of features in the sample. Therefore, 3D imaging artifacts can be reduced or eliminated by milling of the sample using a focused ion beam (FIB) [10]. FIB systems are similar to SEM, the only difference is the use of an ion beam rather than the electron beam for scanning the sample surfaces. A focused beam of metal ions is generated by a liquid metal ion source (LMIS) which can produce ions of ≈5 nm in diameter. Gallium is the most preferred LMIS because of its low melting point, low volatility, and low vapor pressure[11].

When Ga is heated the liquid metal flows down a needle tip to which a voltage is applied. This voltage results in Ga+ ions emission and the resulting beam is accelerated, directed through several apertures, and focused through a series of lenses at the sample surface[11]. Ga+ ions are heavy, and when they meet atomic nuclei in a sample, they cause efficient 'sputtering' (i.e.,

removal of the substrate) at a rate that is dependent on the material itself, and on the beam parameters [10].

3.1. Applications of FIB technology:

a) Scanning Ion Microscopy

When a solid sample is irradiated with focused Ga ion beam, secondary electrons are generated and emitted from the surface can be detected and observed as images. The secondary electrons produce a contrast depending on the crystal orientation of each grain producing a Scanning Ion Microscope (SIM) image. By observing SIM images of metal polycrystals, it is possible to obtain knowledge about the size and distribution of crystal grains. However, resolution is inferior to SEM images (SIM: 4nm, SEM: 0.5nm)[12].

b) FIB-SEM dual-beam microscopy:

FIB-SEM forms a powerful tool for 3D imaging. The SEM column (usually oriented vertically) and an FIB column oriented 45°–55° with respect to the microscope column have their own systems of lenses, apertures, and electronics, and generally operate independently of each other. A sample placed in the evacuated FIB-SEM instrument chamber can be interrogated by either of the beams, or by both beams simultaneously when placed. This allows the user to mill (with the FIB) and image (with the SEM) a specific location on a sample without tilting or moving the stage [13].

c) Deposition:

The FIB can also be used to deposit material (platinum or carbon) on different surfaces. Deposit materials are supplied by gas injection system (GIS) that contains the chemical gas compound which is the precursor form and consists of organometallic molecules. When this compound is exposed to the region of interest, beams decompose the molecules locally and deposit highly pure material onto the surface. Platinum and carbon coatings reduce the curtaining effect and allow automatic beam tuning and slice-thickness control [14].

d) Preparation of TEM samples:

Ultramicrotomy with a thin diamond blade which is used for preparation of the thin sections for TEM has several limitations such as: non-uniform slice thickness, inability to choose specific sites of interest, and difficulty when hard or brittle materials such as dental enamel, ceramics and dental composites are used. FIB milling as an alternative milling method works by ablation of a small amount of material when a primary ion beam hits the sample surface with precision milling down to a nanoscale. However, FIB may cause local heat production along the beam path that could alter or damage the sample. The beam damage could be minimized if the thinning is conducted under cryogenic conditions [15].

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Biomaterials Journal
http://www.biomatj.com
Online ISSN: 2812-5045

Type of the Paper (Review Article)

Characterization of scaffolds

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Citation: ***Reem A. Hany.Characterization of scaffolds*** *. Biomat. J., 2 (2),53 – 67 (2023).*

https://doi.org/10.5281/znodo.5829408

Received: 10 February 2023
Accepted: 15 February 2023
Published: 28 February 2023

Abstract: complete characterization of scaffold structure and properties is essentially the first step in the process of developing successful bone engineering scaffolds. Numerous studies demonstrate that pore size, interconnectivity and porosity affect bone tissue regeneration, and these three design features appear to be the most important structural variables in an initial scaffold screening. Clearly, besides scaffold morphology and mechanical and surface properties, biological characterization of scaffolds by suitable cell culture methods is also required.

Keywords: : *Scaffold, characterization.*

The most important function of a bone tissue engineering (TE) scaffold is its role as a template that allows cells to attach, proliferate, differentiate, and organize into normal, healthy bone as the scaffold degrades. Depending on the final application, scaffold requirements include matching the structural and mechanical properties with those of the recipient tissue and optimization of the micro-environment to support cell integration, adhesion and growth, issues that have become known as structural and surface compatibility of biomaterials. Scaffolds have to fulfil many requirements, such as osteo-conductivity, appropriate rate of biodegradation, interconnected porosity, suitable mechanical strength, and structural integrity. Therefore, the complete characterization of scaffold structure and properties is essentially the first step in the process of developing successful bone engineering scaffolds. Numerous studies demonstrate that pore size, interconnectivity and porosity affect bone tissue regeneration, and these three design features appear to be the most important structural variables in an initial scaffold screening. Clearly, besides scaffold morphology and mechanical and surface properties, biological characterization of scaffolds by suitable cell culture methods is also required.[1]

1. Structural (Architectural) characterization

1.a. porosity

Porosity is a measure of the void space in a material that can be determined from the ratio of the void volume to the bulk material volume. Porosity is known to play a role in determining cell seeding efficiency in addition to the diffusion properties and mechanical strength of a scaffold. However, it is not a unique parameter (i.e. structurally different materials can have identical porosities) and it cannot be used on its own to sufficiently characterize scaffolds.[1]

N.B: No single investigative technique is able to fully characterize the porous nature of scaffolds if they exhibit porosity in different scales, e.g. in some cases from nano to microporosity.

