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Dissolving biomolecules and modifying biomedical implants with supercritical carbon dioxide*

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Abstract: We describe two methodologies for dissolving ionic/polar species in $scCO_2$. Both lead to a broadening of the range of applications for $scCO_2$. Fluorinated surfactants may be used to prepare water in carbon dioxide microemulsions to allow solubilization of ionic and biological species. We outline also the preparation of $scCO_2$ soluble metal precursors that can be impregnated efficiently into polymeric substrates. Further processing by heat or UV light leads to metallic particles distributed throughout a polymer substrate. The clean synthesis of such composites can be applied to the development of improved medical implants.

SOLVING THE INSOLUBLE

In the last decade, supercritical fluids have attracted great interest as environmentally acceptable replacements for a wide range of processes that currently rely on conventional organic solvents. There have been several recent review articles and books describing the potential and current uses of supercritical fluids, ranging from commercial scale extraction to catalytic and asymmetric synthesis of pharmaceutical intermediates [1–4]. Supercritical fluids are versatile solvents that possess a unique combination of gas- and liquid-like properties. Like gases they have high diffusivity and low viscosity, but like liquids they have appreciable densities and can dissolve other species. Moreover, the density and, hence, solvating power of supercritical fluids is tuneable, allowing a degree of control which is not present in conventional solvents. However, supercritical fluids are not "super-solvents". For example, the most commonly used fluid, supercritical carbon dioxide ($scCO_2$), has solvating properties characteristic of both fluorocarbon and hydrocarbon. Thus, polar compounds and charged species are largely insoluble in $scCO_2$. In this paper we describe methods for the solubilization of polar and charged species in $scCO_2$, with particular reference to biological molecules and metals or their salts.

Water in $scCO_2$ microemulsions

One potential method for solubilizing hydrophilic species in $scCO_2$ was to develop surfactants to support water in $scCO_2$ microemulsions (Fig. 1). The key problem was to identify a surfactant capable of supporting water in $scCO_2$ microemulsions. Examples of such microemulsions in supercritical propane had been reported using the surfactant aerosol OT [5]. This work led to a significant body of research into microemulsions in supercritical alkane systems [6]. However, the challenge was to develop such systems in $scCO_2$, a more environmentally attractive supercritical solvent. Much work has focused on the design of surfactants capable of supporting water in CO_2, taking into account factors such as favorable CO_2-tail interactions, properties affecting the curvature of the micellar interface and surfactant volatility [7]. The first successful example of water in $scCO_2$ microemulsions was reported in 1996 and focused

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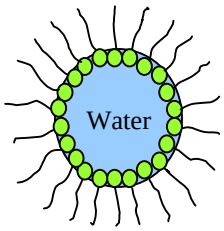

F_3C-[(O-CF_2-$CF(CF_3)$)$_n$-(O-CF_2)$_m$]-O-CF_2-$COO^-NH_4^+$

Fig. 1 Schematic diagram of water in $scCO_2$ microemulsion.

on a commercially available carboxylic acid-terminated perfluoropolyether (PFPE) [8]. The surfactant itself was prepared by the formation of the ammonium carboxylate salt of the PFPE (Fig. 1). The microemulsions formed from this surfactant are optically transparent, thermodynamically stable, and were fully characterized by cloud point studies, on-line FTIR, and UV-visible spectroscopy. Figure 2 shows an FTIR spectrum of an $scCO_2$ solution in which an inactive surfactant is used. Only bands assigned to free (unassociated) water, surfactant, and CO_2 are readily observed. However, in the presence of the active PFPE ammonium carboxylate surfactant, significant differences are observed in the FTIR spectrum (Fig. 3). Most importantly, IR bands appear which indicate the presence of bulk, H-bonded water thus proving the presence of microemulsions. Unfortunately, commercial samples of this original PFPE material (molar mass 800) have recently proved difficult to obtain commercially. Thus, we have begun to investigate alternatives. A range of carboxylic acid-terminated PFPE materials are commercially available from DuPont under the collective brand name Krytox™. They have differing average molar masses of 2500 M_W (Krytox FSL), 5000 M_W (FSM), and 7500 M_W (FSH). The carboxylic acid-terminated molecule must first be converted to the ammonium carboxylate salt to prepare an active surfactant. The conversion process is the same method as that used in our earlier work [8], and FTIR spectroscopy is used to monitor the process for each precursor conversion (Fig. 4).

