Designation: F3659 – 24

Standard Guide for Bioinks Used in Bioprinting[1]

This standard is issued under the fixed designation F3659; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide is a resource for bioprinting tissue-engineered medical products (TEMPs) with bioinks and biomaterial inks. There are existing standards that cover biomaterials and scaffolds in a more general fashion (Guide F2150, Guide F2027, ISO 10993 series). This guide focuses specifically on extrusion bioprinting utilizing bioinks and biomaterial inks with inherent or inducible fluidic properties with or without encapsulated cells used to construct TEMPs. For the remainder of this guide, both bioinks and biomaterial inks will be collectively referred to as bioinks.

1.2 For the purposes of this guide, bioprinting is defined as the three-dimensional printing of materials (bioinks) to fabricate structured constructs for use in biological or medical applications.

1.3 TEMPs may be produced by many different bioprinting modalities, including but not limited to the following: electrospinning, electrospray, extrusion-based, droplet-based, inkjet-based, and laser-assisted bioprinting. Extrusion-based bioprinting is the primary focus of this document since it is currently the most well-understood modality used to construct TEMPs, but other bioprinting modalities are also addressed.

1.4 This guide will focus on bioinks and biomaterials used as inks with inherent or inducible fluidic properties. These inks may or may not contain encapsulated cells. Chemical properties of the inks and other factors that affect printability are addressed.

1.5 Pre-printing and printing considerations are the focus of this guide, but considerations regarding post-printing product stabilization are also addressed.

1.6 This guide will address assessments regarding the sterility and cytocompatibility of bioinks, including chemical and physical benchtop tests, as well as measures of post-printing cell viability.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D7805 Terminology for Printing Ink Vehicles and Related Materials

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F1635 Test Method for ***in vitro*** Degradation Testing of Hydrolytically Degradable Polymer Resins and Fabricated Forms for Surgical Implants

F1983 Practice for Assessment of Selected Tissue Effects of Absorbable Biomaterials for Implant Applications

F2027 Guide for Characterization and Testing of Raw or Starting Materials for Tissue-Engineered Medical Products

F2064 Guide for Characterization and Testing of Alginates as Starting Materials Intended for Use in Biomedical and Tissue Engineered Medical Product Applications

F2103 Guide for Characterization and Testing of Chitosan Salts as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Product Applications

F2150 Guide for Characterization and Testing of Biomaterial Scaffolds Used in Regenerative Medicine and Tissue-Engineered Medical Products

F2212 Guide for Characterization of Type I Collagen as Starting Material for Surgical Implants and Substrates for Tissue Engineered Medical Products (TEMPs)

F2315 Guide for Immobilization or Encapsulation of Living Cells or Tissue in Alginate Gels

F2347 Guide for Characterization and Testing of Hyaluronan as Starting Materials Intended for Use in Biomedical

[1] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.42 on Biomaterials and Biomolecules for TEMPs.

Current edition approved March 15, 2024. Published April 2024. DOI: 10.1520/F3659-24.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

and Tissue Engineered Medical Product Applications
F2450 Guide for Assessing Microstructure of Polymeric Scaffolds for Use in Tissue-Engineered Medical Products
F2475 Guide for Biocompatibility Evaluation of Medical Device Packaging Materials
F2603 Guide for Interpreting Images of Polymeric Tissue Scaffolds
F2739 Guide for Quantifying Cell Viability and Related Attributes within Biomaterial Scaffolds
F2902 Guide for Assessment of Absorbable Polymeric Implants
F2997 Practice for Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis
F2998 Guide for Using Fluorescence Microscopy to Quantify the Spread Area of Fixed Cells (Withdrawn 2023)[3]
F3089 Guide for Characterization and Standardization of Polymerizable Collagen-Based Products and Associated Collagen-Cell Interactions
F3106 Guide for ***in vitro*** Osteoblast Differentiation Assays
F3224 Test Method for Evaluating Growth of Engineered Cartilage Tissue using Magnetic Resonance Imaging
F3259 Guide for Micro-computed Tomography of Tissue Engineered Scaffolds
F3354 Guide for Evaluating Extracellular Matrix Decellularization Processes
F3510 Guide for Characterizing Fiber-Based Constructs for Tissue-Engineered Medical Products
F3515 Guide for Characterization and Testing of Porcine Fibrinogen as a Starting Material for Use in Biomedical and Tissue-Engineered Medical Product Applications

