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REVIEW ARTICLE

Role Of 3-D Bioprinting In Forensics- A Review

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ABSTRACT

3D bio-printing involves directly depositing a mixture of high-density living cells and a bio-ink is printed out in an overlapping process by the 3D bio-printer, which is under the design and control of computer. The term bioprinting describes the simultaneous positioning of biomaterials and living cells in a prescribed layer by layer stacking organization to create engineered tissue and organs. The process of Freeform Reversible Embedding of Suspended Hydrogels (FRESH) is a printing method that extrudes bio inks into a yield-stress support bath that holds the bio inks in place until they cure. In-situ 3D bioprinting is a kind of bio-printing which is directly printed onto or into the damaged tissue or organ. This review specifically focuses the current development of FRESH bioprinting and in situ bioprinting and the various challenges and legal considerations in this field.

Keywords: Gender Determination, Gonial Angle, Mental Foramen, Panoramic Radiographs, Lateral Cephalogram.

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INTRODUCTION:

Tissue engineering involves the use of biomaterials and combine scaffold cells and biologically active molecules into functionally active tissue. The main goal of tissue engineering is to replace necrotic and pathological body tissue or organ with substitutive tissue or organ. [1] While regenerative medicine is a wide field of research which includes tissue engineering that incorporates the body system to promote self-healing with help of biomaterials to recreate and rebuild cells, tissue, and organ. With various advances in the medical field the need for organ transplantation increases simultaneously as there are only minimal donor as well as the higher risk of rejection. Tissue engineering appears appealing in these situations to save the lives of patients. There also various shortcoming faced namely the biocompatibility, control precision and controllability.

A greener technology of 3D printing is innovative in the field of tissue engineering. 3D bio-printing involves directly depositing a mixture of high-density living cells and a bio-ink is printed out in an overlapping process by the 3D bio-printer, which is under the design and control of computer. Thus, the printed structure can have a detailed layout with different structural cell types and can be produced in a large scale due to automated 3D-printing.

The aim of 3D bioprinting is to somehow mimic the natural cellular architecture by depositing materials and cells in a particular fashion which can restore the normal structure and functionality of complex tissues. [2] In 3D bioprinting, cells or biomolecules are printed directly onto a substrate in a specific pattern such that the cells can hold together to form the required 3D construct. 3D printing mostly uses bio-ink such as extracellular matrix proteins, cell suspension and/or hydrogel which is a colloidal in nature with various crosslinked structure through covalent bond. These hydrogels have high biocompatibility and biodegradability, which are mostly made up of collagen, alginic acid, agarose, chitosan hyaluronic acid, PEGDA etc,

The various 3D bioprinting technology includes

1. Extruded 3D bioprinting technology either by chemical, photo, or physical crosslinking.
2. Ink-jet 3D bioprinting technology by piezo-electric or hot-bubble by generation of steam.
3. 3D- Laser Bio-printing technology by using high-energy laser pulse. [3]

There is various application of bioprinting in tissue engineering include in construction of blood vessels with favourable elasticity and biocompatibility to replace necrotic blood vessel and can combine with artificial organ, in construction of Specific individual human skeletal bone and teeth, artificial skin with perfusion and with the same texture. The major set back in 3D bio-printing is the distortion of the solid and liquid-like bio ink due to gravity and loss of ink fidelity. There is also inability to deposit the bio-ink layer which collapses as there is no physical support and as well as increased time for curing of the material leading to loss of rigidity and structural stability. [**Error! Bookmark not defined.**]

To overcome these hurdles development of temporary support structure to allow the bio-ink sufficient time for crosslinking and enhance the rigidity has been made using the embedded 3D bio-printing. In this the support material should have properties like Bingham plastic or Herschel–Bulkley fluid, which acts as a solid until a sufficient shear stress (the yield stress) is applied, at which point it transitions from a solid to a liquid-like behaviour. This provides sufficient time for the bio-ink to cure and stay as support until it cures and becomes a solid.[4] Freeform Reversible Embedding of Suspended Hydrogels (FRESH) 3D bioprinting is an implementation of embedded printing developed specifically to overcome the limitations of printing soft and

low viscosity bioinks. The goal is to be able to take complex 3D tissue and organ models, FRESH 3D prints these models out of a wide range of biocompatible hydrogel and cell-laden bioinks within the support bath where the bioink will gel, and then release the printed construct. [4]

