Applications of Supercritical Fluid in Alloplastic Bone Graft

Chin-Feng Chen*,1, Che-Shoa Chang1,2

* Corresponding author. Tel.: +886-2-2392-2300 Fax: +886-2-2392-2310
E-mail address: d49213003@ym.edu.tw

1 Institute of Clinical Dental Science, National Yang-Ming University, Taipei, Taiwan.
2 Department of Dentistry & Oral-Maxillo-facial Surgery, Taipei Veterans General Hospital, Taipei, Taiwan.

Abstract

In this study the supercritical fluid (SCF) technology is introduced into biomaterial processing, especially the fabrication of alloplastic bone graft. SCF offers very excellent extraction characteristics for some compounds, due to its favorable diffusivity, viscosity, zero surface tension, liquid-like density and solvating power, and other physical properties. The most desirable SCF solvent for extraction is supercritical carbon dioxide (SCCO₂), which is non-toxic, non-flammable, inexpensive, mild supercritical condition (Tc=31.2 °C, Pc=7.4 Mpa), friendly to mankind, and environmentally benign. At present SCCO₂ has been applied for green organic solvent for environmental protection, extraction and purification of organic functional matters high purification and separation of critical materials, cleaning of wafers, substitute for fluorine chlorine hydrocarbon compounds, formations of nano- or micron- particles, substitute for organic solvent, fabrication of special metal organic matter and special function product, foaming material preparation, waste water processing and chemical weapons destruction with supercritical water, and so on. For the porous structure of porcine vertebra with the content of lipids, proteins, and inorganic substances in the cells and intercellular matrix, SCCO₂ is applied to the processing and fabrication of porcine-derived bone graft. SCCO₂ not only removes the lipid content of bone graft, but also sterilizes it. Specific modifiers and cross-linkers are added to enhance the favorable properties of SCCO₂ and eliminate the antigenicity of porcine proteins deeply and then removed by SCCO₂. The initial result of pilot study showed good biocompatibility in an in vitro test which was performed with MG63 osteoblast-like cells. Advanced optimization and in vitro and in vivo tests will be performed in this study and the results will help much in tissue engineering. With optimal modification based on this study, this technology may also help in skin graft, cartilage graft and other biomaterials.

Keywords: Supercritical fluid, biomaterial processing, alloplastic bone graft, carbon dioxide, biocompatibility.

Introduction

Since long-time, it has always been the goal on which the researchers devoted themselves to study about the repair of bone damage area. The traditional method is mainly the use of autogenous...
bone graft to repair the bone damage area, because it simultaneously possesses the properties of osteogenesis, osteoinduction, and osteoconduction. Thus, the autogenous bone graft was considered as the most ideal bone graft. However, in Younger’s study in 1989, it was demonstrated that the autogenous bone graft still resulted in 17.9% ratios of illness complication. Moreover, the sources and quantity of autogenous bone graft are limited, and it cannot be used massively or for many times. Therefore, there are the appearance and the application of allograft as well as alloplasts. Among these bone graft materials, hydroxyapatite (HA)\(^2\text{–7}\), which has the property of osteoconduction, and demineralized freeze-dried bone allograft (DFDBA)\(^8\text{–12}\), which has the property of osteoinduction, are most widely used and studied. There are many studies about these two different bone graft materials, in which also confirmed that HA not only possesses high degree of biocompatibility\(^2\text{–7}\), but also acts as the scaffold of repaired blood vessel or newborn bone tissue in the healing process of bone damage.\(^7\text{–10}\) The porous HA possesses even better property. However, it’s one of imperfection that HA lacks of the osteoinduction characteristic. As for DFDBA, it had been discovered that it possesses quite good osteoinduction characteristic, and was widely applied to the periodontal treatments. But it lacks of the scaffold effect for space maintaining and it is made from the human cadaver, so that its source is also limited and possesses the disadvantages with the probability of approximately 1/6,000,000 for infection of human immunodeficiency virus.\(^13\text{–14}\) Thus, to seek other desired bone graft materials made by animals or artificial materials which simultaneously possess the characteristics of biocompatibility, osteoconduction and osteoinduction and are apt for larger defects with higher safety and lower cost is the primary goal that the researchers devote themselves to study about. For example, the True Bone Ceramics.\(^15\text{–16}\)

In Europe, the bovine-derived and sheep-derived bone graft materials have been developed for years. But the safety is quite questionable because the prion might induce Bovine Spongiform Encephalopathy (BSE) and new variant Creutzfeldt-Jacob disease (vCJD). As in Taiwan, there are quite sufficient source of pigs and a lot of researches about the pigs. For example, the immunogenicity-free skin graft from pathogen-free and immunogenicity-free pigs has been developed in Animal Technology Institute Taiwan (ATIT). Although there might be doubts about aftosa (foot and mouth disease, FMD) in Asia, the viruses, including virus of FMD, are to be deactivated in the SCF process in this project.\(^17\text{–18}\)