Characterization tools for scaffold porosities

Calculated either physical (manual) or automated image analysis

Porosimetry

Porosity assessment via porosimetry is based on the study of the flow of gases or liquids (or both), across a porous structure. Therefore, this method is only suitable for the

detection of open pores that allow fluid transport. Consequently, standard porosimetry methods cannot be used to assess total pore volume.[1]

i. Mercury intrusion porosimetry (MIP)

MIP Are based on the pressurized penetration of a liquid mercury into a porous structure and is a very useful method in characterization of macro-porous materials. Mercury has a high surface tension and therefore forms large contact angles with most other materials (~=130◦). In addition, mercury does not spontaneously penetrate pores by capillary action, and therefore an external pressure must be applied to force mercury into the pores. The pore diameters (size) intruded by mercury can be calculated according to the following equation:

$$D = \frac{-4\gamma\cos\theta}{p}$$

where D is the diameter of the pore, γ is the surface tension of mercury, θ is the contact angle between mercury and the solid and p is the applied pressure.

The direct data acquired is the accumulated volume of mercury entering the porous system. A pore size distribution curve is obtained by relating the log differential intrusion volume to log pore diameter. [2]

Limitations:

1. As with flow porosimetry, closed pores are hidden from the test.
2. Fibrous meshes are susceptible to undergo mechanical deformation under the pressures attained in a mercury porosimetry experiment.
3. Assuming that pores are cylindrical is often an over-simplification. It may not provide an accurate surface area measurement, due to a well-known problem associated with the so-called "bottle neck" effect. This occurs where large cavities exist behind narrow necks, which are considered to be pores having the diameter of the neck.[2]
4. Mercury health hazards: exposure to mercury may cause irritation to the eyes, skin, and stomach; cough, chest pain, or difficulty breathing, insomnia, irritability, indecision, headache, weakness or exhaustion, and weight loss. Workers may be harmed from exposure to mercury.[3]

N.B: Liquid intrusion porosimetry is equivalent to MIP, with the exception that other non-wetting liquids such as oil or water are used. Due to the lower viscosity of the liquids applied here and the pressures needed, smaller pores may be measured, as small as 1 nm.[4]

ii. Liquid displacement method:

Liquid displacement is a method often used to characterize scaffold porosity. In the liquid displacement method, the scaffold is added to a known volume of liquid V1, commonly ethanol, and is often assisted by various techniques to ensure that all pores are completely filled with liquid.

The resulting volume V2 is measured, and the impregnated scaffold is removed from the container. The volume of the liquid in the bath after scaffold removal V3 is recorded, and this volume is equivalent to the void volume. From these measurements, porosity can be calculated according to Equation:

$$\text{porosity} = \frac{V3}{V3 + V2 - V1}$$

N.B: This method was also used with hexane as a displacement liquid due to ethanol potentially shrinking the silk scaffold.[4]

iii. Liquid pycnometry (Archimedes principle):

Liquid pycnometry follows the same steps as the liquid displacement method, with the exception that instead of measuring volumes, weights are measured. The whole procedure takes place in a pycnometer filled with a liquid. First, the weight of the liquid W_1 and the dry weight of the scaffold W_s are measured, then the scaffold is inserted into the liquid. Once this has been achieved, more liquid is added to compensate for the liquid initially displaced by the scaffold until the pycnometer is full again.

The weight of liquid and scaffold together W_2 is recorded, and then that of leftover liquid when the scaffold has been extracted W_3. The porosity can be calculated using Equation: [4]

$$\text{porosity} = \frac{W2 - W3 - Ws}{W1 - W3}$$

iv. Gas pycnometry:

Used to measure the volume of solids based on Boyle's law (PV=K), where P is pressure, V is volume & K is constant. The most basic setup for this approach requires a reference chamber of a known volume connected by a valve to a sample chamber, and a manometer (pressure measuring tool) associated to each chamber. First, the pressure of both chambers is measured. Then, the valve connecting the chambers is opened, allowing the pressure to reach an equilibrium.

Where p1C is the initial pressure of the chamber containing the sample, V1 is the gas volume in the chamber, V2 is the volume of the reference chamber, p2C the initial pressure of the reference chamber, and pO is the equilibrium pressure reached after the valve is opened.

The volume of the sample can be calculated. Gas pycnometry allows quantitative assessment of scaffold porosity. The approach to convert the volume to porosity is by measuring the apparent volume of a cube that the scaffold has been cut into, using a caliper. The measured pycnometer volume is then inverted and divided by the apparent volume, the result being porosity, as shown in Equation: [4]

$$\text{porosity} = \frac{V_{\text{Apparent}} - V_{\text{Pycnometer}}}{V_{\text{Apparent}}}$$

Closed pores cannot be characterized by gas pycnometry.[4]

v. Gas adsorption:

Based on The Brunauer–Emmett–Teller (BET) Theory in which a gas, commonly N2, adsorbs to the surface of a measured solid and provides a quantitative assessment of its specific surface area. The data acquired by BET measurements have the form of isotherm curves providing information on each adsorption layer. These adsorption layers are schematically shown in (Figure 2i), The resolution of this method is extremely high, since it can characterize the surface area of pores as small as 0.5 nm (but only as large as 2 μm).