We have utilized *in situ* FTIR spectroscopy to determine whether a given surfactant forms water in $scCO_2$ microemulsions. Preliminary results show that the higher molar mass surfactants (FSM and FSH) do not allow the formation of microemulsions in $scCO_2$. By contrast, the ammonium carboxylate

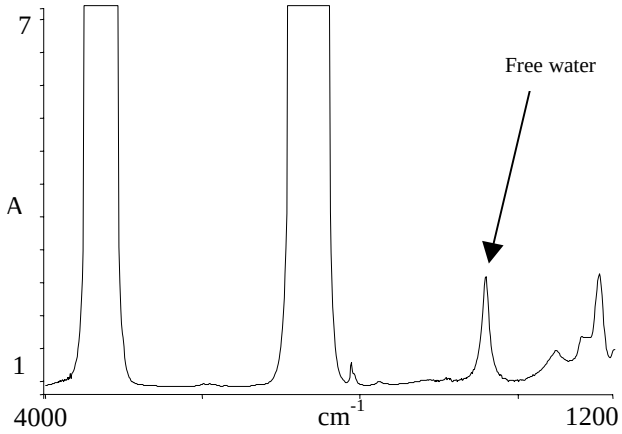

Fig. 2 FTIR spectrum of $scCO_2$ containing water and an inactive surfactant. No microemulsions are detected.

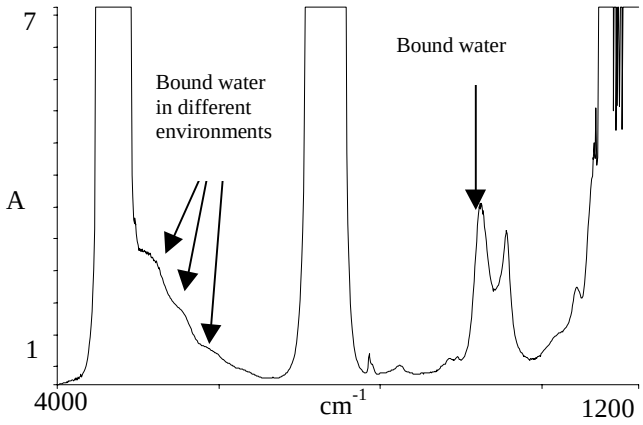

Fig. 3 FTIR spectrum of water in $scCO_2$ microemulsions. Note FTIR bands assigned to hydrogen-bonded water domains.

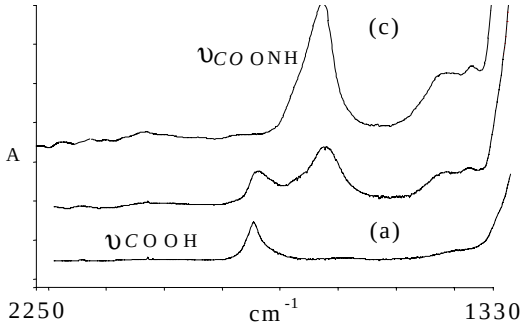

Fig. 4 FTIR spectra showing the conversion from the carboxylic acid terminated Krytox FSL precursor (a) through to the ammonium carboxylate surfactant (c).

salt of Krytox FSL (2500 molar mass) shows substantial bound water peaks in the FTIR spectrum which are indicative of water in supercritical CO_2 microemulsion formation [9]. Our recent experimental studies have therefore focused on this surfactant.

Dissolving ionic and polar species

The aqueous environment of the optically clear water in $scCO_2$ microemulsions can support dissolved ionic species. Our earlier work has shown that dissolved species such as sodium nitroprusside and potassium permanganate can be detected in the aqueous micellar environment by UV/visible spectroscopy [10]. Now, using the same spectroscopic techniques, we have determined that the new Krytox-based surfactant (2500 molar mass) is also able to solubilize these same species. In the absence of the surfactant, no metal complex absorptions are detected. We have utilized these surfactant/$scCO_2$ systems to solubilize complex biological molecules for a number of biomedical and pharmaceutical applications. Previous experiments had demonstrated the solubilization of molecules such as Bovine Serum Albumen (BSA) [8]. We have extended these studies to the solubilization of enzymes such as β-galactosidase. The solubilization of this enzyme in water in $scCO_2$ microemulsions has been proved by UV/visible

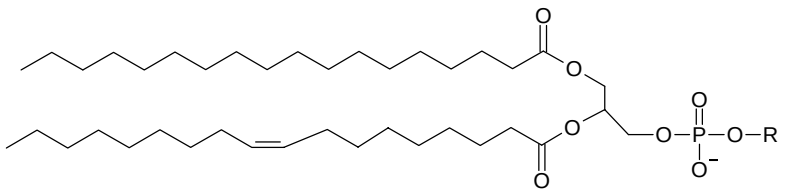

Fig. 5 Schematic structure of a generic phospholipid.