HISTORY:

Since the year 2000 there started the usage of 3D printing in the field of medicine. In the year 2003 Thomas Boland had first created the bio-printer. And in 2006 a lab grown bladder was implanted and in 2009 first blood vessels were 3D bio printed. Since then, various research is done in field of tissue engineering for development of tissue and organs. [5]

BIO-FABRICATION OF CELLS:

The main component of any bio fabrication is the process that requires $1x10^7$ to $2x10^7$ cells per ml quantities to reach about $2x10^8$ cells for a bio fabricated tissue or organoid structure. For this cell bio-fabrication, requires the use of mesenchymal stem cell expansion towards the scaling-up of the number of cells. Cell expansion is an essential step for cell and tissue manufacturing. These tissue products are dependent on growth of adherent cells from standard or cell-stack based flat culture plates. [6]

BIO-INKS:

Bio-fabrication involves the bioprinting of cells and biomaterials i.e., bio inks into 3D construction. Bio-inks is defined as a "formulation of cells suitable for processing by an automated bio fabrication technology that may also contain biologically active components and biomaterials." [7] During the initial stages of development these Bio-inks referred to purely cellular component which were patterned on a hydrogel bio paper. Later with various development in this field they refer to the cell-containing hydrogel which are processed and constructed into 3D organs and structure. These bio-inks need to have rheological properties for transition of bio-ink from gel to solid state at an appropriate time, to support the encapsulation of cells and maintenance of viable cells in the 3D-construct. [8] The most commonly is the extrusion-based bio-printing, in which the bio-ink must flow through the nozzle in a low viscosity state and then remain localized on deposition with additional cross-linking. These cross-linking can be physical or photoinduced with initiators and light. There is also a shear thinning hydrogels that disassemble under shear stress and when removed enables crosslinking, which are made of high molecular weight polymers. [7] The lithography-based bio-fabrication involves layer by layer photo cross-linking using a low-viscosity photo cross-linkable hydrogel precursor. The challenges include a longer processing time, maintaining cells in a suspended state as well as incorporating more than one material.

Recent developments in bio-inks include controlled extrusion of low-viscosity hydrogels printing bio-inks into granular support hydrogels that fluidize around the printing needle during extrusion and subsequently solidify to trap the printed structure in 3D space.

BIO-PRINTING:

Bioprinting is defined as the simultaneous positioning of biomaterials and living cells in a prescribed layer by layer stacking organization to fabricate engineered tissue and organs. There are various types of bio-printing technologies based on their working mechanism and deposition means namely

- Extrusion based bio-printing (utilizing extrusion force by means of pneumatic, mechanical, or solenoid-based system)

- Droplet based bio-printing (utilizing electrical, acoustic or thermal energy to generate droplets)
    - Inkjet bioprinting
    - Electro-hydrodynamic jetting
    - Acoustic droplet ejection
    - Microvalve bio-printing
- Laser based bio-printing (utilizing laser power source either by photopolymerization and process based on cell transfer by laser guidance direct writing)

Through these 3D printing we can produce adult organs and tissue, but these materials are not compatible with the body and so their mechanical properties are not replicated, higher incidence of tissue rejection too takes place. But softer thermoelastic materials are softer and they deform due to gravitational force before they develop cross-links between them. [9]

FRESH BIOPRINTING:

Freeform Reversible Embedding of Suspended Hydrogels (FRESH) is an embedded printing by extruding bio inks within a yield-stress support bath that holds the bio inks in place until cured. FRESH technique involves similar extrusion with soft biological materials with a support bath made up of thermos-reversible gelatin microparticles. The aqueous phase of the support bath support multiple crosslinking strategies, such as change of pH, divalent cation, and UV, to gel different hydrogels and other soft polymeric materials. This support bath minimizes or removes the effect of gravity by embedding the printed material, allowing freeform printing of delicate, unsupported structures that would collapse immediately.

