Sterilization Methods and Their Influence on Physicochemical Properties and Bioprinting of Alginate as a Bioink Component

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ABSTRACT: Bioprinting has emerged as a valuable three-dimensional (3D) biomaterial method to fabricate complex hierarchical cell-containing constructs. Spanning from basic research to clinical translation, sterile starting materials are crucial. In this study, we present pharmacopeia compendial sterilization methods for the commonly used bioink component alginate. Autoclaving (sterilization in saturated steam) and sterile filtration followed by lyophilization as well as the pharmacopeia non-compendial method, ultraviolet (UV)-irradiation for disinfection, were assessed. The impact of the sterilization methods and their effects on physicochemical and rheological properties, bioprinting outcome, and sterilization efficiency of alginate were detailed. Only sterile filtration followed by lyophilization as the sterilization method retained alginate’s physicochemical properties and bioprinting behavior while resulting in a sterile outcome. This set of methods provides a blueprint for the analysis of sterilization effects on the rheological and physicochemical pattern of bioink components and is easily adjustable for other polymers used in the field of biofabrication in the future.

INTRODUCTION

Biofabrication has emerged as a new and rapidly growing field with applications in tissue engineering and regenerative medicine. Generally, biofabrication aims at the generation of hierarchical three-dimensional (3D) and cell-laden scaffolds deploying additive manufacturing. The systematic combination of polymer—either of synthetic or natural origin—cells, and growth factors leads to so-called bioinks that must fulfill certain characteristics to be taken into consideration for application in a challenging biological environment. The reader is directed to excellent review articles on recent developments in the field of biofabrication.

For successfully bridging the gap from research to applied clinical practice, reliable and effective sterilization of the applied polymers is critical during early-stage bioink development. Treatment with 70% ethanol has been suggested as a method for disinfection in biofabrication. However, as a noncompendial method, it is only of limited use for potential clinical translation. Sterility of biomaterials and implants is mandatory, as sadly documented by implant-related infections related to increased patient morbidity and fatalities. Sterilization methods include ISO norms (ISO 11737: Sterilization of medical devices) or sterilization monographs described in different pharmacopeias (USP, EP, BP, JP). However, due to the requirements of bioinks, sterilization processes for medical devices (ISO norm) or small molecules and pharmaceutical relevant polymers in solution (pharmacopeial methods) cannot be directly transferred. One reason is the temperature stability, which is in general higher for metals used for load-bearing implants than for polymers as used in bioinks, causing degradation or chemical changes. Consequently, it is important to evaluate the suitability of the sterilization method in line with the physicochemical and mechanical properties of the sterilized material. Although the number of publications dealing with biofabrication has been significantly increasing over the last few years, to the best of our knowledge, only one study investigated sterility and its influence on material properties. In this study, the authors evaluated three sterilization methods on four frequently used biopolymers and identified ethylene oxide (EO) treatment as the most promising one. However, this method is not practicable in every laboratory due to its high safety requirements and high-cost involvement. Furthermore, EO is challenged in light of the clean air act and under review by Food and Drug Administration’s (FDA) Environmental Protection Agency (EPA).

Here, we focus on pharmacopeia compendial, potentially high throughput, and cost-effective sterilization methods applicable
in routine laboratory work. To this end, we selected sodium alginate as the bioink component, a commonly used bioink in 3D bioprinting, and compared the effects of the sterilization treatment on physicochemical and rheological properties, bioprinting, and sterilization efficiency.

**RESULTS AND DISCUSSION**

Two compendial sterilization methods, autoclaving (powder and liquid) and sterile filtration, were compared for their sterilization outcome on the polysaccharide alginate (Table 1). For comparison, disinfection with ultraviolet (UV)-light was selected as the noncompendial method.