In this interdisciplinary study we introduce the SCF technology into the fabrication of alloplastic bone graft materials. SCF technology has widely been applied in food and pharmaceutics industry in recent years.\(^19\text{–20}\) SCF is referred to the condition of fluid which temperature and pressure surpass its critical temperature and critical pressure.\(^19\text{–20}\) The matter usually possesses gas phase, liquid phase and solid phase which everybody knew very well. However, when its temperature and pressure surpass its critical temperature and critical pressure, the substance enters the so-called supercritical fluid condition. Before passing the critical point, there exists obviously the interface between gas and liquid. While it reaches the critical point, this interface namely vanishes. Some matter may changes to other color from colorless when it arrives at the supercritical
condition. If it passes through the reduced pressure or the temperature decrease again, it can also return to gas and liquid biphase. The physical property of SCF is situated between the gas phase and the liquid phase. For example, the viscosity of SCF approaches to gas, while the density of SCF approaches to liquid. Because its density is high, it may transfer more matter than gas. Because its viscosity is low, the power needed for transportation is lower than liquid. Also, the diffusion coefficient is higher than liquid 10 to 100 times, so that the mass transfer resistance is far lower than liquid, because the mass transfer of SCF is quicker than liquid. In addition, SCF possesses gas-like surface tension, so that it is very easy to permeate through the porous structure. Besides physical properties, its chemical properties also differ from gas and liquid. For example, carbon dioxide does not possess the extraction power under the gas condition. While it enters the supercritical condition, carbon dioxide turns into organophilic, thus has the ability to dissolve organic matter. This dissolving capability differs with the temperature and the pressure. There are many matters that can serve as SCF. Among these matters, carbon dioxide is the most preferred one, because its critical temperature is only 31.2 °C, close to the room temperature, and its critical pressure approximates 72.8 a.t.m., which is also not too high. It possesses neither toxicity for normal condition nor spontaneous combustion property, and has wide sources and inexpensive price. At present SCCO\textsubscript{2} has been applied for green organic solvent for environmental protection, extraction and purification of organic functional matters high purification and separation of critical materials, cleaning of wafers, substitute for fluorine chloride hydrocarbon compounds, formations of nano- or micron-particles, substitute for organic solvent, fabrication of special metal organic matter and special function product, foaming material preparation, waste water processing and chemical weapons destruction with supercritical water, and so on.\textsuperscript{19-20} For example, Taiwan I-Mei Foods Co., LTD. utilizes SCCO\textsubscript{2} to the cleaning of rice, removing heavy metal and agricultural chemicals remaining. German and Italy’s food industry utilize SCCO\textsubscript{2} to remove the caffeine ingredient of tea and coffee bean, as well as to extract effective component of herbal medicine. Moreover, a very interesting example is that Danish wood industry uses SCCO\textsubscript{2} to impregnate the antiseptic into the deep wood for enhancing the resistance of corrosion and termite and mosquito.\textsuperscript{20}

In recent years the SCF technology has widely been applied in food and in the drugs manufacture industry.\textsuperscript{19-20} However, there were very few researches about the applications of SCF technology on the graft materials. Fages et al., a French research team, utilized SCCO\textsubscript{2} to remove the fat of sponginess bone of sheep spine, again utilized hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), sodium hydroxide (NaOH), Sodium dihydrogen phosphate (NaH\textsubscript{2}PO\textsubscript{4}), as well as ethanol for a series of processing in order to remove partial protein ingredient of the sheep bone and decrease the antigenicity. They also confirmed that such product possesses good biocompatibility and the property of osteoconduction. Afterwards they developed a commercial commodity, called as OXBONE\textsuperscript{TM}.\textsuperscript{18,21-24} However, in their studies, SCCO\textsubscript{2} was used only to remove the lipid content, in which they didn’t utilized all the advantages and characteristics of SCF, such as the high penetrability and the high dissolving power. Besides, the procedures were very complicated. Thus, we utilize SCF with another method in this study to the fabrication and processing of alloplastic
bone graft.

Material & Method

In this study we make use of SCCO$_2$ with specific modifiers and specific cross-linkers to treat the porcine vertebra fragments in order to remove the lipid content and remaining undesired chemicals, decrease the antigenicity of protein content, preserve the inorganic structure, and sterilize it at the same process. With the removal of the lipid content, the probability of contamination during healing period will be decreased. With the decrease of antigenicity, the inflammatory reaction of host during healing period will be reduced. With the sterilization and disinfection properties of SCF at the same process, the probability of infection of bacteria and virus will be eliminated. And with this interdisciplinary combination application, the cost of bone graft will be reduced definitely and the safety of bone graft will be enhanced.

