Materials Science and Engineering C 61 (2016) 324–332

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

The influence of sterilization on nitrogen-included ultrananocrystalline diamond for biomedical applications

Wei Tong [a], Phong A. Tran [b,c], Ann M. Turnley [d], Morteza Aramesh [a,e], Steven Prawer [a], Milan Brandt [f], Kate Fox [f,*]

[a] School of Physics, University of Melbourne, Parkville, Victoria, Australia
[b] Department of Chemical and Biomolecular Engineering, University of Melbourne, Parkville, Victoria, Australia
[c] Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, Queensland, Australia
[d] Department of Anatomy and Neuroscience, University of Melbourne, Parkville, Victoria, Australia
[e] School of Chemistry, Physics, and Mechanical Engineering, Queensland University of Technology, Brisbane, Queensland, Australia
[f] Centre for Additive Manufacturing, School of Aerospace, Mechanical and Manufacturing Engineering, RMIT University, Melbourne, Victoria, Australia

ARTICLE INFO

Article history:
Received 23 July 2015
Received in revised form 6 December 2015
Accepted 18 December 2015
Available online 21 December 2015

Keywords:
Diamond
Nitrogen-included ultrananocrystalline diamond
Sterilization
Cell culture
Bioactivity
Charge injection

ABSTRACT

Diamond has shown great potential in different biomedical applications, but the effects of sterilization on its properties have not been investigated. Here, we studied the influence of five sterilization techniques (solvent cleaning, oxygen plasma, UV irradiation, autoclave and hydrogen peroxide) on nitrogen-included ultrananocrystalline diamond. The chemical modification of the diamond surface was evaluated using X-ray photoelectron spectroscopy and water contact angle measurements. Different degrees of surface oxidation and selective sp^2 bonded carbon etching were found following all sterilization techniques, resulting in an increase of hydrophilicity. Higher viabilities of *in vitro* mouse 3T3 fibroblasts and rat cortical neuron cells were observed on oxygen plasma, autoclave and hydrogen peroxide sterilized diamond, which correlated with their higher hydrophilicity. By examination of apatite formation in simulated body fluid, *in vivo* bioactivity was predicted to be best on those surfaces which have been oxygen plasma treated and lowest on those which have been exposed to UV irradiation. The charge injection properties were also altered by the sterilization process and there appears to be a correlation between these changes and the degree of oxygen termination of the surface. We find that the modification brought by autoclave, oxygen plasma and hydrogen peroxide were most consistent with the use of N-UNCD in biological applications as compared to samples sterilized by solvent cleaning or UV exposure or indeed non-sterilized. A two-step process of sterilization by hydrogen peroxide following oxygen plasma treatment was then suggested. However, the final choice of sterilization technique will depend on the intended end application.

1. Introduction

Recent reports have established diamond as an attractive candidate biomaterial due to many of its excellent properties, such as superior hardness, low coefficient of friction, high corrosion resistance, good biocompatibility and excellent charge injection properties [1–8]. Among the different types of diamond, ultrananocrystalline diamond (UNCD), which is composed of ~5 nm sp^3 diamond grains in a matrix of sp^2 carbon [9] has drawn much attention [10]. Compared with other types of diamond, UNCD films exhibit a nanotopography which appears to facilitate cell–substrate interactions in line with the nanoscale extracellular matrix of tissue. These surfaces have been reported to modulate the functions of many kinds of cells such as osteoblasts [11], fibroblasts [12], cortical neurons [6,13], and cortical stem cells [14] while also showing potential for biosensing post-surface functionalization [15,16]. When UNCD is fabricated with nitrogen within the gas mixture during growth, the UNCD films exhibit high conductivity due to the increase of the density of states associated with π bonding within grain boundaries. This electrically conductive nitrogen-included ultrananocrystalline diamond (N-UNCD) has a high electrochemical charge injection [7]. Accordingly it is a highly sought after biomedical material for use in high end biomedical applications, for example, as the neurally-interfaced stimulating electrodes of the high acuity retinal prosthesis developed as part of the Bionic Vision Australia's Bionic Eye Program which aims to restore sight to suffers of the retinal disease, *retinitis pigmentosa*. [7,17,18].

For all biomedical implants, sterilization is mandatory after device fabrication and prior to clinical implantation. Accordingly, it is insufficient to study the properties of biomaterials without considering the impact of sterilization and it is necessary to establish the influence of sterilization techniques before clinical use. Different sterilization techniques have been reported to influence both the surface chemical and topological properties of many biomaterials, such as titanium alloys and ultrahigh molecular weight polyethylene, consequently changing their biological related performance [19–22]. Although some sterilization techniques may induce negative effects on material properties

* Corresponding author.
E-mail address: kate.fox@rmit.edu.au (K. Fox).

http://dx.doi.org/10.1016/j.msec.2015.12.041
0928-4931/

[22], others may give rise to potentially beneficial changes when they are utilized in biological applications. [19].

The effects of sterilization on the chemical properties of N-UNCD films have not been thoroughly investigated and are poorly understood. Here, we assess five common sterilization treatments (solvent cleaning, oxygen plasma, UV irradiation, autoclave and hydrogen peroxide sterilization compared to as received) to ascertain the effects that each technique may have upon the surface chemistry of N-UNCD films. The corresponding impact on the cell responses of the modified surfaces was evaluated using mouse 3T3 fibroblast and primary rat cortical neuron cultures. Then, the examination of apatite formation on sterilized substrates in simulated body fluid was used to predict their *in vivo* bioactivity. Finally, electrochemical characterization was performed to measure the charge injection capacities for neural stimulation.

