New Insights on Biopolymer Sterilization Using Supercritical CO_2 Technology

Gonçalo C. Soares[a], Miguel Silva[a], Sandra Borges[a], Sara Baptista-Silva[a], Ricardo Freixo[a], David A. Learmonth[b], Mariana C. Vallejo[b], Rui. A. Sousa[b], Ana L. Oliveira[a]*

[a]Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia, Porto, Portugal
[b]Stemmatters, Biotecnologia e Medicina Regenerativa SA, Parque de Ciência e Tecnologia Avepark, Zona Industrial da Gandra, Guimarães, Portugal.
* aloliveira@porto.ucp.pt

CATÓLICA FACULTY OF BIOTECHNOLOGY PORTO

Introduction/Resume

The development of biomaterials represents a challenge to existing medical sterilization technologies, since they are often composed of natural polymers which are sensitive to high temperatures. Supercritical CO_2 ($ScCO_2$) has recently been identified as an effective technique for the sterilization of thermally and hydrolytically sensitive polymers.[1] $ScCO_2$ sterilization can be achieved at low temperature and presents several advantages such as inertness, non-toxicity, high penetration ability and non-flammability.[1] According to EN 556–1, a guaranteed sterility assurance level (SAL) is required.[2] It has been demonstrated that $ScCO_2$ combined with low molecular volatile additives can markedly improve bacterial endospores' inactivation to reach the required SAL[3] Although progress has been done in this field demonstrating the efficacy of $ScCO_2$ sterilization of several biopolymers, many are still to be studied. In this work, $ScCO_2$ was used for sterilizing two proteins, silk sericin and fibroin and a polysaccharide, gellan gum. The applied $ScCO_2$ sterilization process was modified from Herdegen *et al.*[4], according to each of the studied polymeric system. The biomaterials were inoculated with *Bacillus cereus*, a spore-forming bacteria, and after the $ScCO_2$ process, the microbiological analysis indicated an effective sterilization.
Characterisation of biomaterials before/after $ScCO_2$ sterilization, was performed by Scanning Electron Microscopy (SEM), Fourier Transformed Infrared (FTIR), NMR and Fast Protein Liquid Chromatography (FPLC) for proteins. According to these techniques the physical integrity, molecular structure and biological activity of the tested materials was maintained and no signs of degradation were detected. The proposed $ScCO_2$ treatments proved efficient for sterilizing the studied biopolymers without compromising its properties or performance.

Materials and Methods

$ScCO_2$ has been used to sterilize silk fibroin in the form of porous scaffolds and membranes while silk sericin and gellan gum were tested in the form of powder, after freeze-drying. In order to evaluate the sterilization efficacy, each sample have been inoculated with a bacterial solution of *Bacillus cereus* NCTC 2599, with a density superior to 10^6 colony forming unit (CFU)/ mL.

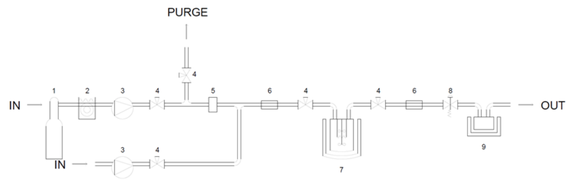

Figure 1. Schematic diagram of $ScCO_2$ equipment. Legend: 1. CO_2 bottle; 2. Thermostatic bath; 3. Injection pumps; 4. Needle valves; 5. Mass flow meter; 6. Heater resistors; 7. Pressure vessel with a stirrer and a refrigeration circuit, surrounded by a heater resistor; 8. Back pressure regulator valve with heater resistor; 9. Collection cup with heater resistor.

Table 1. Process conditions used for the $ScCO_2$ sterilization process.

#Cycles	Pressure range (bar)	Δ Time (min)	Temperature (ºC)	Stirring (rpm)	Sterilization aid
1	0-245	20	38	500	2% (v/v) H_2O_2 30%
2	80-245	20			
3	80-245	20			
4	80-245	20			
5	80-245	20			
6	80-245	20			

Results

$ScCO_2$ sterilization proved to be effective against *Bacillus cereus*, which was guaranteed by a 10^{-6} reduction of CFU/mL, according to SAL assay (**Table 2**).

Table 2. Microbiological results of the different studied materials after 9 days of incubation at 37 ºC.

	Control		After Sterilization	
Materials:	CFU/mL	Log(CFU/mL)	CFU/mL	Log(CFU/mL)
Sericin	$2.4x10^7 \pm 5.6x10^6$	7.4±0.10	$<1.0x10^1$	<1.0
Fibroin	$8.0x10^6 \pm 7.1x10^5$	6.9±0.04	$<1.0x10^1$	<1.0
Gellan gum	$5.0x10^7 \pm 1.3x10^7$	7.7±0.12	$<1.0x10^1$	<1.0

FTIR analyses revealed that any physicochemical alteration occurred by means of $ScCO_2$ sterilization. As can be seen in **Figures 2-4**, FTIR bands are coincident before and after $ScCO_2$ sterilization for every sample tested out.

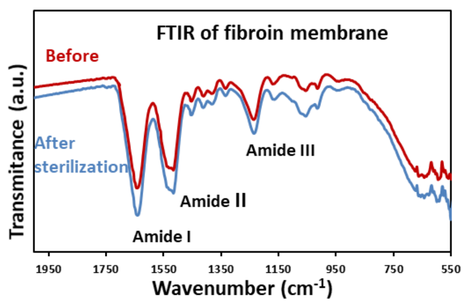

Figure 2. FTIR corresponding to the fibroin membrane.