Freeform Reversible Embedding of Suspended Hydrogels (FRESH) 3D bioprinting is a newer concept which utilizes the embedded 3D bio-printing and can print 3D tissue and organ models. FRESH embodies three unique aspects:

(i) a support bath that acts as a viscoplastic material with Bingham plastic-like rheological behaviours to achieve freeform fabrication,

(ii) a customizable aqueous phase of the support bath compatible with the multiplexing of gelation mechanisms, and

(iii) support bath liquification for non-destructive print release under biologically compatible conditions. [9]

The Forgacs research group in 2006 used simple collagen gel to hold the spheroids in position till they were fused. To achieve more development, few modifications was adapted namely spheroids were placed within the moulds and within agar-based hydrogel layers.

Hydrogel-based bio-inks needs to be crosslinked in-situ i.e., printing alginate in calcium chloride liquid baths for ionic cross-linking and photo crosslinking using UV irradiation of printed methacrylate gelatin. But these hydrogel inks are mechanically weak, and they need support even after gelation to retain their structure. Shiwarski JD et al in 2021 had used UV curable diacrylate matrix, Pluronic F-127 a sacrificial support that can be removed after printing. In the meanwhile, Feinberg and co-workers were developing and experimenting a FRESH 3D bio-printing approach using a yield support bath made up of Bingham plastic with rheological properties. [4]

FRESH 3D printing was specifically designed to achieve a gel-like support material that can transition from solid to liquid like with material properties that would favour for hydrogel deposition within it. Thus, the aim of FRESH 3D printing was to use unmodified biological hydrogels such as type I Collagen and decellularized ECM with high density cell-laden bio-inks for fabrication of functional tissue and organs with

life-like properties.

In a study by Ma.Y. et al in 2021, for FRESH bio-printing made few changes in the bioprinter such as developed a large volume extruder, increasing the build volume in z-axis, improvising the syringe pump extruder and the quality of bio-printing. In their study they used alginate an extract from the seaweed to create a heart model tissue due to its high-fidelity printability. [10]

Another Printing process namely Freeform Reversible Embedding (FRE) printing process which is like FRESH except the ink employed isn't a hydrogel. In this method printing of functional and cellularized tissue and this method expands the size of FRESH-printed scaffolds to organ scale.

FluidForm Inc. manufactures a research-grade support bath for FRESH bio-printing known as Life Support and is popularly distributed by companies such as Advanced Biomatrix, CELLINK and Allevi. [4]

Furthermore, modifications include agarose-based support bath termed as Suspended Layer Additive Manufacturing (SLAM) and Constructs Laid in Agarose Slurry Suspension (CLASS) and alginate-based support baths. Hinton TJ et al in their article reported development of a method to 3D print PDMS elastomer in a hydrophilic support bath, designed to enable true freeform fabrication of complex structures. They termed freeform reversible embedding (FRE), FRE printing provides a framework for additive manufacturing of a range of soft polymeric materials. This work is based on their previous results using gelatin and Carbopol-based microparticulate supports but seeks to achieve a number of additional advances important to improving PDMS 3D printing. Firstly they have exclusively used Sylgard 184 PDMS (Dow Corning), which is the de facto standard in the microfluidics and tissue engineering fields and thus the most relevant for translating 3D PDMS printing to these research areas. Second, they have investigated a range of Carbopol formulations to determine how changes in chemistry and molecular weight impact surface structure of PDMS filaments extruded within these materials. Third, they have varied the temperature during curing of the Sylgard 184 to evaluate how long the material can remain in a pregelled state in the Carbopol without losing dimensional stability. Fourth, they have used changes in salt concentration to modulate the yield stress of the Carbopol to aid removal of delicate PDMS prints from the support bath.[11]

In the previous studies by Hinton et al and Bhattacharjee et al., the support bath manifest yield stress behaviour with the used of jammed microparticles. The designed support bath acts as a solid below a threshold applied shear stress but transition to liquid like behaviour above this threshold. With the help of this principle a syringe needle is inserted to the support bath and transverse through it during the printing process. [12] The support bath liquefies around the needle because it exceeds the yield stress and when the needle moves away the support bath resolidifies as the shears stress drops. Through this mechanism a bio ink or any other biological material extruded through the needle, displaces the liquefied support bath and when the needle departs the support bath resolidifies leading to immobilization of the extruded material and allows sufficient time to form cross-links and this nullifies the effect of gravity by providing a support structure.