2. Materials and methods

2.1. Preparation of diamond films

Nitrogen-included ultrananocrystalline diamond (N-UNCD) films were deposited on polished silicon wafers (n-type Si (100), MMRC Pty Ltd) using an Iplas microwave plasma-assisted CVD system as previously described [7]. Before the film deposition, silicon substrates were cleaned with acetone, methanol and isopropyl alcohol and then seeded by ultrasound in a ~5 nm nanodiamond/methanol solution. The seeded Si substrates were placed in the MPCVD chamber and a gas mixture of 79% argon, 20% nitrogen and 1% methane (all gases, BOC Australia, purity 99.999%) was introduced into the chamber. For diamond film growth, the microwave power was kept at 1000 W, chamber pressure at 90 Torr and the stage temperature at 900 °C for 17 h. After the growth, the chamber was cooled down in an atmosphere of argon. Consistent with previous reports [7], the N-UNCD surface appears to be composed from fins-like nanostructures, typically about 100 nm in length giving the surface a grassy appearance (Fig. 1). The resulting N-UNCD film thickness is approximately 6–8 μm and the conductivity is ~46 S/cm.

2.2. Sterilization

After preparation, N-UNCD films were divided into groups and sterilized by five different techniques.

- Solvent cleaning (Solvent): Randomized samples were chosen and cleaned by ultrasound in acetone, methanol and isopropyl alcohol, each step for 5 min. Samples were then dried in nitrogen gas, packed and sealed for characterization.
- Oxygen plasma (Oxygen): The diamond films for oxygen plasma sterilization were placed in a Diener plasma cleaner. The samples were exposed to a 3:1 argon:oxygen plasma at 40 sccm with a power of 50 W for 5 min, then packed and sealed for later experiments.
- UV exposure (UV): Samples were placed in a laminar flow hood with a UV-C germicidal lamp and exposed to ultraviolet light (254 nm) for 17 h, then packed until use.
- Autoclave (AC): Samples were sterilized by steam autoclave at 120 °C for 25 min, then packed and sealed until being used.
- Hydrogen Peroxide (Sterrad): The diamond films were placed in individual customized tissue culture plates to enable gas flow to the samples and sterilized using hydrogen peroxide sterilization (STERRAD 100S system; Advanced Sterilization Products, Irvine, Calif). The cycle time was 1 h and the temperature was less than 50 °C.

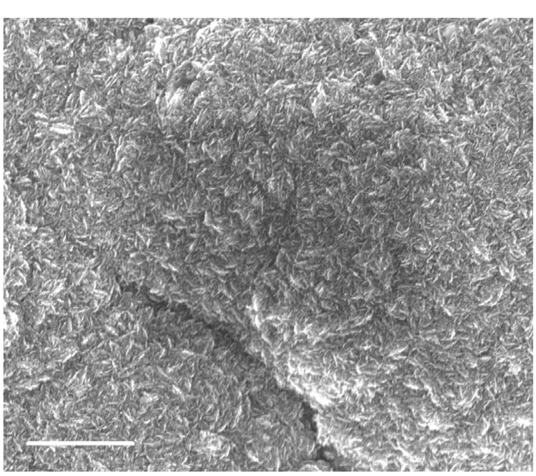

Fig. 1. High-resolution scanning electron microscope image of the as-received N-UNCD surfaces. Scale bar: 1 μm.

Non-sterilized diamond films (as received, AR), fabricated by the same technique served as a control in all experiments.

2.3. Surface characterization

Scanning electron microscope (SEM) images and focused ion beam (FIB) milling was performed to observe the surface topography of the prepared samples using an xT Nova Nanolab 200FIB/SEM. The surface chemical composition of diamond films before and after sterilization was determined by X-ray photoelectron spectroscopy (Thermo-Fisher K-Alpha), using a Mg Kα radiation source at a power of 300 W. Contact angle measurements were carried out using a contact angle measuring system (DataPhysics, OCA-20 contact angle meter and tensiometer) at room temperature.

2.4. Fibroblast viability assays

N-UNCD films were cut into 3×3 mm^2 coupons to use for the experiments. Mouse 3T3 fibroblasts (CCL-92, ATCC, VA, USA) were used here as this cell line has been widely used to test cytotoxicity and biocompatibility of materials for biomedical applications. Samples were inserted to each well of a 96-well plate and fibroblasts were seeded in Dulbecco's Modified Eagle Medium (DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin (P/S) all obtained from Sigma) at the density of 10,000 cells/cm^2. Borosilicate glass coverslips of 5 mm in diameter (Fisher Scientific) were degreased in acetone, ethanol and etched in 1 M NaOH for 1 h followed by autoclaving sterilization and used as a reference. The plate was placed in a cell culture incubator (37 °C, 5% CO_2) for 2 days. At the end of the indicated time, cell viability was determined using Alamarblue assay (Life Technologies) following the manufacturer's instructions. Viable cell numbers were then calculated using a pre-established standard curve. The cells on the samples were also incubated with Calcein-AM (Life Technologies) which stains live cells and imaged using an inverted fluorescence microscope (Olympus IX71, Olympus Australia, Victoria, Australia). Experiments were done in at least duplicate and repeated three times.

2.5. Cortical neuron in vitro testing

Given that diamond is rapidly progressing in the field of neural engineering, it was essential to assess the effect of sample sterilization on the diamond surfaces using a neural cell population as neural cells are often considerably more susceptible to cell death than the more hardy fibroblasts. Therefore the survival of the neurons was tested on sterilized and as-received N-UNCD.

N-UNCD films were cut into 3×3 mm^2 coupons and three samples from each experimental group were placed into a 24-well plate for cell seeding. Cultures of cortical neurons were obtained by isolating the cerebral cortices from one-day-old rats, as previously described [23]. The meninges were removed and the cortical tissue was dissociated