N.B: The curve showing the relation between pressure and volume of a given mass of gas when the temperature is constant is called its isothermal curve. It is represented by the equation. PV= constant. [4]

Methods to visualize and subsequently quantify porosity in scaffold structures are:

i. Scanning electron microscopy (SEM):

Scanning electron microscopy is a method in which the surface of a sample is examined, providing information on its morphology. Commonly, to achieve this, an electron beam is directed at the sample, which then excites the atoms on the surface, causing secondary electrons to be emitted. These are then detected, and an image can be constructed. Samples are often nano sputtered with gold, platinum, or other conductive materials to render the surface conductive and to avoid charging effects. Further preparation steps are often taken in order to ensure the sample is dry.

An advantage of SEM over physical methods is that it can qualitatively assess cell growth on surface layers. SEM has also been used to characterize scaffold porosity by converting images to binary (back scattered), whereby fibers are black and pores are white or vice versa.

Limitation: These images are a 2D projection of a 3D pore structure. Its limitation is what might appear to be a closed pore in 2D could actually be an open pore in 3D.

One extremely different approach consisted of using a focused ion beam (FIB) to remove surface layer coating and progressively take 2D SEM images of the subjacent layers,

allowing for subsequent 3D tomography. It was concluded that the 2D imaging was insufficient in providing insight into the true morphology of the scaffold, however, the 3D method caused results with much noise and was not compared with other 3D characterization methods.[4]

ii. TEM:

Transmission Electron Microscopy (TEM) is a microscopy technique that is equivalent to light microscopy, except it uses electrons instead of photons, i.e., light, thus allowing for a much higher resolution due to the easily achievable small wavelengths.

Limitation: However, the optimal thickness for studied samples is in the low nm range, rendering it unsuitable for many scaffolds, and only having the potential to examine individual fibers containing nanomaterials.

iii. AFM:

Is a technique that is used to map the topography and to study the properties of materials on micro and nanoscales. AFM uses a probing tip at one end of a cantilever to interact with the material (sample). The interaction between the sample and the tip gives rise to either attractive or repulsive forces.

It can operate in contact, non-contact or tapping mode. The forces exerted on the cantilever by the surface of the sample can then be characterized by a laser-detector setups. These forces give information about the topography of the sample with resolutions of under 1 nm. If the tip and the sample are close to each other, the attractive force deflects the cantilever towards the sample, and when the tip is brought into contact with the sample, the repulsive force detects the cantilever away from the sample.

A laser beam detects these deflections, any deflection will cause changes of the direction of the reflected beam. The most popular ways of obtaining topography imaging are contact and tapping modes. The tapping mode, in comparison with the contact mode, presents the advantage of reducing friction forces when scanning (soft) samples.

One advantage of AFM is that it allows for the characterization of not only morphological, but of mechanical properties.[4]

iv. Confocal laser scanning microscopy (CLSM):

CLSM is a technique that allows for filtering and increased resolution of light microscopy. It can be combined with fluorescence microscopy to increase the resolution and is therefore another powerful tool to image cell growth. Due to the ability of CLSM of obtaining 2D images at different depths, a 3D image can be reconstructed.

Limitation: Confocal microscopy requires fluorescence staining of collagen, which may affect cell–matrix interactions because staining can change the density of ligands on the surface of scaffold.[5]

v. Computed tomography (μ -CT):

Micro Computerized tomography (CT) is a technique in which X-rays are used to create a 3D image of the structure of a sample. It is a **qualitative** and **quantitative**, **non-destructive** method for obtaining the fiber diameter and alignment, porosity shape, pore size and interconnectivity and distribution all from one measurement by providing a complete slice by slice 3D scaffold representation. Pixel has a side size in the μm range. This allows for the characterization of μm-scale structures. μ-CT can access both connected and isolated pores enabling the **total void volume** to be determined.

Can scan all types of materials in hydrated or dried state (i.e., polymers, ceramics, metals, and composites) obtained through various fabrication methods (e.g., membranes, fibers, porous scaffolds, particles). However, the visualization of certain materials is somewhat problematic. Among them, polymers that exhibit low X-ray absorbency. [6]

To improve their contrast in CT imaging—in either dry or hydrated state—several staining agents typically used in histology were tested as barium chloride, iodine, potassium iodide, and silver nitrate as contrast. 1.5 wt.% barium chloride is the ideal amount in order to preserve the initial morphology and improve the image contrast.[6]

Nano-CT uses the same technology as micro-CT, with the pixel sizes being in the nm range, making it a tool capable of characterizing many features of nanoscale scaffolds, however, many research institutions lack access to nano-CT equipment.[4]

N.B: Other imaging techniques, such as scanning electron microscopy (SEM) associated with EDX, atomic force microscopy (AFM), or confocal microscopy (CFM), provide important information regarding surface morphology, topography, and chemical composition. Nonetheless, their use is associated with several drawbacks, such as the need to destroy the sample to obtain suitable geometries that can be further analyzed, and the registered data provide information only with respect to the surface of the sample (SEM, AFM) or thin 3D sections (CFM).[6]

1.b. Permeability

Successful bone TE depends on the scaffold's ability to allow nutrient diffusion to and waste removal from the regeneration site, therefore, permeability is a key parameter for the design of scaffolds. Permeability is directly related to the degree of pore interconnectivity. Several permeability measurement systems have been developed for determining the permeability of scaffolds.