With the use of FRESH technology, we can customize the support bath material in accordance with the bio-ink material such as there is no interferences between both the material. Thus, the FRESH bioprinting and embedded bioprinting can be developed in future to achieve patient specific adult organs and tissue.

IN-SITU BIOPRINTING:

In-situ 3D bioprinting is a kind of bio-printing which is directly printed onto or into the damaged tissue or organ. Construction of the tissue ex-situ and introduction in-situ may lead to the spread of infections and have demonstrated to have better functionality and integration with the host.

Two types of bio-printers are used commonly for in situ bioprinting namely:

1. Bedside bioprinter
2. Handheld bioprinter

The selection of bio ink for in-situ bioprinting is very essential to obtaining higher printing resolution of <100μm, faster crosslinking time, tissue regeneration and similar mechanical and physical properties between the constructed tissue and the target host tissue. These can be mesenchymal stem cells, amniotic fluid derived stem cells, photosensitive polymer-based hydrogels, nano-hydroxyapatite, viscoelastic material-based bio ink, conductive bio ink and so on.

Di Bella et al. in 2018 in their study had used a Gelatin methacrylate (GelMA) and Hyaluronic Acid (HA) methacrylate bio ink with mesenchymal stem cells (MSC) for treatment of damaged cartilage tissue in sheep. During the early stages cartilage regeneration was evident but lateral integration to the host tissue was not accomplished due to weak adhesion. [13, 14]

Cheng et al.,2020 had developed MSC containing fibrin Hyaluronic Acid bio inks to create skin precursor sheets for porcine full thickness burns after their failure with fibrin-based bio inks and alginate-based bio-inks in murine and porcine wound to induce granulation tissue formation and re-epithelialization in 2018. The MSC- fibrin treated porcine wound exhibited lesser inflammation, scarring and contraction with good re-epithelialization and had overcome their previous drawback. [15]

Ying et al., 2020 for healing of skin had designed a tuneable pore forming GelMA/ polyethylene oxide emulsion bio ink with 3T3 fibroblast. This porous bio ink helped to achieve liquid and oxygen transport, increased cellular proliferation with good elasticity but there have been no reports on the toxicity caused by free radicals generated. To achieve oxygen transport, we can employ peroxide or fluorocarbons into the bio inks for shorter duration not enough till the completion of wound healing. Wang et al in 2022, had developed a microalgae laden bio ink with photo assistance to facilitate a sustained oxygen transport after in situ bioprinting. Thus by increasing the oxygen supply we can achieve tissue repair by accelerating the angiogenesis as well as extracellular matrix synthesis. The challenge faced by this is the continuous light exposure which is essential to provide the sustained oxygen supply to the tissue. [16]

Biomaterial ink refers to the acellular materials that are developed to overcome the high cost and time with stem cell or cell laden bio inks for bio fabrication. Ma et al., in 2020 developed a cartilage repairing biomaterial ink which consisted of HAMA as well as acrylate-terminated 4-armed polyethylene glycol (PEG) dissolved in phosphate buffered saline and then subjected to bio-printing. The resultant bio fabricated structure was successful in repairing the cartilage defects and resembled the native cartilage tissue with glossy and smooth appearance along with its microstructure but lacked the biomechanical properties of the same. [17]

Quint et al. in 2021 incorporated growth-factor biomaterial ink for muscle recovery, this muscle ink, formed GelMA hydrogel after photopolymerization slowly released VEGF to surrounding tissue for a duration of more than 3 weeks. Incorporating this muscle ink in murine model led to functional muscle recovery, reduced fibrosis and anabolic response compared to untreated injured muscle.

Zhou et al., in 2021 had used conductive hydrogel biomaterial ink in live rat livers and post-mortem pig hearts and the advantage of this conductive hydrogel is that helps in cardiac and nerve regeneration through propagation of electric signals to cells. These conductive biomaterial ink used in in-vivo and ex-vivo bioprinting had revealed to have good lateral adhesion with the host tissue. [18]

Keriquel et al., (2010) in their study used nanohydroxyapatite (n-HA) as biomaterial ink for calvaria defects repair and found that they were biocompatible with osteoblast but there was no sufficient repair found. The draw back with hydroxyapatite is that they are unable to replicate the mechanical properties of bone tissue while having similar porosity with the native bone. [19]

IN-SITU BIOPRINTER:

Bed side mounted bioprinters use medical imaging such as MRI and CT or a combination of various imaging technique to create a specific and unique design with appropriate dimension to create 3D models.