2.2 *ISO Standards:*[4]
ISO/ASTM 52900 Additive manufacturing—General principles—Fundamentals and vocabulary
ISO 7198 Cardiovascular Implants and Extracorporeal Systems—Vascular Prostheses—Tubular Vascular Grafts and Vascular Patches
ISO 9000 Quality management systems—Fundamentals and vocabulary
ISO 9001 Quality management systems—Requirements
ISO 10993-1 Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process
ISO 10993-2 Biological evaluation of medical devices—Part 2: Animal welfare requirements
ISO 10993-3 Biological evaluation of medical devices—Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity
ISO 10993-4 Biological evaluation of medical devices—Part 4: Selection of tests for interactions with blood
ISO 10993-5 Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity
ISO 10993-6 Biological evaluation of medical devices—Part 6: Tests for local effects after implementation
ISO 10993-7 Biological evaluation of medical devices—Part 7: Ethylene oxide sterilization residual
ISO 10993-9 Biological evaluation of medical devices—Part 9: Framework for identification and quantification of potential degradation products
ISO 10993-10 Biological evaluation of medical devices—Part 10: Tests for irritation and skin sensitization
ISO 10993-11 Biological evaluation of medical devices—Part 11: Tests for systemic toxicity
ISO 10993-12 Biological evaluation of medical devices—Part 12: Sample preparation and reference materials
ISO 10993-13 Biological evaluation of medical devices—Part 13: Identification and quantification of degradation products from polymeric medical devices
ISO 10993-14 Biological evaluation of medical devices—Part 14: Identification and quantification of degradation products from ceramics
ISO 10993-15 Biological evaluation of medical devices—Part 15: Identification and quantification of degradation products from metals and alloys
ISO 10993-17 Biological evaluation of medical devices—Part 17: Establishment of allowable limits for leachable substances
ISO 10993-18 Biological evaluation of medical devices—Part 18: Chemical characterization of materials
ISO 10993-19 Biological evaluation of medical devices—Part 19: Physico-chemical, morphological and topographical characterization of materials
ISO 10993-20 Biological evaluation of medical devices—Part 20: Principles and methods for immunotoxicology testing of medical devices
ISO 10993-22 Biological evaluation of medical devices—Part 22: Guidance on nanomaterials
ISO 11135 Sterilization of health care products—Ethylene oxide—Requirements for the development validation and routine control of a sterilization process for medical devices
ISO 11137-1 Sterilization of health care products—Radiation—Part 1: Requirements for development, validation and routine control of a sterilization process for medical devices
ISO 11137-2 Sterilization of health care products—Radiation—Part 2: Establishing the sterilization dose
ISO 11607-1 Packaging for terminally sterilized medical devices—Part 1: Requirements for materials, sterile barrier systems and packaging systems
ISO 11607-2 Packaging for terminally sterilized medical devices—Part 2: Validation requirements for forming, sealing and assembly processes
ISO 11737-1 Sterilization of health care products—Microbiological methods—Part 1: Determination of a population of microorganisms on products
ISO 13019 Tissue-engineered medical products—Quantification of sulfated glycosaminoglycans (sGAG) for evaluation of chondrogenesis

[3] The last approved version of this historical standard is referenced on www.astm.org.
[4] Available from International Organization for Standardization (ISO), ISO Central Secretariat, Chemin de Blandonnet 8, CP 401, 1214 Vernier, Geneva, Switzerland, https://www.iso.org.

ISO 13408-1 Aseptic processing of health care products—Part 1: General requirements
ISO 13408-2 Aseptic processing of health care products—Part 2: Sterilizing filtration
ISO 13408-3 Aseptic processing of health care products—Part 3: Lyophilization
ISO 13408-4 Aseptic processing of health care products—Part 4: Clean-in-place technologies
ISO 13408-5 Aseptic processing of health care products—Part 5: Sterilization in place
ISO 13408-6 Aseptic processing of health care products—Part 6: Isolator systems
ISO 13408-7 Aseptic processing of health care products—Part 7: Alternative processes for medical devices and combination products
ISO 13485 Medical devices—Quality management systems—Requirements for regulatory purposes
ISO 14644-1 Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness by particle concentration
ISO 14644-2 Cleanrooms and associated controlled environments—Part 2: Monitoring to provide evidence of cleanroom performance related to air cleanliness by particle concentration
ISO 14644-3 Cleanrooms and associated controlled environments—Part 3: Test methods
ISO 14644-4 Cleanrooms and associated controlled environments—Part 4: Design construction and start-up
ISO 14644-5 Cleanrooms and associated controlled environments—Part 5: Operations
ISO 14644-7 Cleanrooms and associated controlled environments—Part 7: Separative devices (clean air hoods, gloveboxes, isolators and mini-environments)
ISO 14644-8 Cleanrooms and associated controlled environments—Part 8: Classification of air cleanliness by chemical concentration (ACC)
ISO 14644-9 Cleanrooms and associated controlled environments—Part 9: Classification of surface cleanliness by particle concentration
ISO 14644-10 Cleanrooms and associated controlled environments—Part 10: Classification of surface cleanliness by chemical concentration
ISO 14644-13 Cleanrooms and associated controlled environments—Part 13: Cleaning of surfaces to achieve defined levels of cleanliness in terms of particle and chemical classifications
ISO 14644-14 Cleanrooms and associated controlled environments—Part 14: Assessment of suitability for use of equipment and materials by airborne chemical concentration
ISO 14644-15 Cleanrooms and associated controlled environments—Part 15: Assessment of suitability for use of equipment and materials by airborne chemical concentration
ISO 14698-1 Cleanrooms and associated controlled environments—Biocontamination control Part 1: General principles and methods
ISO 14698-2 Cleanrooms and associated controlled environments—Biocontamination control Part 2: Evaluation and interpretation of biocontamination data
ISO 16379 Tissue-engineered medical products—Evaluation of Anisotropic Structure of Articular Cartilage Using DT (Diffusion Tensor)—MR Imaging
ISO 17668-1 Sterilization of health care products—Moist heat—Part 1: Requirements for the development, validation and routine control of a sterilization process for medical devices
ISO 20399-1 Biotechnology—Ancillary materials present during the production of cellular therapeutic products-Part 1: General requirements
ISO 20399-2 Biotechnology—Ancillary materials present during the production of cellular therapeutic products-Part 2: Best practice guidance for ancillary material suppliers
ISO 20399-3 Biotechnology—Ancillary materials present during the production of cellular therapeutic products-Part 3: Best practice guidance for ancillary material users
ISO 21560 General requirements of tissue-engineered medical products
ISO 21973 Biotechnology—General requirements for transportation of cells for therapeutic use

2.3 *USP Standards:*[5]
USP <61> Microbiological Examination of Nonsterile Products
USP <71> Sterility Tests
USP <85> Bacterial Endotoxins Test
USP <161> Medical Devices—Bacterial Endotoxin and Pyrogen Tests
USP <1043> Ancillary Materials for Cell, Gene, and Tissue-Engineered Products