Table 1. Overview of the Sterilization and Disinfection Methods, Abbreviations, and Their Specifications

| Method                     | Abbreviation | Description                                      | Refs |
|---------------------------|--------------|--------------------------------------------------|------|
| UV-light irradiation (UV) | UV           | irradiation with UV-C at 254 nm                   | 18, 19 |
| sterile filtration (filt. + lyo.) | filt. + lyo. | filtration (≤0.22 μm membrane)                  | 20   |
| autoclaving (solution; autoclaved) | sterilization in saturated steam | at 121 °C for 15 min | 20   |

First, we analyzed the molar mass distribution of alginate by gel permeation chromatography (GPC) measurements in an aqueous environment after the sterilization or disinfection process (Figure 1A). Compared to the nonsterilized alginate polymer, a low molecular tailing at larger retention volumes (5–7 mL) was detected in the RI signal after the autoclaving process (Figure 1A). This finding was corroborated by differential pressure changes, which were lower for autoclaved samples (Figure 2B). Moreover, the resulting number average molar mass ($M_n$) was statistically significantly lower by 17 and 19 kDa for autoclaved alginate as a powder or in an aqueous solution, respectively (Figure 2C). The weight average molar mass ($M_w$) was found to be statistically significantly lower after autoclaving alginate as an aqueous solution. The $M_w$ dropped from 77 ($±5.2$) kDa for native alginate to 53 ($±5.6$) kDa for the autoclaved alginate. Consequently, dispersity ($D$) was only affected by autoclaving alginate as powder, which resulted in a value of around 2.0 ($±0.03$) and a broader, more heterogeneous mass distribution (Figure 2D). These findings corroborate the observations for gelatin and gelatin methacrylate reported by O’Connell et al.15

To investigate if the observed changes in the molar mass distribution lead to macroscopic effects on printability, in-depth rheology studies were performed. Oscillatory (Figure 2A,B), as well as rotatory measurements (Figure 2C,D), revealed a distinct impact on material properties. Considerably lower values for the storage ($G'$) and loss modulus ($G''$) were found for autoclaved samples compared to UV-treated and filtered solutions in the performed amplitude sweep (Figure 2A). We hypothesize that the short-chain fragments that were generated during moist-heat treatment by autoclaving act as a plasticizer, resulting in a reduced chain entanglement of the polymer. Consequently, both the stiffness as well as the energy stored during deformation were reduced, thereby at least in part influencing the printability of alginate. Importantly, all alginate solutions used in the present study did not form gels at 8% (w/v), as demonstrated by $G'' > G'$ notwithstanding the sterilization method. The performed

![Figure 1](https://dx.doi.org/10.1021/acsomega.9b04096)  
**Figure 1.** (A) Normalized refractive index and (B) differential pressure detector response signal obtained via GPC at 35 °C using 0.05 M phosphate-buffered saline (PBS) at pH 6.8 as eluent. (C) Calculated $M_n$, $M_w$, and (D) $D$ values based on calibration with bovine serum albumin. Analysis of variance (ANOVA) was performed, and samples with a $p$-value $<0.05$ were considered as statistically significant and are marked with an asterisk (*).
frequency sweeps confirmed the results and revealed an unexpected rheological behavior for the alginate autoclaved as a powder at frequencies above 10 rad/s (Figure 2B). The elastic component of the alginate material was found to collapse by the order of 4–5 magnitudes with respect to the last value of the linear region (∼10 rad/s). This finding was reproduced with additional samples of autoclaved alginate as a powder (n = 3). We assume that the breakdown of elastic components is related to significant degradation of the material during the sterilization process. Moreover, autoclaving caused the disappearance of an explicit yield point present in other samples at around 10 Pa (Figure 2C). Increasing the shear rate from 0.1 to 100 s⁻¹ revealed shear thinning from 132.9 (±33.6) to 2.8 (±0.1) Pa·s for untreated, from 183.7 (±56.8) to 3.7 (±0.4) Pa·s for UV-irradiated samples, and from 59.1 (±8.6) to 3.9 (±0.3) Pa·s for sterile-filtered samples (Figure 2D). This rheological behavior is typically observed for polymer solutions due to the increasing orientation of the polymer chains with an increasing shear rate.4,7 The viscosity was significantly lower for autoclaved samples and only a minor shear-thinning effect due to the amount of low molecular chain fragments was observed. The viscosity decreased from 6.1 (±0.9) to 2.2 (±0.1) Pa·s for alginate autoclaved as an aqueous solution and from 11.8 (±4.5) to 0.9 (±0.1) Pa·s for alginate autoclaved as powder.

Figure 2. Rheological investigations of aqueous alginate solutions 8% (w/v) at 25 °C. (A) Amplitude sweep from 0.1 to 100% with a constant angular frequency of 10 rad/s. (B) Frequency sweep from 0.1 to 100 rad/s with a constant amplitude of 1%. (C) Shear stress sweep and (D) shear rate sweep from 0.1 to 100 Pa and 0.1 and 100 s⁻¹.