Characterization tools for scaffold permeability

i. Intrinsic permeability testing

The physical principle used for the measurement of permeability is based on the measurement of the pressure drop caused by the introduction of the scaffolds in a fluid (e.g. water or cell culture medium). Immediately prior to intrinsic permeability testing, each scaffold's length and diameter is measured using digital calipers. Scaffolds are then carefully wrapped with Teflon® tape and placed into a custom-built flow-rate-controlled permeability chamber. The chamber is connected with a flow-through blood pressure transducer and syringe pump.

Deionized water was then pumped through the sample at a constant flow rate, and the pressure drop across the sample was recorded until the system reached steady state. Intrinsic permeability can be easily determined by applying simple mathematical relations. According to Darcy's law which states that states that the flow rate through a sample is proportional to the applied pressure, intrinsic permeability k [m2] can be obtained by: [7]

$$K = \frac{Q \mu L}{A (Pb - Pa)}$$

where Q is the flow rate (m^3/s), A is the cross-sectional area (m^2), Pb - Pa is the drop pressure between two points, μ is the dynamic fluid viscosity (Pa s), and L is the scaffold thickness.

ii. MRI methodology

High-resolution MRI is used for characterizing the permeability and fluid velocity. The sample is sealed in a specially constructed MRI probe consisting of a flow chamber surrounded by a solenoid RF coil (electromagnetic coil produces uniform magnetic field) and positioned in the MRI scanner.

Cross-sectional fluid velocity images are generated using a phase-encoding MR imaging technique. A whole-body MR scanner (equipped with a spectrometer is used to obtain MR image as a 3-D complex matrix.[8]

1.c. Crystallinity

Processing parameters are known to affect the polymer crystallinity. When processing PCL using solution electrospinning the extremely rapid removal of solvent may be expected to result in little opportunity for crystal nucleation and hence poor crystal

structure. In contrast, drawing of the fibers in the whipping region of the jet enhances the crystallinity and orientation of the polymer chains.

The crystallinity of scaffolds can be characterized by:

i. X-ray powder diffraction (XRD)

Is a rapid analytical technique primarily used for quantitative determination of crystal phase content and composition and can provide information on unit cell dimensions. Based on the ability of crystals to diffract X-rays in a characteristic manner allowing a precise study of the structure of crystalline phases. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings.

It is used to study the crystalline content, identify the crystalline phases, spacing between lattice planes, and epitaxial growth of crystallites. Since every material has its unique diffraction patterns so materials and compounds can be identified by using a database of diffraction patterns. The wide angel XRD (WAXD) (diffraction angle greater than 5) technique is used to determine the degree of crystallinity of polymers and fibers. It can also be used to determine the chemical composition or phase composition of a film, the texture of a film, the crystallite size and presence of film stress.[9]

2. Mechanical characterization

Clearly, understanding the correlation between pore structure, porosity, and scaffold mechanical properties is crucial in the process of optimization of scaffold architecture. The regeneration process is also influenced by the mechanical properties of the scaffold. An artificial substrate conveys to cells' physical signals (e.g., stiffness) that regulate many processes in regeneration, such as cell proliferation and migration. For this reason, the mechanical compatibility of the material is fundamental in determining the outcome of the regeneration process and the scaffold would rather resemble the mechanical properties of the native tissue.[10]

Accurate measurement of mechanical properties of scaffolds for biomedical applications is essential, to guarantee they can withstand the forces during surgical operation and those exerted by physiological activities and/or by tissue growth. [10]

The mechanical properties of scaffolds can be characterized by:

- The standard mechanical tests are **uniaxial tension, compression, and indentation**. In most of the cases, compressive mechanical testing is used to measure the mechanical strength of a scaffold. For a tensile test to be accurate and repeatable it is important to report macroscopic dimensions (gauge length and cross sectional area), the strain rate, the applied load, as well as whether they have been performed at room temperature or under physiological conditions (at 37 C, in PBS or culture media).
- Uniaxial tensile testing gives information about the Young's Modulus or stiffness (E) in tension (slope of the initial linear stress-strain), yield strength (end of linear elastic region, beginning of non-linear plastic region), fracture stress/deformation, and fracture energy (toughness) per volume.
- Other techniques such as AFM-based nanoindentation or bending tests have also been reported to measure the local stiffness, hardness and flexural properties. [10]

i. Compressive strength testing:

Studies usually report the measurement of compression strength via uniaxial testing. Specimens are compressed between two fixed steel plates at a rate of typically 1.0 mm/min. In case of swollen samples, the platens may be permeable to allow the escape of water as the sample is compressed. The load versus displacement curve was obtained through a computerized data-acquisition system.

The compressive strength of the structure may be calculated as as the maximum applied load (carried at the 0.2% offset) divided by the cross-sectional area of the sample. Thus, the compression strength test characterizes the mechanical properties of porous ceramics, hydroxyapatite, bioactive glass and composite scaffolds. The area under the

stress–displacement curve obtained in a compression strength test is usually considered to access the work of fracture, a measure of the toughness of the scaffold. [10, 11]

ii. Tensile strength testing

Mechanically characterized by uniaxial tensile tests at a constant cross-head speed using an Instron (universal) Testing System. The simplest measurement that yields the elastic modulus of a specimen, in which the sample is grasped at two ends and pulled while axial strain and stress are simultaneously measured. Samples are tested at a specific strain until **specimen failure**. By analyzing the obtained stress-strain curves, the Young's modulus (MPa) can be calculated.