2.4 *Other Documents:*
21 CFR 210 Current Good Manufacturing Practice in Manufacturing, Processing, Packaging, or Holding of Drugs; General
21 CFR 211 Current Good Manufacturing Practice for Finished Pharmaceuticals
21 CFR 610.12 General Biological Products Standards—Sterility
21 CFR 820 Quality System Regulation
21 CFR 1271 Human Cells, Tissues and Cellular and Tissue Based Products
21 CFR 1271.210 Human Cells, Tissues and Cellular and Tissue Based Products; Suppliers and Reagents
FDA Guidance on 10993-1 Guidance for Industry and Food and Drug Administration Staff: Use of International Standard ISO 10993-1, "Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process" https://www.fda.gov/media/85865/download
FDA Guidance on GMP for Combination Products Guidance for Industry and FDA Staff Current Good Manufacturing

[5] Available from U.S. Pharmacopeial Convention (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

Practice Requirements for Combination Products, https://www.fda.gov/media/90425/download

3. Terminology

3.1 *Definitions:*

3.1.1 *bioink, n*—a biologically active single or multicomponent formulation with inherent or inducible fluidic properties that contains one or more of the following: cells, biological components, and materials suitable for bioprinting.

3.1.1.1 *Discussion*—In this definition, the term "biologically active" is used to differentiate between an ink and a bioink.

3.1.2 *biomaterial, n*—a synthetic or natural substance or composite used for a biological or biomedical application.

3.1.3 *bioprinting, v*—three-dimensional printing of materials (bioinks) to fabricate structured constructs for use in biological or medical applications.

3.1.4 *crosslink, v*—union of high-polymer molecules by a system involving primary chemical bonds that is done either by addition of a chemical substance (crosslinking agent), exposing the mixture to heat, or by subjecting the polymer to high-energy radiation (UV or EB). **D7805**

3.1.5 *extrusion, n*—additive manufacturing process in which material is selectively dispensed through a nozzle or orifice. **ISO/ASTM 52900**

3.1.6 *hydrogel, n*—a water-based open network of polymer chains that are crosslinked either chemically or through crystalline junctions or by specific ionic interactions. **F2603, F2450**

3.1.7 *photopolymerization, v*—additive manufacturing process in which liquid photopolymer in a vat is selectively cured by light-activated polymerization. **ISO/ASTM 52900**

3.1.8 *support material, n*—a process aide material that allows the bioprinting of an object with bioinks that can't hold a shape or dimensions (such as an overhang). The support medium is a liquid or liquid-like solid compatible with the bioink.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *bioprinter, n*—a machine that additively manufactures objects from materials (bioinks) that may or may not contain cells or biologically active components for use in biological and medical applications. Can also be referred as printer in this standard.

3.2.2 *cell sedimentation, v*—settling of the cells at the bottom of the printing syringe/cartridge during bioprinting.

3.2.3 *crosslinking agent, n*—a component added that facilitates the linking of polymer chains, resulting in changes to the chemical and physical properties of the bioink.

3.2.4 *embedded printing, v*—bioprinting within a support medium, such as a hydrogel or semisolid matrix, that is used to provide support during the printing of the object. This support medium may or may not be removed as a later step.

3.2.5 *fugitive bioink, n*—transient or temporary materials used in 3D printing and bioprinting that can be rapidly removed to form internal voids or channels within a printed construct. Examples of removal methods are leaching, washing, temperature dissolution, chemical dissolution, and enzymatic processing.

3.2.6 *printability, n*—the ability of a material to be processed into three-dimensional structures with predefined geometries by a (bio)printer.

4. Significance and Use

4.1 *Standard Structure*—This standard has been organized according to a typical bioprinting operational workflow. In this way the standard has three main sections: pre-printing considerations, printing considerations, and post-printing considerations. There is also a section on bioprinting modalities and additional considerations of product release, containers, and transport. Certain processes will appear across multiple sections, for example cytocompatibility or crosslinking, as these issues have considerations that take place prior to printing, during the printing process, and following the printing process. Contents of main sections are listed below.

4.1.1 *Pre-Printing Considerations*—Pre-printing considerations include: bioink common applications, support material, and bioink selection considerations. Bioink selection considerations include: formulation of bioinks, bioink properties, changes in properties resulting from formulations, sterility, cellular component, and fugitive element considerations. Within the bioink properties there are considerations related to the viscoelastic properties, chemical properties, structure of polymer and functional groups, purity of material, mechanism of crosslinking, and degradation considerations. Contents of the section on pre-printing considerations are listed below.

4.1.2 *Printing Considerations*—Printing considerations include: printability, specifically bioink considerations, cellular component, support material, stabilization, and aseptic printing considerations. Within the cellular component section considerations cover cell viability, temperature, cell shearing, cell distribution, sedimentation, and concentration in the bioink. Contents of the section on printing considerations are listed below.

4.1.3 *Post-Printing Considerations*—Post-printing considerations include: post-print bioink, stabilization, and considerations for removal of provisional components and materials. Post-printing stabilization focuses on the modalities (such as crosslinking, temperature-induced self-assembly, and evaporation) as well as stabilization effects on the cell health, function, and modification of properties. The considerations for removal of provisional components and materials include the types of components, timing and method of removal, and the removal effects. Contents of the section on post-printing considerations are listed below.

4.1.4 *Additional Considerations*—Additional considerations include: discussion on product release, container, and transport considerations. Container considerations focus mainly on the storage stability of bioinks, transfer of bioinks to the print cartridge, as well as container closing integrity and assessing the quality of the container. Contents of the section on additional considerations are listed below.