Figure 3. Three-dimension (3D) printed line patterns after different sterilization techniques compared to untreated alginate. (A−F) represents a nine-line pattern and (G−H) a five-line pattern. (A, G) Preset model (B, H) native, (C, I) UV, (D, J) sterile-filtered + lyophilized, (E, K) autoclaved as a solution and (F, L) autoclaved as powder. Printing was performed on a BioX printer (CELLINK, Gothenburg, Sweden) at 25 °C and 120 kPa air pressure. Solutions were stained with Sicopharm Cochineal Red (BASF, Ludwigshafen, Germany) for better contrast.
Autoclaved as a powder (Supporting Videos S4, S5, S9, and S10). The given line structure. This revealed a slight interlacing of the lines at the turning points of needle for the extrusion of new lines. Moreover, all samples treated, and sterile-samples) were preserved and retained their shape. Native, UV-irradiated, and sterile-alginates (native, UV-irradiated, and sterile-filtered samples) were preserved and retained their shape. Native, UV-treated, and sterile-filtered alginates solutions were printable with accuracy trade-offs at the points where the printer positioned the needle for the extrusion of new lines. Moreover, all samples revealed a slight interlacing of the lines at the turning points of the given line structure. This finding may be attributed to the absence of CaCl₂ for stabilization of the 3D printed structure by alginate cross-linking. As cross-linking is time- and concentration-dependent and furthermore depends on the available surface in contact with the cross-linking agent, the addition of CaCl₂ was avoided to allow direct comparison of the printing outcome without postprinting modification.

Sodium alginate, approved by the FDA26 as a food-additive, is considered as noncytotoxic.27 To investigate if degradation products formed during the sterilization process of alginate influence cell viability, the metabolic activity of NIH 3T3 cells was monitored deploying WST-1 proliferation assay with increasing alginate concentrations (Figure 4). It is important to note that no gel formation occurred due to the use of calcium-free Dulbecco’s modified Eagle’s medium (DMEM). No significant differences between the differently sterilized alginites were observed, indicating good cell compatibility of all alginites after the sterilization process. Important to note is that only the influence of the material was investigated while bioprinting was not investigated.

In a final step, we evaluated the sterility of all samples discussed above according to the European Pharmacopeia. Beforehand, the inhibitory effects of all polymer samples were excluded by validation with various type of strains prescribed in the European Pharmacopeia. Sterility testing revealed the existence of *Bordetella bronchiensis* in the unsterilized starting material and in the UV-treated alginate sample (Figure S1). In contrast, the sterile-filtered and subsequently lyophilized alginate sample remained sterile after 14 days of incubation in liquid media. These findings are of major importance as disinfection by UV-light is frequently used in the field of biofabrication.

**CONCLUSIONS**

By analyzing pharmacopeia compendial methods for sterilization of the model bioink component alginate, we developed sterilization strategies suitable for sensitive polymers applied in biofabrication. First, sterilization of a potential bioink should be investigated in the early phase of bioink development. Second, UV-light treatment as a noncompendial method was not effective in terms of sterilization outcomes and should be avoided. Based on the examined methods, we recommend sterile filtration at low polymer concentrations followed by lyophilization to obtain both sterile and printable bioinks.

**MATERIALS AND METHODS**

**Sodium Alginate Solutions.** Sodium alginate (Protanal LF 10/60 FT) was obtained from FMC BioPolymer (Philadelphia, PA). Sodium chloride (NaCl), monobasic sodium phosphate (NaH₂PO₄), and dibasic sodium phosphate (Na₂HPO₄) were purchased from Sigma-Aldrich (Steinheim, Germany) and were used without further purification. The lyophilized powders were stored at −20 °C prior to use.

Sodium alginate solution was prepared by dissolving sodium alginate powder either native or sterilized in MilliQ water for 24 h at 35 °C and 600 rpm in a Thermomixer comfort (Eppendorf, Wesseling-Berzdorf, Germany).