N.B: If the sample is anisotropic, additional uniaxial tests in the other two coordinate directions can be used. Stresses can be applied in two (biaxial) or three (triaxial) dimensions simultaneously.[10, 11]

iii. Fatigue

Fatigue can be tested using an Instron testing machine under uniaxial cyclical loading.

iv. Nano-Hardness

Nano-indentation is the most common characterization technique used, chiefly because it allows the hardness of specific areas of an electro-spun membrane to be evaluated with very fine spatial resolution and with minimal preparation.

However, there are problems with precision and sometimes accuracy when the technique is applied to polymer surfaces, especially fibrous and porous ones. Many polymers are too soft to be investigated using nanoindentation, while viscoelastic behaviour (creep) and difficulty in accurately characterizing tip shape prevents accuracy using the traditional analysis of Oliver and Pharr.

Compared with other methods, modulus values of polymers measured by nanoindentation are often much larger or even negative in extreme cases, because of the effects of creep. Recent attempts to characterize the creep response of polymeric materials suggest that the modulus depends on the speed of the indentation and thus there is no single well-defined value for modulus in these materials. The most suitable technique used for the nano-indentation of polymers, which can be related to electrospun membranes include AFM.[2]

N.B: specimens for nano-hardness measurement must be non-porous.

Dynamic indentation

Attempts to characterize the viscoelastic behaviour of polymer samples have also been made using dynamic indentation with an oscillating tip. Storage (elastic part) and loss (viscous part) moduli were determined in studies of different polymer materials.

A hemispherical indenter impacts a disk at velocities from 100 mm/s to 5000 mm/s, typically deforming the specimen to failure. Displacement of the impact head is measured using a high speed video camera and Digital Image Correlation (DIC). We are not aware of studies involving dynamic indentation on porous surfaces but this method does have potential for characterizing storage and loss moduli of electrospun membranes. [2]

Problems faced when applying nano-indentation to fibrous membranes

- There are obvious difficulties for determining the moduli of porous samples using nano-indentation. These include ensuring both an ideal contact between tip and fibre and that the fibre is adequately supported to prevent it bending or slipping away from the probe.
- A more common method is to test a single fibre, which has been spun onto a hard plate. In this situation it was found that the substrate on which the fibre is mounted can affect the measurement if the fibre diameter is below 300 nm.
- Problems involving tip contact with a small-radius fibre; the probe may not contact at 90◦ or it may slip slightly.[2]

Atomic force microscope based on indentation

Imaging is not the only feature from atomic force microscopes. AFM devices can also be used as a "mechanical" machine. In these experiments, an AFM-tip or a colloidal probe

is extended and retracted towards/from the sample of interest. Such motion takes place under controlled displacement speeds. During this process, the deflection of the cantilever is determined as a function of the displacement of the scanner, and the force sensed by the cantilever is calculated using **Hooke's law**. The force-distance curves recorded in this way can be divided into three clearly distinguishable segments (approach, contact with the sample, and retraction). They can be described as follows:

1. The approach curve delivers information about the existing repulsive or attractive forces between the tip/colloidal probe and the sample (e.g., electrostatic, van derWaal forces). These types of measurements have been crucial for the understanding of molecular and colloidal interactions.

2. The second part of the curve, during contact between the cantilever and the sample, provides information about (e.g., Young´s Modulus, stiffness, relaxation time, hardness and viscosity).

3. Finally, the segment representing the retraction motion relates to adhesive forces. The maximum adhesion parameter, or pull-off force, is indicative of the stickiness of the sample.[2]

3. Characterization of surface wettability [12]

Hydrophobic materials are typically characterized by large contact angles (CAs) often (>90°), whereas hydrophilicity is characterized by low CAs (<90°). Routine water CA measurements using the **sessile drop technique** feature the deposition of droplets of water using a syringe and needle controlled by a syringe pump to deposit the water at a nominal flow rate or to produce a standardized volume. Once the water droplet falls onto the experimental material below, a back-lit image of the droplet on the surface in profile is captured by a **high-resolution camera/video contact analyzer**.[24] The contact angle is measured 5 times from different positions on each scaffold and an average value is calculated.[23]

A change in the **contact angle** can be a useful indicator of successful surface modification or blending of the scaffold to improve the wettability, however, the contact angle is also dependent on the surface roughness and porosity. When a droplet of water is placed on a fibrous mesh only a fraction of the water comes into contact with the fibers which decreases the liquid–solid interactions and increases the liquid–air interactions leading to typically higher contact angles than for smooth surfaces. In addition, when measuring contact angles of meshes it is often difficult to extrapolate the circular part of the drop profile with the surface when it is irregular.[12]

For these reasons the water contact angle of PCL meshes/scaffolds can vary greatly and the tested specimen should be non-porous, flat smooth solid surface which presents a challenge in biomaterials research. When PCL is modified with collagen, gelatin or plasma the contact angle reduced to zero meaning it often only serves as a qualitative guide to the success of a modification process when used in this way.[12]