5. Bioprinting Modalities

5.1 Several factors drive the selection of bioprinting technologies, including but not limited to cost, ease of use, material capabilities, resolution, and fabrication speed. The operation of any of the bioprinting platforms needs bioinks with a particular set of properties. Specific parameters must be considered when designing a bioink for a particular bioprinting method, for example, extrusion bioprinting needs shear-thinning materials. Generally, within these modalities the resolution and speed are primarily governed by the bioink properties (for example, photoinitiator concentration, optical density) and processing parameters (for example, laser intensity, energy, exposure duration). Although general characteristics of typical bioprinting modalities are described herein, processes and capabilities may vary across specific implementations.

5.2 *Extrusion-Based Bioprinting*—Extrusion-based direct-write 3D printing (EDP) techniques, including bioplotting and microextrusion printing, consist of robotically controlled deposition of a viscous material as continuous filaments (**1**).[6] The material is stored in a reservoir and is dispensed through a nozzle onto a substrate as either a self-supporting construct or, in the case of embedded printing, within a bath of another supporting material. The driving mechanism for material extrusion is generally either pneumatic or mechanical. The 3D construct is created layer-by-layer by depositing two-dimensional layers by moving the nozzle and substrate relative to one another. The subsequent layers are printed by moving the stage or the nozzle in the z-direction, with the deposited layer serving as the foundation for the next layer. The 3D construct can also be created freeform by depositing bioinks through some combination of x-, y-, and z-direction motion,

[6] The boldface numbers in parentheses refer to the list of references at the end of this standard.

for example during embedded printing. The 3D construct is stabilized by various processes including material phase transition, physical, and chemical crosslinking mechanisms. A few systems use multiple print heads to facilitate serial deposition of several materials. EDP processes are compatible with a wide range of fluid properties and can create constructs with varying resolution, sizes, and cell densities.

5.3 *Droplet-Based Bioprinting*—Droplet-based bioprinting techniques, including inkjet printing, acoustic jet printing, and micro-valve printing, involve creating 3D constructs by layer-on-layer deposition of controlled volumes of fluid droplets in targeted spatial locations. The droplets can be produced in two modes, a continuous mode where the ongoing generation of droplets produces a jet, or in a droplet-on-demand mode where the droplets are generated only when needed for printing. Thermal or acoustic forces are used to eject droplets of the fluid through a print head with a valve. The print head is heated electrically or contains a piezoelectric crystal that produces acoustic waves to produce pulses of pressure that force the droplets from the valve at regular intervals. The print heads can be combined in an adjustable array format to facilitate simultaneous printing of multiple cells and materials. 3D constructs may be created by printing binder droplets onto a powder bed (called binder jetting) or a photopolymer/monomer/oligomer which is subsequently crosslinked (called material jetting). These techniques enable high-throughput printing, high resolution, inexpensiveness, and high reproducibility, but suffer from limited material availability due to the requirement for the biological material to be in liquid form. Droplet-based approaches can also be sensitive to the density or volume fraction of suspended particles (including cells), which can affect the droplet formation process.

5.4 *Vat Photopolymerization-Based Bioprinting*—Vat photopolymerization-based bioprinting processes including stereolithography (SLA), digital light processing (DLP), and two-photon polymerization (2PP) involve selective crosslinking of photosensitive bioinks using an appropriate mode and wavelength of irradiation. The bioink is contained in a vat, and photopolymerization is achieved by layerwise scanning of a laser beam (SLA), layerwise projection of images using a digital micromirror device (DLP), or by direct writing using ultrashort laser pulses (2PP). Vat photopolymerization-based processes can typically offer a higher resolution and faster speed than many other bioprinting processes. Bioink properties (for example, rheology, thermal conductivity), ribbon properties (for example, optical transparency, thermal conductivity, thickness of bioink coating), and processing parameters (for example, laser intensity, energy, and exposure duration) impact the resolution. Due to the requirement of photosensitivity, the current library of bioinks suitable for vat photopolymerization is limited compared to that of other bioprinting processes.

5.5 *Electrostatic Direct Writing (EDW) Bioprinting*—Electrostatic direct writing (EDW) techniques including melt electrowriting, electrohydrodynamic printing, and near-field electrospinning use an electric field for the generation of a continuous, stable fluid jet that is collected onto a computer-controlled translating collector. The fluid (for example, melt, solution, gel) extruded at a low flow rate through a nozzle experiences a force due to the applied voltage between the nozzle and the collector. Once this force overcomes the fluid surface tension, a continuous jet is drawn towards the collector. Appropriate levels of fluid flow rate, voltage, and collector distance are used in EDW processes to prevent the whipping instability in the jet, typically associated with traditional electrospinning, as it is collected onto the collector. The relative motion between the nozzle and collector is governed by a computer-aided toolpath characteristic of additive manufacturing. Depending on the nature of the material, solidification of the fluid jet can be achieved via cooling, solvent evaporation, or chemical crosslinking using coagulation baths. Bioink properties (for example, rheology, thermal conductivity), ribbon properties (for example, optical transparency, thermal conductivity, thickness of bioink coating), and processing parameters (for example, laser intensity, energy, and exposure duration) impact the resolution. It should be noted that EDW processes are not typically suitable for processing bioinks containing living cells due to high solution temperature, solution volatility, and/or process-induced shear stresses.