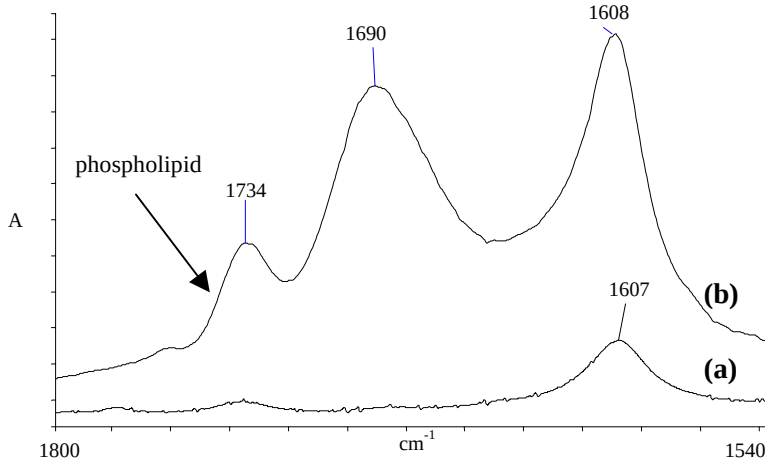

Fig. 6 FTIR spectra. (b) water in $scCO_2$ microemulsions containing phospholipid species. The carbonyl vibration of the phospholipid is clearly visible; (a) in the absence of surfactant the phospholipid is insoluble in $scCO_2$.

spectroscopy. Another class of important biological molecules is the phospholipids (Fig. 5). These make up the major proportion of cell membranes in the body, and are believed to be important in the attraction of calcium and subsequent production of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], the mineral component of mature bone. We have shown (Fig. 6) that phospholipids can be solubilized by use of water in $scCO_2$ microemulsions. FTIR spectroscopy confirms the presence of a significant concentration of the phospholipid within the aqueous domain. Now, we are investigating the possibility of using such a supercritical solution to deliver phospholipids to the active sites of porous biomedical implants to encourage calcium uptake and hydroxyapatite growth after implantation. $ScCO_2$ is the ideal medium for such impregnation since the gas-like diffusivity ensures rapid and uniform impregnation. In addition, the use of $scCO_2$ allows solvent-free processing which is highly desirable in the preparation of biomedical materials. Any surfactant residues will be removed following further treatment with $scCO_2$. Preliminary results show promise, phospholipids have been deposited on metal substrates, and further results will be reported in the future.

We have demonstrated that water in $scCO_2$ microemulsions may be used to dissolve a broad range of ionic and polar molecules. The next part of our paper deals with the synthesis and preparation of metal species that are soluble in $scCO_2$ and their utilization in the modification of polymeric medical implant components.

THE SOLVATION OF METAL COMPLEXES

Vapor pressure is the most important indicator of solubility in a supercritical fluid; the more volatile a substance the more soluble it is likely to be. Compounds with high lattice energies, such as metal salts, are largely insoluble in $scCO_2$ due to their lack of volatility. Recently, there has been an increasing interest in the use of $scCO_2$ for the clean-up of both solid and liquid matrices contaminated with heavy metal salts. Normally, such processes are carried out with conventional organic solvents containing a complexing agent to extract the heavy metal species. The key impetus for using $scCO_2$ is the very efficient penetration of matrices by the gas-like $scCO_2$, and the elimination of conventional solvents and solvent residues from the process. However, such extractions require that the $scCO_2$ be modified with a complexing agent that allows *in situ* chelation/extraction of the heavy metal contaminant. This technique was first demonstrated in the extraction of Cu^{2+} from an aqueous solution using a $scCO_2$ soluble complexing agent (lithium bis (trifluoroethyl) dithiocarbamate) [11]. In general, the solubilization of a metal ion requires that the metal charge be shielded from CO_2 so that the fluid 'sees' a hydrocarbon or fluorocarbon shell. The most commonly used ligands for the solubilization of metals in $scCO_2$ are β-diketonates, dithiocarbamates, organophosphates, and crown ethers [1].