Skardal et al., in 2021 have designed a bioprinter with three axis movement and multiple sets pressure driven nozzle for the delivery of alternating layers of hydrogel solution and cross-linker solutions. A team from the University of Lowa used pressure driven nozzle in their design so that the co-axial nozzle delivers hydrogel and crosslinker simultaneously. Along with this a second dispensing arm to deliver cell spheroids and thus reducing the total printing time but the drawback is when printing in situ on planar defect surface. [20]

Ma et al in 2020 integrated a 6-degree-of-freedom (6-DOF) robot and fast calibration tool to improve in situ printing accuracy and was found to be favourable for performing a variety of surgical procedure in operating rooms. The biomaterial ink deposited was cross-linked using UV light. [17] While Urciuolo et al., in 2020 had used low energy near infrared laser light to cross-link for in vivo applications. [26]

Handheld bioprinters avoid the usage of medical imaging and CAD modelling thus is more cost effective, simple, and lesser time associated with the bioprinting process. One of the most commonly available bioprinter is Biopen, this allows for design modifications to occur in real-time as well as can adapt to slight changes in the tissue microenvironment.

Di Bella et al. employed an upgraded the Biopen to repair cartilage defects in sheep by having a multi-inlet extruder nozzle which allowed the authors to print cell-laden materials enclosed in a protective biomaterial shell to limit shear stress-related cell damage. In addition, the handheld printer included a UV light source to facilitate photo crosslinking of bio inks, two chambers to hold core/shell bio inks, and a motorized control system. Other alternative to photo-curing will be printer that facilitate enzyme-activated and ionic activated materials. In this the biomaterial and cross-linking solution are loaded individually and then extruded concurrently through a microfluidic cartridge allowing the bio ink for rapid gelation. [14]

Vimex is currently developing as minimally invasive bioprinter for cartilage injury, arthroscopic printing tool along with video camera enables surgeon to visualize and construct the tissue. [21]

APPLICATIONS IN FORENSIC ODONTOLOGY

Producing three-dimensional models in Forensic Odontology may serve either of two purposes:

models so produced can provide a three-dimensional model to present in court as an exhibit, or they can be used in analysis. Printed three-dimensional exhibits may be of special utility where it becomes important to illustrate a pattern of bony injury, for example. The pattern of fracture may provide important information about the process that caused it, and this may be of particular interest to a court. Kettner et al. report the usefulness of comparing a printed exhibit of an injured skull with the alleged instrument of causation, a hammer. They point out that such models may be useful in the analysis phase where religious or cultural beliefs prevent maceration of the skull. [22] In analysis, a particularly valuable use of this technology would be in preserving the three-dimensional aspects of a bite mark injury after death for later comparison, as it removes the external pressures and distortions that may arise during the taking of a physical impression of a bitten area. Regions including faces, female breasts and buttocks are particularly difficult regions in which to secure accurate impressions without introducing uncontrolled elements of physical distortion.

CHALLENGES:

- printing with cells requires further research for manufacturability and potential clinical translation
- Printing resolution is also a major challenge to overcome, as the average resolution of 3D bioprinted constructs ranges from tens to hundreds μm, but the native tissue anatomy requires a resolution of 5–10 μm at the minimum.
- Proper environment for cell culture, which is necessary for cell differentiation, tissue maturation, functional vessel network integration, and overall mechanical stability.
- Further improvement in mimicking the native cell environment is also required for personalized applications in clinical research.
- Biomaterials used for bioprinting must also match the physical, chemical, and biological properties of patient tissue for clinical translation.

FUTURE DIRECTIONS:

To alleviate construct immunogenicity, biomaterials, including gelatin and gelatin methacryloyl, dECM, and polyethylene glycol, can be utilized to improve biocompatibility. Additionally, the inclusion of conductive polymers, sacrificial hydrogels, or adjunctive anti-inflammatory compounds during printing may also be beneficial in integrating bioprinted tissues with the host.