5.6 *Laser-Assisted Bioprinting*—Laser-assisted bioprinting refers to a set of direct writing processes including laser-induced forward transfer (LIFT), biological laser processing (BioLP), and matrix-assisted pulsed laser evaporation direct writing (MAPLE DW) that utilize a pulsed laser to deposit bioinks onto a substrate. Central to the process is a glass or quartz ribbon that is coated with the bioink. The nanoseconds laser with UV or near-UV wavelength scans the ribbon causing rapid volatilization of the bioink and ejection of droplets or a plume which transfers material onto a receiving substrate. The receiving substrate may be coated with a supportive material to enable cellular adhesion and sustained growth after cell transfer from the ribbon. Laser-assisted bioprinting processes can typically enable single cell deposition and achieve picoscale to microscale resolution. Bioink properties (for example, rheology, thermal conductivity), ribbon properties (for example, optical transparency, thermal conductivity, thickness of bioink coating), and processing parameters (for example, laser intensity, energy, and exposure duration) impact the resolution.

6. Pre-Printing Considerations

6.1 Prior to performing a bioprint, there are important pre-print considerations to take into account. For example:

6.1.1 Establish if the bioink will include live cells. If so, is the same bioink that includes the cells being used to provide the scaffold structural portion of the print, or will a separate bioink be used for structural printing and the cells subsequently deposited within the scaffold either during the print (perhaps on a layer-by-layer basis or via a dual-head printer)?

6.1.2 Establish if a bioink with cells will provide the desired structural properties needed for the print.

6.1.3 Establish if the bioink with cells will provide the appropriate cell density required for the given application or if subsequent cell culture and proliferation will be required.

6.1.4 Establish the effects of the printing process on cell viability.

6.1.5 Establish how the print will be removed from the print bed (that is, will the print require crosslinking in order to give it the mechanical properties necessary to remove it from the print bed).

6.1.6 Establish if the print will need to undergo post-processing. If so, will the process selected kill cells in the print, and if so, should such prints be printed without cells, fixed, and subsequently seeded with cells?

6.2 *Bioink Common Applications*—Common bioink applications include bioprinting 3D tissue constructs (ISO 21560, 21 CFR 1271). The most frequently reported bioprinted tissues are cartilage (Test Method F3224, ISO 13019), bone (Practice F2997, Guide F3106, ISO 16379), and vasculature (ISO 7198) (**2**). Other applications will likely be developed in the future.

6.3 *Support Material Considerations*—In general 3D printing, material supports are often printed with a thinner infill/shape and function to support overhanging parts of the print (typically 45° overhang or greater). The thinner infill makes them easier to break off after the print is complete. Depending on the bioink, the bioink may not be able to support itself without an external support material (for example, collagen bioinks that are printed into a gelatin support bath—this freeform reversible embedding of suspended hydrogels is called "FRESH" printing (**3**)) or being crosslinked.

6.4 *Bioink Selection*—Bioink types vary in their source, composition, and optical, rheological, mechanical, chemical, and other properties (for example, viscosity, gelation time, etc). Bioink selection is critical in the bioprinting process as the user needs to choose a suitable bioink that will provide the intended biochemical and physical cues to promote cellular growth, development, and proliferation (Practices F748 and F1983; Guides F2027 and F2150). The selection of the bioink will also dictate the type of bioprinter and bioprinter modality that will be required for manufacturing the desired construct.

6.4.1 *Formulation of Bioinks*—Bioink formulations vary according to their base components, which include hydrogel-based, protein-based, polysaccharides, decellularized extracellular matrix-based (dECM), synthetic polymer-based bioinks, and combinations thereof. Each of these methods (Guide F3354) has its own benefits and drawbacks involving construction of the scaffolding system and regeneration/replacement of the natural extracellular matrix. Currently, the most common bioink formulations in 3D bioprinting involve using collagen-based substances (like gelatin) or alginate, a naturally occurring polysaccharide acquired from brown seaweeds, which is characterized by an overall negative charge and lack of integrin-binding sites, enabling easy crosslinking with cations such as calcium (Guides F2064 and F2315). To facilitate cell growth, chemically modified alginates with arginyl-glycyl-aspartic acid (RGD) peptides are typically used, as these peptides display integrin-binding capabilities. Together with the alginate's crosslinking properties, this formulation enables the construction of tissue-mimetic 3D matrices composed of living cells and other biodegradable materials.

6.4.1.1 *Concentration of Components*—As mentioned in 6.4.1, bioinks are often composed of alginate and gelatin at a variety of concentrations. These components are typically dissolved separately in buffers such as sterile phosphate buffer saline (PBS) at temperatures around 25 °C. Optimization of the components' concentrations are application, materials, and printing system specific. For example, concentrations of the components affect the viscosity which can impact printability of the materials and fidelity of the printed structures; in addition, at higher concentrations the stiffness of the material may be deleterious to cells (**4**). Importantly, these compositions will vary based on the cell systems (that is, cell-based strategies such as cell clusters, microvessels, etc.) and polymerization methods used. After cells are mixed with the alginate solution, the resulting alginate-cell mixture is often placed into a calcium chloride solution at low concentrations (1 to 2 % $CaCl_2$). Then, the gelatin, methylcellulose, or other polymerizing agents are used to increase viscosity of the bioink to prepare its printability. Table 1 displays the main bioink components, their properties, typical concentrations, and functions.