On **porous substrates** an equilibrium between drop and surface is not reached, thus a **dynamic contact angle** is measured. It was experimentally proven that the **advancing** and **receding** contact angles, and **the contact angle hysteresis** of rough and chemically heterogeneous surfaces, are determined by interactions of the liquid (using syringe-needle method). The "advancing contact angle", θa, and "receding contact angle", θr, values are also measured to express the effect of the actual surface roughness and chemical heterogeneity of the substrates. θa indicates the contact angle when the volume of the droplet is expanded through a syringe (or a dispenser) the three-phase contact line is advanced on a fresh substrate. Meanwhile, θr designates the contact angle when the volume of a preformed droplet on a substrate is withdrawn by applying the suction of a portion of liquid from the droplet through a needle, showing the minimum contact angle value before the three-phase line is broken inwards. **Contact angle hysteresis (CAH)** is the difference between θa and θr. CAH = 0 and θ = θa = θr on ideal, atomically flat and chemically homogeneous surfaces. In practice, CAH is around 5–20° on most of the practical surfaces. CAH

value depends on the magnitude of surface roughness and the surface chemical heterogeneity of solids.[27]

Measurement setups employ a camera recording images of the drop and measuring the contact angle from the images. Once the liquid is released onto the substrate surface the instrument starts image acquisition of the drop at different rates. Drops falling on the substrate initially oscillate, stable measurement of the contact angle is only possible after the kinetic energy has dissipated. The image analysis of contact angle instruments also measures the base diameter of the drop and the drop volume over time. In this way not only the contact angle is evaluated for each frame taken, but also the remaining quantity of liquid on the substrate. All contact angle measurements are carried out under standard climate conditions, 23 °C and 50% relative humidity.[25]

Similarly to contact angle analysis**, XPS** has been used only qualitatively to identify new chemical species for surface modified PCL including increased oxygen presence after air plasma, increased nitrogen after inclusion of collagen, –OH and –CO after argon or oxygen plasma.[12]

4. Biological characterization

Scaffolds for bone tissue engineering need to be characterized **in-vitro** by cell culture methods before **in-vivo** and **clinical studies** take place. The cost of in vivo animal studies and the loss of animal lives continue to motivate the development of in vitro screening assays.

In vitro biological characterization of scaffolds:

The in vitro investigations can be divided into two levels:

a. **The first level involves analyzing cyto and structural compatibility of the scaffold materials with selected cell lines**

The first question regarding application of scaffolds for bone TE is the cyto compatibility of the newly developed material. In screening tests, the effect of porous scaffolds on the functions of cell types is investigated by **continuous cell lines**. Different cell types are available for measuring cell behavior on biomaterials for bone regeneration, for example, mouse calvaria osteoblast-like cells and human osteosarcoma cell lines. During various incubation periods of 24 h up to 3 days. Essential parameters are determined, like:

1. Cell **morphology**, are determined using **light or scanning electron microscope imaging.**
2. **The number of attached cells** (by intracellular LDH activity or BrdU assay).
3. **The cell viability** (by mitochondrial activity, MTT or WST-1) are analyzed.

Dynamic bioreactors concepts as **spinner flasks** and **rotating wall vessel** have been developed to mimic the native micro-environment during cell culturing. **Spinner flasks** support the 3D cell culture by continuously stirring cell suspensions. 3D scaffolds can be integrated within the spinner flask systems that permit the seeding and penetration of cells within the scaffolds.

Due to the continuous stirring, spinner flasks generate local high shear regions that damage cells or the integrity of MCS. Therefore, **rotary wall vessel bioreactors** were developed to reduce shear stress by rotating the cell culture chamber rather than stirring the cell culture media. Both bioreactor systems enable enhanced mass transport of nutrients.[1]

b. **The second level considers the interactions of cells and scaffolds, cell attachment, cell proliferation and osteogenic cell differentiation**

There are a number of different types of in-vitro osteogenesis assays currently used that attempt to predict in-vivo performance listed here in historical order

1. In vitro apatite forming ability measured by a simulated body fluid test.

(Its idea is that materials forming an apatite layer on their surfaces are able, in principle, to bond to bone. They also speculated that this apatite formation is reproducible in-vitro

and invented an acellular simulated body fluid (SBF) in which the ion concentrations and pH were nearly equal to these factors in human blood plasma. They found that the in-vivo apatite formation was successfully reproduced in-vitro when these materials were simply soaked in SBF at 36.5 °C. They proposed that the bone-bonding capability of a given material could be evaluated by examining the apatite-forming capability on its surface in SBF).[13]

2. Invitro osteogenic differentiation assays involving seeding of human or rodent osteoprogenitor cells such as MSCs, calvarial bone progenitors, or cells lines derived from an osteosarcoma and evaluating their differentiation via bone protein expression (bone sialoprotein, osteocalcin), alkaline phosphatase and mineral content which represent typical markers to identify osteoblasts.[13]

3. Imaging to determine the cell behavior on the scaffold including cellular attachment, spreading & differentiation.

- **SEM** it can qualitatively assess cell growth on surface layers.
- **μ-CT & Nano-CT** has excellent penetration depth. However, X-ray radiation is ionizing and can damage tissue or samples.
- **MRI** has excellent imaging penetration depth and safety. MRI can provide anatomical, functional, and cellular information.
- **Immunofluorescence microscopy & Confocal laser scanning microscope** has high sensitivity and excellent spatial resolution. Also, various types of biomarkers can be easily used with optical imaging to monitor intracellular signaling and cellular interactions.
- **Multimodal imaging** can be one strategy to overcome limitations of each imaging method and complementarily offer morphological, functional, and molecular information about tissue-engineered constructs. In addition, the multimodal imaging strategy tends to utilize synergetic features of different imaging techniques. Recently, combinations of imaging modalities such as MRI/CT and MRI/fluorescence have been explored for visualization of engineered tissue constructs in preclinical and clinical applications. In addition, contrast agents are also essential for multimodal imaging and various types of multimodal imaging contrast agents have recently been developed.[26]

5. Chemical characterization

Chemical characterization of scaffolds and chemical composition can be obtained by:

i. Fourier Transform Infrared Spectroscopy (FTIR) [14, 15]

FTIR is a useful and convenient tool for determining the chemical composition of scaffolds. It is used to study and identify chemical substances or functional groups in solid, liquid, or gaseous forms. When used with a total internal reflectance (ATR) accessory it provides a quick, semi-quantitative method for confirming the presence of additives as nano-hydroxyapatite (nHA) or gelatin. The ATR method is normally considered non-destructive, however, good contact between the sample and ATR crystal requires applying significant pressure which will damage delicate scaffold morphologies.