At Nottingham, we have developed an interest in solubilising metal complexes in $scCO_2$ in order to facilitate their impregnation into polymeric materials. Others have explored this process as a method for dyeing polymeric fibers [12,13]. Our approach has been to develop FTIR methods for *in situ* monitoring of polymer impregnation from a supercritical fluid solution, using organometallic species as molecular probes [14]. These studies provide an understanding of the conditions required for impregnation. The degree of polymer impregnation depends upon how the metal complex partitions between the $scCO_2$ phase and the polymer under a given set of conditions. We have, therefore, developed a range of organometallic species with different solubilities in $scCO_2$. Our goal is to provide a method for modifying polymeric substrate properties and forming composites. Thus, some of these complexes are designed to decompose under controlled conditions of heat, light, and reduction with hydrogen to yield nanometer-sized metallic particles.

To do this requires precursors that are soluble in $scCO_2$, but will also decompose cleanly to the desired metal or metal oxide, and free ligand residues. Such properties are inherent in the majority of precursors used in chemical vapor deposition such as metal β-diketonates, a class of volatile complexes in which the metal is surrounded by a fluorocarbon or hydrocarbon shell. We have utilized a series of organometallic silver complexes of the form Ag(hfpd)L (Fig. 7) (hfpd = 1,1,1,5,5,5 hexafluoro-2,4-pentanedionate and L are multidentate amines, multidentate glymes, phosphines, or thioethers). The silver precursor is dissolved in $scCO_2$ and allowed to diffuse into the polymer substrate. Upon depressurization of the system, the CO_2 rapidly escapes as a gas, and the infused precursor is trapped in the polymer. Decomposition leads to nanometer-sized metal particles. In addition, free ligand residues are produced that may be extracted efficiently from the polymer using a $scCO_2$ flow system to yield the desired polymer/silver composite.

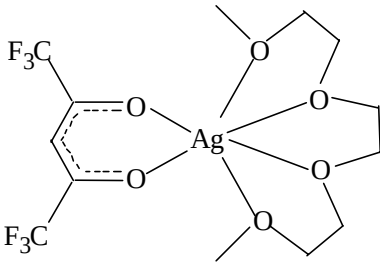

Fig. 7 CO_2-philic groups screen metal charge. Fluorination confers greater CO_2 solubility.

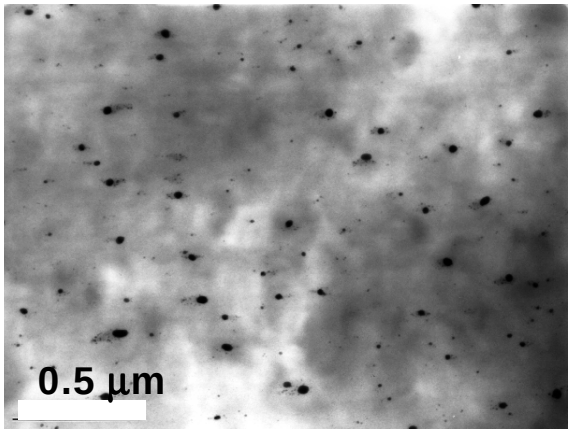

Fig. 8 Transmission electron micrograph. $ScCO_2$ impregnation of an UHMWPE film produced this composite of silver nanoparticles dispersed throughout the polymer matrix.

Transmission electron microscopy (TEM) of a very thin sliced (microtomed) section of a typical sample reveals the silver particles (Fig. 8). The distribution of metal is homogeneous throughout the sample, and the particle size is uniform with loading typically from 1–3% by weight. The use of conventional solvents for this process would result in contamination of the substrate with solvent residues that can be difficult to remove. The use of $scCO_2$ is particularly advantageous since no solvent residues remain after processing. Others have developed similar techniques with a view to preparing composites for catalysis or electronics applications [15]. We have investigated one application in particular, the modification of ultra high-molecular-weight polyethylene (UHMWPE), which is the material most frequently used in orthopedic implants.