ETHICAL AND LEGAL CONSIDERATION:

The study revealed that, from the NEST-Ethics perspectives of tissue engineering, the second level of arguments, those referring to the techno-moral change, receive much less attention. The terms “hard impacts” and “soft impacts” come in handy for better understanding of these second level arguments. Hard impacts are quantifiable consequences of the new technology on the well-being of lives and soft impacts cover the category of effects that are not easy to quantify, such as changes to experience, habits and perceptions. [23]

Firstly, the perception of “naturalness”, where the process of bringing ultimately a foreign artefact in to the human body, introducing a dynamic product in to a dynamic structure and the dynamic interaction between the two are highlighted as facts to ponder, given that the exact behaviour of the engineered tissue in in vivo conditions is not completely known and once introduced and the process started, cannot be completely reversed. Secondly, the lack of consideration of soft impacts in the ethical framework discussed earlier, including the discussion of distributive justice. Thirdly and finally, the lack of completeness in taking into account the values and concerns of envisioned users, the general public.

<u>Regarding source and donation of cells:</u>

Four clusters of ethical issues were identified, namely (1) the source of cells, (2) the donation of cells, (3) the

use of animals in laboratory, and (4) morally problematic techniques, of which the first two will be briefly reviewed in this section

Regarding clinical translation:

Pre-clinical

Firstly, the irreversible impacts and consequences of implanting the artificial construct or organ in to the body, partially if not completely irreversible, puts pressure on knowing the exact reaction of human body to these artificial artefacts. Once the procedure is complete, the patient has to live with the consequences of it throughout one's life, which warrants for a detailed pre-clinical study, not only of the construct or organ but also the individual elements. Secondly, it is highly difficult to assess and deliberately decide which element of the bioprinted product provokes a particular bodily response, if some abnormality or uneasiness is detected after transplantation.

Clinical

Two components in patient selection are reported to be most important, namely (1) Inclusion of patients with least comorbidities (healthiest individuals) or those with highest number of comorbidities (sickest individuals), as each of these group will respond differently and give different data, and (2) Obtaining competent and informed consent from the selected individuals, bearing in mind the adequacy of social support, appropriate mental health and willingness to lose some degree of personal privacy, as the results mandates publication. In the first step of patient inclusion, the poorest sections of the society should not be taken for granted. [24]

LEGAL CONSIDERATION:

This includes patenting and related problems, regulatory policies, government interventions and decisions. While discussing about the field of tissue engineering as a new paradigm in medicine (which holds good for bioprinting also), Trommelmans et al. rightly pointed out that addressing one or two major tenets/issues legally will not solve all the legal problems that the technology faces in totality. To give an analogy, the very idea that addressing legally the bioethics related to use of stem cells and donation of cells will solve all the legal problems facing the whole technology, should be dismissed. The disqualification of the need for a special legal framework for bioprinting will lead to complications later. Bioprinting deserves a more organized and complete legal attention, given its huge potential in coming years.

- Patenting and IP rights dominate any new technology with a very high potential for commercialization and no wonder, bio-printing is one such technology
- Government intervention in research and novel technologies is imperative as it will determine the very future of the technology itself. Policy decisions and appropriate regulatory concerns have to be made and addressed, even at an earlier stage of technology development. With bioprinting, the technology which has the potential to save lives and revolutionize the medical field deserves special attention in having a legal framework
- Good Manufacturing Practices (GMP), that aides in assuring best product quality and consistency. Some constituents of GMP are: maintaining a clean and hygienic bioprinting area, controlled environmental conditions thus preventing cross contamination or infection, clearly defined processes, documentation and effective control, good documentation practices, recording deviations and retention and maintaining records of manufacture including distribution (that enable the complete history of a batch to be traced) in a comprehensible and accessible form
- Since bioprinting is a very promising technology, it poses a greater risk of falling into wrong hands. To prevent this from happening, discussion is needed if licensing would help. [25]

CONCLUSION:

FRESH is a customizable approach enabling the fabrication of biological and synthetic constructs from soft materials via embedded printing within a yield-stress support bath. By pairing specific microparticle support baths with an aqueous buffer to support bioink specific gelation mechanisms, FRESH supports a broad range of print materials and cells for advanced biofabrication. There are various legal constraints but development in the field of FRESH bioprinting can be a promising in the field of tissue engineering.

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