6.4.1.2 *Function of Each Component*—When considering which bioink formulation to use, it is important to consider the function of the independent components as well as the compounding functions within a bioink. For example, as mentioned in 6.4.1, alginate is one of the commonly used aspects of current bioink formulations due to its biocompatibility and instantaneous ability to crosslink with cations such as calcium. Importantly, alginate is structurally like natural extracellular matrices and provides a stiffness to the matrix which functionally facilitates cell differentiation and growth. Gelatin, methylcellulose, or alginate are frequently mixed to improve overall viscosity of the bioink construct, as well as improving unstable/erratic degradation of the matrix, facilitating cell-cell interaction, and increasing cell viability (Guide F2739) (**5**). Gelatin is primarily used due to its identical composition to naturally occurring collagen. Moreover, gelatin can functionally mimic the native ECM and provides temporary support to the bioink, allowing for the production of channels, vessels, or other vasculature to promote cellular networking. Another well-known, highly used bioprinting formulation includes the use of collagen (Guides F2212 and F3089), a key element to the native ECM (particularly in cartilage) that confers unique biocompatibility and prevents immunological rejection. Lastly, gelatin methacryloyl (GelMA) plays an important role in bioink formulations as well. This component confers suitable rheological and mechanical properties, improving the crosslink ability of hydrogel formations.

6.4.2 *Bioink Properties*—While bioink properties vary depending on their respective formulations, the key fundamental principles are their crosslinking capabilities, which provide mechanical strength and stiffness to mimic cell and tissue mechanical properties, and the rheological properties of the materials used, which can assist in bioink printability while also improving cell viability and function (**7**). Enhancing crosslinking capabilities (typically through the use of bioink components such as GelMA) allows for significant cell viability (in many cases, >90 %) and induction of differentiation among various cell types to mimic *in vivo* tissue systems. In some cases, cell proliferation and viability has been observed for two or more weeks, which demonstrates how favorable

TABLE 1 Major Components of Bioink Formulations Including Their Properties, Typical Concentrations, and Functions (6)

Component	Properties	Concentrations	Functions
Alginate (F2064, F2315)	Naturally occurring, non-toxic, biodegradable, non-immunogenic linear, highly biocompatible with other substances	~1–4 % w/v	Enables the entrapment of water and other molecules, provides defense mechanism for encapsulated cells, and is highly compatible with other components, enabling different hydrogel components depending on desired properties/cells used
Gelatin	Identical composition to naturally occurring collagen	Between 8–15 % w/v	Functions to mimic the innate cellular ECM to provide temporary support for the creation of channels, vessels, or vasculature
GelMA	Confers excellent rheological properties; biocompatibility and tunable biodegradation	Between 4–10 % w/v	Improves crosslink-ability of hydrogels for tissue engineering via a two-step crosslinking process (reversible thermal gelation and permanent photo-crosslinking)
Hyaluronic Acid (F2347)	Anionic, non-sulfated glycosaminoglycan present in connective and neural tissues	0–1 % w/w	Useful for skin tissue engineering as it is a major component in connective and neural tissues
Cellulose	Obtained as fibers from natural resources, confer mechanical properties to hydrogels; confers biocompatibility due to abundance of hydroxyl groups	9 mg/mL final concentration or 2 % w/v	Can be used to prepare hydrogels with a variety of structures/properties
Collagen (F2212, F3089)	Main component of mammalian extracellular matrix (i.e., connective tissues as cartilage); uniquely biocompatible and has low immunogenicity	3–70 mg/mL final concentration	Improves biocompatibility of hydrogel mixture and prevents immunological rejection, a significant limitation to current clinical use cases of hydrogels
Polyethylene glycol (PEG)-derivatives	Confers hydrophilicity to the hydrogel; good mechanical stability	~1 % w/v	Useful for vascular tissue, bone tissue, and/or cartilage tissues
Agarose	Comparable to gelatin according to thermal behavior, mimicking the ECM; low gelling temperature; high mechanical strength	1.5 % w/v	Provides great support for chondrogenic differentiation in MSCs
Polyethylene glycol (PEG)	Confers hydrophilicity to the hydrogel	20 % base polymer; 10 % PEG crosslinker	Facilitates exchange of cell nutrients and waste due to inherent molecular structure
Chitosan	Naturally obtained, biodegradable polymer similar to many other components of the ECM; low mechanical properties, slow gelation, and antimicrobial activity; some are insoluble in water	3–5 % w/v	Widely used in bone tissue repair engineering applications. Positively charged, enabling hydrophobic interactions with gel components to promote cell encapsulation
Fibrinogen (Fibrin) (F3515)	Main ECM protein in blood clots and forms when fibrinogen is enzymatically crosslinked by Thrombin into fibrin; confers biocompatibility through multiple cell and growth factor binding domains	1–100 mg/mL	Improves biocompatibility of hydrogel mixture, rapid enzymatic gelation that does not damage cells
dECM (decellularized extracellular matrix) (F3354)	Multiple components of extracellular matrix typically derived from a specific tissue (e.g., bladder, muscle, skin); uniquely biocompatible and has low immunogenicity	3–70 mg/mL final concentration	Improves biocompatibility of hydrogel mixture and prevents immunological rejection, a significant limitation to current clinical use cases of hydrogels, tissue specific factors that can improve regeneration

bioinks are for promoting cell growth. PEG/gelatin/hyaluronic-based bioinks display exceptional cellular adhesion properties as well, as they mimic the natural extracellular matrix and enhance cell viability to a greater degree. Numerous natural (and some synthetic) bioink formulations are biodegradable and have little impact on cellular microenvironment. Concerning rheological properties, the viscosity of a bioink is an important aspect that can dramatically influence fluid behavior and shape retention after printing. Both of these characteristics can result in different physical, electrostatic, and/or biological interactions (7), and using crosslinkable materials. Hydrogel bioinks form an ECM-like microenvironment of interconnected polymer chains into a crosslinked network, where the pore size, pore interconnectivity, and polymer chemistry influence cell motility and the diffusion of nutrients and metabolites (8). Other important bioink factors relate to the bioprinting process, specifically, the printing parameters, structure, and resolution, which all impact the final printing results. For example, bioprinting techniques such as organ-on-a-chip (OoC) platforms, which consist of a patterned ECM-like tissue model embedded with cellular components, replicate normal tissue and organ function to mimic the biological behavior of cells and human systems (8). Importantly, a significant element of the OoC platform is the bioprinting resolution, which can

vary from ~10 µm to over 200 µm depending on the experiment and models used. Lower resolutions create microfluidic encasements and connecting channels, enabling circulation across the chip, while larger bioprinting resolutions offer precise patterning of cell-laden constructs and are useful for high-throughput experiments such as drug screening (**8**).