**Cell Culture Material.** Dulbecco’s modified Eagle’s medium (DMEM; high glucose, no calcium, no glutamine; catalog no. 21068028), fetal bovine serum (FBS; ref 10270-106), and Nunc 96-well plates were purchased from Thermo Fisher Scientific (Schwerte, Germany). Penicillin G and streptomycin solutions were purchased from Biochrom AG (Berlin, Germany). Watersoluble tetrazolium salt 1 (WST-1) was purchased from Roche (Basel, Switzerland).
Sterilization. Autoclave sterilization of sodium alginate was performed in a DX-90 2D autoclave (Systec, Linden, Germany) according to the European Pharmacopoeia for 15 min at 121 °C for solutions and powders. Sterile filtration was performed by filtering the sodium alginate solution (1%, w/v) through a Filtropur S 0.2 μm polyethersulfone (PES) syringe filter (Sarstedt, Nümbrecht, Germany) under a Safe 2020 clean bench (Fisher Scientific, Schwerte, Germany). The filtered samples were then frozen at −80 °C and lyophilized in syringes equipped with a Filtropur S 0.2 μm syringe filter for 48 h at 11 μbar. UV-light sterilization of sodium alginate powder was performed under a TL-900 universal UV lamp (Camag, Muttenz, Switzerland) at 254 nm wavelength and a distance of 2 cm for 1 h.

Sterility Testing. Sterility testing was conducted at the Institute for Hygiene and Microbiology of the University of Würzburg using the Steritest Symbio pump (Merck, Darmstadt, Germany). The laboratory is accredited according to the standard DIN EN ISO 17025. Specimen handling and processing were performed using RheoCompass software version 1.23.378 (Anton Paar, Graz, Austria). All GPC measurements were performed in a Malvern Viscotek GPCmax system with an integrated isocratic pump, autosampler, and a Viscotek 305 TDA triple detector (Malvern Panalytical, Kassel, Germany). A Phenomenex BioSep SEC-s4000 and SEC-s2000 column in series with 5 μm particle size and 145 Å pore size (4.6 × 300 mm²), and a guard column at 298 K and 0.05 M PBS at a pH of 6.8 were used. The flow rate was adjusted to 0.3 mL/min. Prior to each measurement, the samples were filtered through Whatman Puradisc 0.2 μm PES syringe filter (GE Healthcare, Chicago) to remove particles. Data were processed using OmniSEC software version 5.12 (Malvern Panalytical, Kassel, Germany).

Cytotoxicity. NIH 3T3 fibroblasts were seeded in a microplate at a concentration of 2000 cells per well in calcium-free DMEM and incubated overnight at 37 °C and 5% CO₂. The following day, 80 mg/mL stock solutions of alginate in MilliQ water, treated with different sterilization techniques, were diluted with calcium-free DMEM to 10, 5, 2.5, and 1.25 mg/mL and added 1:1 to the fibroblasts in triplicates resulting in final alginate concentrations of 5, 2.5, 1.25, and 0.625 mg/mL respectively. After 24 h of incubation at 37 °C and 5% CO₂, the solutions were aspirated and the cells were washed twice with PBS. Afterward, each well was treated with a mixture of 10 parts DMEM and one part WST-1. Then the absorbance was measured at 450 nm (soluble formazan product) and 630 nm (background noise) with a Spectramax 250 microplate reader (Molecular Devices, San José, USA).

Statistical Analysis. For statistical analysis, analysis of variance (ANOVA) was performed with OriginPro 2017 (OriginLab Corporation, Northampton, USA). Samples with a p-value <0.05 were considered as statistically significant and are marked with an asterisk (*).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04096.

3D bioprinting process of the native alginate solution in a 9 line pattern (MP4)
3D bioprinting process of the UV irradiated alginate solution in a 9 line pattern (MP4)
3D bioprinting process of the sterile filtered and lyophilized alginate solution in a 9 line pattern (MP4)
3D bioprinting process of the autoclaved alginate solution in a 9 line pattern (MP4)
3D bioprinting process of the alginate solution autoclaved as powder in a 9 line pattern (MP4)
3D bioprinting process of the native alginate solution in a 5 line pattern (MP4)
3D bioprinting process of the UV irradiated alginate solution in a 5 line pattern (MP4)
Sterile sample (left), sample containing B. bronchiseptica (middle), and test bacterium (S. aureus, right) in soy casein pepton (PDF)
3D bioprinting process of the sterilized filtered and lyophilized alginate solution in a 5 line pattern (MP4)
3D bioprinting process of the autoclaved alginate solution in a 5 line pattern (MP4)
3D bioprinting process of the alginate solution autoclaved as powder in a 5 line pattern (MP4)

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Notes

The authors declare no competing financial interest.

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