FTIR is also useful for determining the protein conformation (which is protein held together by different bonds and folded into a variety of three-dimensional structures. The folded shape, or conformation, depends directly on the linear amino acid sequence of the protein) based on characteristic shifts of amide groups indicative of hydrogen bonding.

Samples should therefore be ground in a mortar to reduce the average particle size to 1 to 2 microns. About 5 to 10 mg of finely ground sample are then placed onto the face of a KBr plate, a small drop of mineral oil is added and the second window is placed on top. With a gentle circular and back-and-forth rubbing motion of the two windows, evenly distribute the mixture between the plates. The mixture should appear slightly translucent, with no bubbles, when properly prepared.

IR Advantages:

1. All kinds of material can be analyzed.

2. IR can provide a molecular fingerprint that can be used when comparing samples. If two pure samples display the same IR spectrum it can be argued that they are the same compound.

3. IR is most useful in providing information about the presence or absence of specific functional groups.

4. Fast, easy and less expensive.

5. Very small amount of sample is required.

ii. X-ray Photoelectron Spectroscopy (XPS) [16, 17]

The most commonly used surface chemical analysis technique for polymers and biomedical materials is XPS. It can identify the elements that exist within a material (elemental composition) or are covering its surface.

XPS is a powerful measurement technique because it not only shows what elements are present, but also what other elements they are bonded to. Each element produces a set of characteristic XPS peaks. These peaks correspond to the electron configuration of the electrons within the atoms, e.g., 1s, 2s, 2p, 3s, etc. The number of detected electrons in each peak is directly related to the amount of element within the XPS sampling volume.

Advantages of XPS:

1. Non-destructive.
2. Surface and elemental sensitivity.
3. Characterize all elements (except H & He).

Disadvantages of XPS:

1. Expensive.
2. Samples must be compatible with high vacuum environment. So, if your sample will outgas when placed under vacuum, XPS is not the right test for your needs.

iii. SEM & Energy Dispersive X-ray Spectroscopy (EDX) [18]

Scanning electron microscopy (SEM) is an effective method in analysis of organic and inorganic materials on a nanometer to micrometer (μm) scale. In scanning electron microscopy (SEM) the surface of a specimen rather than its interior is scanned with an electron beam.

Energy Dispersive X-ray Spectroscopy (EDS) works together with SEM to provide qualitative and quantitative results. The device consists of variable pressure system with the ability to hold any samples (even wet or samples with minimum preparation). The EDS added the advantages of evaluating the composition of various elements in the sample by converting the intensity of x-ray ratio to chemical compositions in a few seconds.

6. Characterization of Scaffold Degradation

i. Characterization of the Biodegradation Process In-vitro [19]

In vitro **hydrolytic degradation** study is typically carried out in PBS at a pH of approximately 7.2 or simulated body fluid (SBF) at 37 °C so as to mimic the aqueous in vivo environment. **Oxidative degradation** of polymers is studied by immersing the polymer in PBS containing CaSO4 and H_2O_2. For an in vitro **enzymatic degradation** study, the sample is typically incubated with lysozyme or MMPs in a simulated physiological environment of pH7.4 and 37 °C.

Due to the long degradation time of certain synthetic polymers, an accelerated in vitro degradation study is sometimes carried out to predict long-term properties of a scaffold by elevating the temperature and/or adjusting the pH conditions. However, note that the degradation mechanisms in acid, alkaline and neutral environments are different, and the elevated temperature can often affect the crystallinity of the polymer. For these reasons, the relevance, applicability, and validity of an accelerated degradation study need to be carefully reviewed.

Optical and electron microscopy techniques are typically used to study the size, shape, and surface morphology of tissue engineering scaffolds. Light microscopy is

sometimes sufficient to measure the changes in size and shape of the scaffold structure. When the scaffold is significantly thick, light sectioning techniques such as laser scanning confocal microscopy, light sheet or multiphoton methods are needed to obtain a clear image of the features of interest.

During degradation, the morphology of the scaffold changes significantly due to liquid imbibition, surface erosion and molecule rearrangement. When such changes are hard to detect with light microscope, then scanning electron microscopy, transmission electron microscopy, and atomic force microscopy are helpful. Mass or weight loss is one of the three main polymeric factors that is directly affected by degradation.

It is commonly characterized by measuring the dry weight of the sample before and after a certain degradation period using Equation:

$$\text{Mass loss\%} = \frac{W0 - Wt}{W0} \times 100\%$$

where Wt is the dry weight of the sample after a certain degradation period and W0 is the initial dry weight of the sample.

N.B: The test is destructive due to the process of sample dryness, therefore, the sample cannot be used to measure degradation at different time intervals.