The substantial goal of this study is to produce a novel bone graft of higher safety and lower cost from an useless-like discarded material. So, in this study, porcine vertebrates were used for the reason that porcine researches and quantity are very rich in Taiwan and the availability and quality are also very good in Taiwan. Thus, an SCF extraction system (ISCO SFX 210 extraction system) was used to process the fragments of porcine vertebrates. The porcine vertebrates were brought from common market and were divided into fragments of 16 mm homogenous cubic size. The first group of fragments was processed statically by SCCO$_2$ under 45 °C and 40 MPa for 10 minutes. Ethanol and glutaraldehyde were added as the specific modifiers and specific cross-linkers. And then be processed dynamically at the flow rate 2 kg/h for 30 minutes. The second group of fragments was processed under 45 °C and 40 MPa statically for 10 minutes and dynamically at the flow rate 2 kg/h for 30 minutes. The third group as the control group was processed at the subcritical condition under 31 °C and 7 MPa statically for 10 minutes and dynamically at the flow rate 2 kg/h for 30 minutes. These three groups were then tested with MG63 osteoblastic-like cell lines for the biocompatibility.

Result & Disscussion

The biocompatibility shows that the first group has the best affinity and biocompatibility to the cells while the third group has the worst outcomes. MG63 osteoblastic-like cells may proceed with proliferation well on the first group of fragments.

In Fages’ study, cancellous bone of vertebrae from a single sheep was treated with SCCO$_2$ for 12 h, at 250 bars and at 50°C for the removal of lipid content. A pilot plant (Separex, Champignueulles, France) applied for batch extraction and separation was used. They were either frozen at 20 °C or treated with supercritical CO2 and hydrogen peroxide. The extraction vessel had a volume of 2.5 L. All the operations were remote controlled except for loading and unloading. The operating conditions were: CO$_2$ flow rate 2 kg/h; pressure 250 bars; extraction temperature 50 °C; time 10 min/g of bone. After being unloaded from extraction vessel, be immersed in a 35 % H$_2$O$_2$
solution at 40 °C for 24 h. And then be treated in NaOH solution (1 M) for 1 h at room temperature. And then be buffered 15 min in NaH$_2$PO$_4$ (12 g/L), before being rinsed for 3 h in an ethanol solution (95 %) followed by absolute ethanol for 2 h. The composition of the processed bone was: mineral phase 63.5 ± 0.5 %; proteins 24.43 ± 0.5 %; lipids 0.5 ± 0.01 %. This novel bone graft showed good compatibility. However, SCF was utilized only to remove the lipid content of sheep bone in Fages’ study and the characteristics and advantages of SCF were not be completely utilized. For example, the high permeability and the material processing capability.

Ethanol is generally added into SCCO$_2$ as the specific modifiers. It may change the solubility of polar and non-polar substance in SCCO$_2$. For example, the solubility of polar substance will rise with the existence of modifier. GA is added into SCCO$_2$ as the specific cross-linkers. It may cross-link the protein content and decrease the antigenicity and is widely used in the cross-linking of collagens and proteins. Besides, GA and ethanol are generally and clinically used for the sterilization and disinfection of instruments. Viral inactivation of alloplastic and allogenic bone grafts are generally attained by β or γ irradiation, ethylene oxide or autoclaving. None of these methods have proved to be totally satisfactory, either caused by the resistance of some viruses to physico-chemical processing or caused by the loss of inductive or mechanical properties of the graft material. In Fages’ study, they also evaluated the sterilization and disinfection effect of SCCO$_2$ and the other procedures. Their results showed that SCCO$_2$ contributed to 4.02 to 6.58 logs reduction of the viruses; the ethanol 4.21 to 6.6 logs; totally 14.22 to 24.48 logs. In recent years, SCCO$_2$ has been proved the capability of sterilization and disinfection of materials and instruments. In this study, SCCO$_2$ combined with specific modifier and specific cross-linker will enhance the capability of sterilization and disinfection. The efficiency of SCCO$_2$ for sterilization and disinfection has to be verified in the future.

Summary

In this proposal we make use of SCCO$_2$ with specific modifiers and cross-linkers to treat the porcine vertebra fragments to remove the lipid content and remaining undesired chemicals, decrease the antigenicity of protein content, preserve the inorganic structure, and sterilize it at the same process. With the removal of the lipid content, the probability of contamination during healing period will be decreased. With the diminishing of immunogenicity, the inflammatory reaction of host during healing period will be reduced. With the sterilization and disinfection properties of SCF at the same process, the probability of infection of bacteria and virus will be eliminated. And with this interdisciplinary combination application, the cost of bone graft will be reduced definitely and the safety of bone graft will be enhanced. Additional advanced in vitro and in vivo tests have to be executed in the future for the verifications. Even with optimal modification in the future, the SCF may be applied to other graft materials, such as skin graft, cartilage graft, and other biomaterials. To sum up, this interdisciplinary project will contribute to the knowledge base of bone regeneration and tissue engineering, applications of SCF in biomedical materials, and sterilization and disinfection of other instruments and materials.
References


11. Iou GT, Chang CS, Su CY. A Comparative Study on Microcirculatory Changes and Bone Formation of Porous HA-implanted Cavities or Porous HA/DFDBA-implanted Cavities By SEM. Master Thesis of Institute of Clinical Dental Science, School of Dentistry, National Yang-Ming University, Taiwan. 2002.


25. Ma TY. Studies of the structure and property of Genipin- or Carbodiimide-crosslinked biological materials. Master thesis of Institute of Chemical Engineering, National Central University, Taiwan. 2000.