6.4.2.1 *Viscoelastic Properties*—Viscoelasticity is the property of materials that exhibit both viscous and elastic characteristics when undergoing deformation. High viscosity implies that the material will deform slowly when a force is applied, while high elasticity implies that once the force is no longer applied, the material will revert to its initial state prior to applying the force.

(1) Viscosity and Shear Thinning—Shear thinning is a property of non-Newtonian liquids, where the viscosity under static conditions is decreased when shear force is applied. An optimal bioink undergoes a time-dependent recovery to its static values of viscosity when the shear force is relieved (thixotropic behavior). The shear-thinning properties of ink formulations will have different requirements when applied to different printing technologies.

(2) Extrusion and Ink Jet—Requires an ink formulation with suitable shear thinning that has lower viscosity during extrusion to travel down the orifice, but is still viscous enough to maintain its structure once deposited before further curing process takes. In embedded extrusion printing, the bioink does not need to be viscous to maintain its structure once deposited because the support bath provides that function. Shear thinning properties are important for reducing shear force loads on cells in cell-laden formulations.

(3) DLP/SLA—Requires viscosity values that allow free flow when the building platform is moving on the Z axis. Highly viscous materials may contribute to low resolution, longer printing durations, or printing failure.

(4) Assessments—It is imperative to evaluate viscosity and shear thinning when preparing new or known ink formulations. Viscosity can be assessed by using a rheometer (**9**) or alternatively, a viscometer. Shear thinning properties can be evaluated using a rheometer. When evaluating shear thinning, one should measure viscosity in relevant shear rates that are applied on the sample during printing, especially if the sample includes shear rate sensitive biomolecules.

6.4.2.2 *Chemical Properties*—The functional properties of a bioink before, during, and post-printing are driven by its chemical and physical characteristics. These include the chemical structure of the material(s), which dictates the rheological properties before printing and during printing, as well as determines the stabilization and solidification mechanism of the printed constructs. Chemical characteristics of additional components, including for example crosslinkers and photoinitiators, drive the stabilization process during and post-printing.

6.4.2.3 *Structure of Polymer and Functional Groups*—The polymers used in bioinks can include a linear or branched polymeric chain which is composed of bio-inert/biocompatible materials. Examples may include: polysaccharides (chitosan (Guide F2103), hyaluronic acid (Guide F2347), alginate (Guides F2315 and F2064), etc.); proteins (collagen, gelatin, etc.); or synthetic polymers (polyethylene glycol, polycaprolactone, poly(lactic acid), etc.). These polymers can be tethered with functional groups depending on the necessary crosslinking mechanism, either physical or chemical.

6.4.2.4 *Purity of Material*—The polymer(s) should have a suitable purity which does not hinder functions of the bioink, that is, crosslinking or polymerization reaction that leads to a relatively homogeneous and repeatable product (or scaffold) (Guide F2027). The level of impurities should also be assessed per the intended use, that is, toxicity. Identity and levels of impurities in the material should be known and relevant specifications should be set.

6.4.2.5 *Mechanism of Crosslinking*—Crosslinking is a chemical process where one polymeric chain is connected to another polymeric chain in order to achieve certain physical and/or biological properties. The connection can be either via a covalent bond, ionic bond, coordinative bond, hydrogen bonds, and van der Waals interactions.

(1) Chemical Crosslinking—In a chemical crosslinking process, a covalent bond is formed between two polymeric chains. A crosslinking agent (a molecule that bears multiple reactive end groups) can be used in order to facilitate the bond formation. The reaction can be either via chain growth mechanism (for example, radical polymerization or photopolymerization where vinyl or acrylic resins are used) or via a step polymerization reaction (for example, esterification or carbamylation or amide bond formation). Crosslinking can be formed in conjunction with the polymerization process or between two existing polymers that bear "dangling" reactive groups. Enzymes can also be used to facilitate crosslinking between proteins with adequate amino acids which are necessary for a more specific and localized bond formation (for example, transglutaminase can be used to crosslink between a glutamine and a lysine).

(2) Physical Crosslinking—In a physical crosslinking process, a non-covalent interaction is formed between two polymer chains. These interactions can be electrostatic between two moieties that bear opposing electric charges. They can also include hydrogen bond interaction between a hydrogen bond donor and a hydrogen bond acceptor or coordination bonds between metal ions and metal chelating moieties.

(3) Assessment of Crosslinking—The crosslinking properties of bioinks can be considered critical process parameters as they affect the printing efficiency and final product properties. Among the properties for evaluation are kinetics of crosslinking, that is, for example rate of achieving certain G', and mechanical properties of the cured scaffolds, that is, G', G'', Young's modulus (**9**). Standard assay conditions should be developed, for example, using a rheometer. In cases where residual chemical crosslinker may affect safety or functionality, it may be assessed using chemical characterization testing (ISO 10993-18).