Fourier transform infrared (FTIR) spectroscopy can be used to detect the cleavage or scission of functional group along the polymer chain during degradation, and Ultraviolet visible spectroscopy (UV-Vis) can be used to detect the decomposition of the polymer's carbon structure.

Degradation occurring initially in the non-crystalline regions, the level of crystallinity in synthetic polymeric scaffold is expected to increase (increased crystalline/amorphous ratio) during early periods of degradation. These changes can be characterized by X-ray powder diffraction patterns (XRD).

ii. Characterization of the Biodegradation Process In-vivo [19]

Characterization of a retrieved scaffold explant from an animal or human patient involves **weighing, sectioning, staining** and **analyzing the histological features** of the sample. Pathological analysis of the implant site and surrounding tissue using histological staining is another helpful tool in monitoring the inflammatory and immune responses and their effect on scaffold degradation. Hematoxylin and eosin staining is useful for visualize cellular infiltration and scaffold degradation.

Mass loss is one of the most direct characteristics that researchers can use to monitor the in vivo degradation of a scaffold. But the harvested or explanted specimen first needs to have all attached tissue removed. This decellularization process uses a series of freeze/thaw cycles in liquid nitrogen followed by immersion in a 37 °C water bath. When the specimen is clean and free of all adhering tissue, it will be dried and weighed.

Magnetic resonance imaging (MRI) allows tracking of the morphological changes caused by the degradation of the scaffold in-vivo. It is safe, has a good tissue penetration depth and soft tissue contrast. Cells are labelled with ferumoxytol and used MRI to investigate the degradation of the HAp scaffold in a bone defect.

Micro computed tomography (micro-CT) has high resolution and deep penetration and can provide images from the macroscale to the nanoscale. conjugation of gold nanoparticles into a collagen scaffold to enhance the contrast and track the degradation profile of the scaffold.

7. Characterization of drug release scaffolds

Current ways of maintaining therapeutic levels of medications within the bloodstream are limited to repeated administration of drugs either via the oral or parenteral route. This is inconvenient and puts patients at risk of accidental or intentional overdoses. To improve this, it is crucial to develop a delivery system that, once administered, can continue to release drugs in a controlled and sustained manner to achieve safe delivery and maintenance of therapeutically appropriate drug levels long term. [20]

Polymeric micro/nanoparticle or micro/nanofibrous scaffolds have been investigated extensively as carrier vehicles for delivery of therapeutic agents. These scaffolds can deliver drugs to a specific predetermined site while avoiding systemic distribution of their cargo. Compared with their particulate counterparts, micro/nanofibrous scaffolds display several advantages:

(i) their physical structure mimics naturally occurring extracellular matrix (ECM), thus supporting cell adhesion, proliferation, migration and differentiation better than particulate scaffolds.

(ii) they exhibit a higher surface-area/volume ratio and higher interconnected porosity with tunable pore sizes, enabling them to release bio-factors such as proteins or genes and facilitate nutrient and oxygen diffusion as well as waste removal. [20]

Drug loading techniques

Hydrophilic drugs like doxorubicin and chloroquine are effectively encapsulated within hydrophilic polymers including gelatin and PVA, whereas hydrophobic drugs such as paracetamol and ibuprofen (IBU) are better incorporated into and released from hydrophobic polymers like PCL, PLGA and PLA. The long-term release of hydrophilic drugs is, however, more challenging compared with that of hydrophobic drugs.

This is because hydrophilic drugs exhibit poor dispersion within hydrophobic polymers, which usually make up at least part of the carrier vehicle and are highly soluble in the release media (usually water based), leading to a higher risk of burst release. Different drug-loading techniques including surface modification, blending, emulsion and coaxial electrospinning have been employed to encapsulate therapeutic molecules into various scaffolds.[20]

Drug release techniques

The drug, for example ketoprofen or antibiotic, is loaded by immersing precisely weighed amount of scaffolds in drug–ethanol solution in a small glass vial for 48 h at room temperature. Then, the mixture is filtered. The concentration of ketoprofen solution after filtering was determined by using a spectrophotometer at 267 nm. The relative amount of loaded ketoprofen by the scaffolds (A) is calculated from the equation: [21]

$$A = V(C_0 - C_1)/W$$

where V is the volume of ketoprofen solution (mL), C0 is the initial concentration of ketoprofen (mg/mL), C1 is the concentration of ketoprofen solution after adsorption (mg/mL), and W is the weight of the scaffolds (g).

Drug loaded scaffolds are then suspended in 50 mL of phosphate buffered solution at pH 7.4 contained in a glass bottle. This dissolution medium is stirred at 100 rpm in a horizontal laboratory shaker and maintained at 37°C in a water bath. Samples are periodically removed for testing and the volume of each sample is replaced by the same volume of fresh medium. The loss of drug content by doing so at each time point is calculated spectrophotometrically to get the correct drug release profile. [21, 23]

Furthermore, the release of the drug from the investigated scaffolds obeyed quasi-Fickian diffusion mechanism. This mechanism is based on hydrolysis as the polymer is hydrated, swell and then the drug diffuses through the swollen matrix system to the exterior, which ultimately slows down the kinetic release. It is also noted that by increasing drug content in the scaffold (5 to 10 and 20 %) the drug release was increased. This might be due to that higher drug content resulted in higher concentration difference between scaffold and the release medium which cause a higher drug release rate.[22]

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