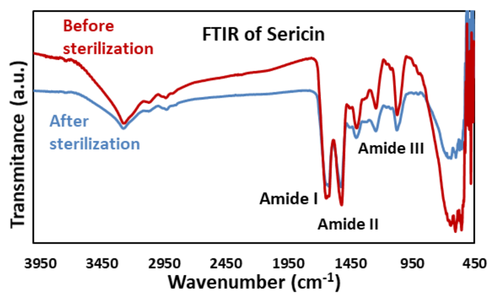

Figure 3. FTIR corresponding to sericin.

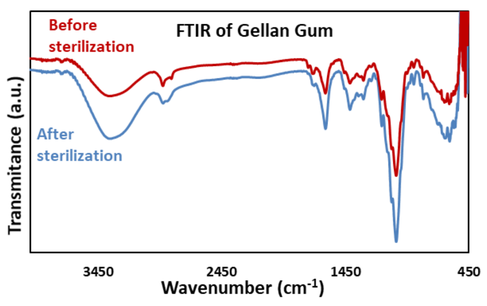

Figure 4. FTIR corresponding to gellan gum.

This $ScCO_2$ sterilization process also proved to be gentle with protein samples, since FPLC chromatograms (**Figures 5 and 6**) show no signs of degradation.

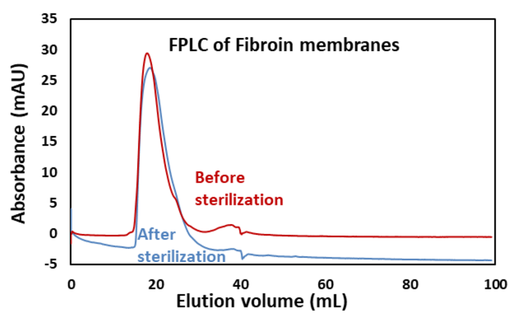

Figure 5. FPLC chromatogram corresponding to fibroin membranes.

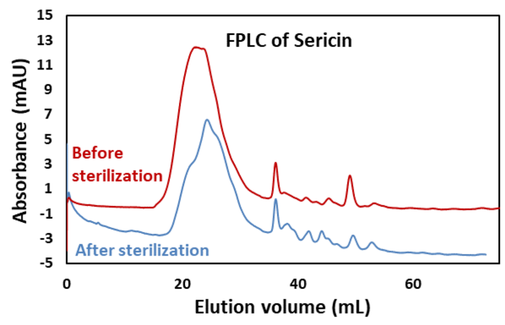

Figure 6. FPLC chromatogram corresponding to sericin.

^{1}H NMR analyses revealed that no detectable structural alteration to the repeat tetrasaccharide backbone of gellan gum occurred during $ScCO_2$ sterilization, as clearly seen in **Figures 7** (pre-treatment) **and 8** (post-treatment).

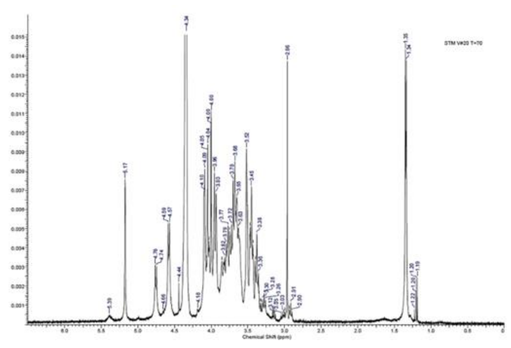

Figure 7. ^{1}H NMR spectrum of gellan gum before $ScCO_2$ sterilisation.

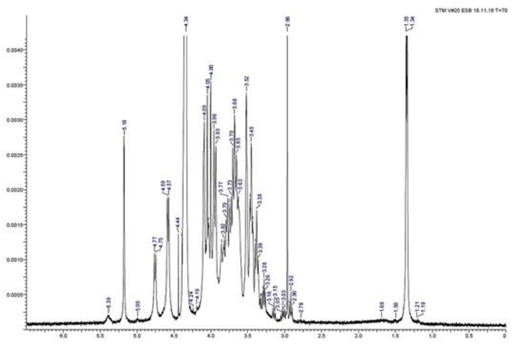

Figure 8. ^{1}H NMR spectrum of gellan gum after $ScCO_2$ sterilisation.

Conclusions

$ScCO_2$ sterilization, under the addition of 2% hydrogen peroxide, successfully inactivated *Bacillus cereus*. Physicochemical properties and structural integrity and stability of fibroin, sericin and gellan gum were not compromised by this sterilization process. Thus, this $ScCO_2$ sterilization process poses as a promising and viable alternative to the most conventional sterilization techniques.

Acknowledgements

National Funds from FCT - Fundação para a Ciência e a Tecnologia through project UID/Multi/50016/2013; FCT post doc fellowship to Baptista-Silva S. (SFRH/BPD/116024/2016), Program FCT Investigator to A. L. Oliveira (IF/00411/2013), project SERICAMED (IF/00411/2013/CP1167), and through "Biotherapies- Bioengineered Therapies for Infectious Diseases and Tissue Regeneration", funded by Norte2020.

References

1. Sikin, A. & Rizvi, S. Recent patents on the sterilization of food and biomaterials by supercritical fluids. *Recent Patents Food. Nutr. Agric.* **3,** 212–225 (2011).
2. EN 556-1 Beuth Verlag, Burggrafenstr.
3. Bernhardt, A. *et al.* Improved Sterilization of Sensitive Biomaterials with Supercritical Carbon Dioxide at Low Temperature. *PLoS One* **10,** 1–19 (2015).
4. Herdegen, V. *et al.* Sterilization of medical products from collagen by means of supercritical CO2. *Chem. Eng. Technol.* **37,** 1891–1895 (2014).