6.4.2.6 *Degradation of Bioink*—Some polymers could be susceptible to degradation. However, the degradation should be assessed on a case-by-case basis based on factors such as the geometry of the device or construct, anatomical location, duration of contact, and/or mechanical loading. Some polymers may be susceptible to one or more of the following degradation

mechanisms such as hydrolysis, enzymatic, and oxidative. For example, typical formulations of polymers such as PEG are generally less prone to degradation by hydrolysis, whereas polylactic acid (PLA) is more prone to degradation via hydrolysis. In either case, degradation kinetics and degradation products should be evaluated. The degradation profile of the post-cured material/device should be considered based on the indication of use and the term of use (permanent or temporary) (Guide F2902, Test Method F1635, ISO 10993-9, ISO 10993-13, ISO 10993-17).

6.4.3 *Changes in Properties Resulting from Formulations*—Bioinks can be formulated as a single or multi-components, at different pHs and with different concentrations of salts and/or buffers. The relative solubility of each component and specific electrostatic interactions between components, for example between polymers or between polymer and photoinitiator, can have significant impact on the rheological properties of the bioink and its compatibility with the printing process. Scaffold properties stem directly from the formulation components. These properties should be evaluated and tailored specifically to meet the target specifications of the scaffolds. For example, the type and amount of polymers and crosslinkers will determine how rigid or stiff the end material may be. They also determine the degree of swelling and stability of the printed scaffolds. The relationship between formulation components and the scaffold properties should be thoroughly investigated prior to final formulation selection. Careful characterization of these relationships will prove handy during the development phases of scaffolds in order to meet specific target properties.

6.4.3.1 *Example: Formulation Modification to Influence Biological Response*—Recent research on different bioink formulations has illustrated that functional modifications of these formulations are an important aspect to consider based on the cell type or cell system used. For example, bioink formulations utilizing PEG substantially inhibit bacterial adhesion to the matrix by physical mechanisms, increasing microbial resistance of the bioink (**10**). Functionalizing PEG-based bioinks with osteogenic or chondrogenic stimuli has also shown promise for the differentiation of human mesenchymal stem cells (HMSCs) (**11**). Collagen is another common element of bioink designs, and can be used in combination with alginate to provide structural and mechanical integrity (**12**). Collagen type I is useful for bioink formulations due to its abundance of integrin-binding domains. These binding domains enhance cell attachment and improve cell growth, in addition to enhancing cell adhesion. The activity of collagen-based bioinks can be augmented with the addition of ECM microparticles which can impact printability (**13**).

6.4.3.2 *Example: Formulation Modification to Influence Rheology*—Bioink formulations are often modified to influence their rheology both to improve printing performance and the properties of the final product. An example is the addition of hyaluronic acid (HA), a non-sulfated glycosaminoglycan widely distributed in connective, epithelial, and neural tissues. One key aspect when considering bioink constructs is the viscosity; higher bioink viscosity results in better cell shape retention, but higher shear forces in some formulations significantly reduces cell viability (**14**). Recent works have shown HA as a promising component in bioink formulations due to its viscoelastic properties which provide resistance to shear forces/stressors that typically diminish cell viability (**14, 15**). Use of HA in conjunction with gelatin may increase viscosity and the distribution of cells (adipocytes in this case) throughout the matrix (**15**). Thus, one should select bioink components based on the desired outcome, considering the cell types or cell systems and bioink physical properties.

6.4.3.3 *Example: Formulation Modification to Influence Mechanical Properties*—As mentioned in 6.4.1.2 and Table 1, varying bioink contents can alter mechanical properties. For instance, HA is particularly useful in skin tissue engineering due to its presence in native connective and neural tissues, and HA derivatives, including acrylated HA and tyramine-conjugated HA, can immobilize bio-active peptides and/or enhance gelation speed (**16**). Likewise, PEG alone confers high hydrophilicity to bioinks, facilitating the nutrient exchange to the cells, while PEG derivatives, such as PEG diacrylate (PEGDA) and PEG methacrylate (PEGMA), enhance the photocrosslinking capabilities of hydrogels to provide better mechanical stability after bioprinting. These properties are particularly true with PEGDA.

6.4.4 *Sterility*—Bioinks should be sterile or have a low bioburden. Because most biological materials, such as bioprinted cell-laden constructs, are not amenable to terminal sterilization, maintaining sterility using sterile materials and aseptic processing during printing is often necessary (**17**). Like traditionally manufactured biologics, the maintenance of sterility of bioink and bioprinted products needs to be demonstrated through appropriate testing. The manufacturer of biological products must perform sterility testing of each lot of each biological product's final container material of other material, as appropriate (21 CFR 610.12).

6.4.4.1 *Sterilization Approach*—Typical sterilization methods can be considered, including sterilizing filtration, ethylene oxide, steam, and radiation. The method of choice should take into consideration the bioink properties and tolerance to the specific sterilization method. Due to the nature of common components included in bioink formulations, it is expected that a significant part of the bioink formulations will be sensitive to ethylene oxide, steam, and radiation, leaving sterilizing filtration as the method of choice in many cases. It is expected that the sterilization method that will be chosen should follow the relevant ISO standards including:

(1) Aseptic Filtration—ISO 13408-2.
(2) Ethylene Oxide—ISO 11135.
(3) Steam—ISO 17668-1.
(4) Radiation—ISO 11137-1 and ISO 11137-2.

6.4.4.2 *Assessments*—Assessment of the bioburden control measures is important to confirm a successful process and that the product meets the intended use (ISO 11137-1, ISO 11737-1, USP <71>, USP <85>, USP <161>). Sterility or bioburden should follow the relevant standards, such as USP <61> or USP <71>. The sterility test must be appropriate to the material being tested such that the material does not interfere with or otherwise hinder the test; the sterility test must be validated to demonstrate that the test is capable of reliably and consistently