$ScCO_2$ impregnation of medical implants

Total hip replacement is a common orthopedic operation with over 500 000 total hip replacements performed *per annum* worldwide, in addition to tens of thousands of shoulder/elbow replacements and tibial knee inserts. The total hip implant is generally made up of two separate parts, the femoral stem and the acetabular socket, and has changed little since its first use in the 1950s (see Fig. 9). It is common practice to use a stem made of titanium alloy and a cup made of UHMWPE.

UHMWPE has proven to be the best *in vivo* bearing surface material. However, the failure of these implants is still common and is caused by gradual wearing of the polymer surface. As the "ball" moves around in the "socket" the polymer degrades at the load-bearing surface through the process of

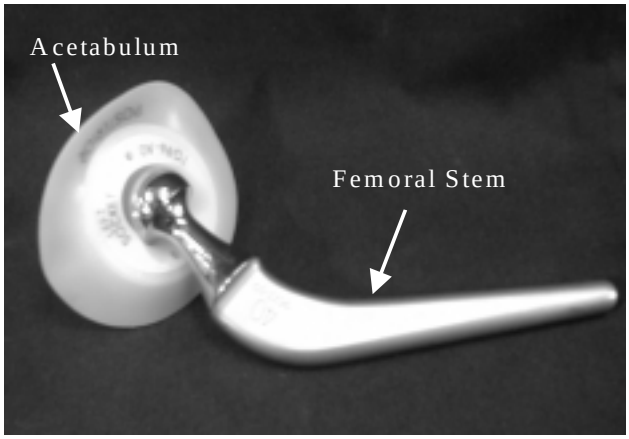

Fig. 9 A modern total hip implant. The femoral stem must bond well to the femur, and the acetabulum (socket) must provide enough movement of the femoral head (ball) to allow leg rotation while avoiding degradation of the polymeric component.

adhesive wear (predominantly). This leads to production of submicron-sized particles of UHMWPE which the body simply cannot remove. Whereas the body tolerates bulk UHMWPE, particulate debris causes severe problems. Macrophage cells, the biological equivalents of vacuum cleaners, unsuccessfully try to digest and remove the particles, and this leads to a severe toxic response and ultimately to rejection of the implant.

Our aim has been to develop supercritical fluid methods to improve the adhesive wear properties of the UHMWPE surface. It is well known that the use of certain metals, metal oxides, and metal sulfides as fillers in polymeric substrates can significantly improve the wear lifetime. We have applied our silver precursors to the preparation of silver/UHMWPE composites by impregnation from a $scCO_2$ solution. Our results (Fig. 8) show that such composites can indeed be prepared. Preliminary mechanical testing on the composites indicates that there is an improvement in the adhesive wear properties. In addition, the presence of silver particles should also lead to visibility of the acetabular component in standard X-ray imaging. UHMWPE can be difficult to see in X-ray images. Indeed, the particulate debris (if formed) may also be visible.

Biological responses

We now have a route to the preparation of such composites but, will they be tolerated *in vivo*? There are several simple tests that we can perform to determine the cell response to a material. Our experiments have focused on the interaction of a mouse macrophage cell line with our polymer composite surfaces.

The role of the macrophage is to respond to toxins and to consume any encountered foreign body material. In brief, the cells are suspended in a liquid culture medium and are seeded onto the polymer surface. After a given time period, the cell culture is fixed and the cellular interaction with the surface is determined using scanning electron microscopy (SEM). We have carried out such cell culture analyses for a wide range of supercritically impregnated UHMWPE samples. Our initial results showed that the macrophages exhibit a toxic response to the modified polymer surfaces. The cells are seen to spread across the polymer surface and have damaged membranes, illustrating an unfavorable response (Fig. 10). However, our investigations revealed that the cells were responding primarily to residues of free ligand released during the decomposition process. We have modified our decomposition process to introduce a brief, but effective, supercritical fluid extraction step to completely remove all of the free ligand residues and traces of organometallic which had not decomposed. On repeating the cell culture studies, we found that the macrophages now responded very well to the composite surface and exhibited no change in morphology from the original cell suspension (Fig. 11).

During an immune response macrophages become activated releasing superoxide that then reacts further to produce hydrogen peroxide. We have measured this release, in the presence of a peroxide sensitive fluorescent dye, to gain a more quantitative approach to our biological response measurements. In brief, the greater the activation of the macrophages to the toxic ligand residues the greater the

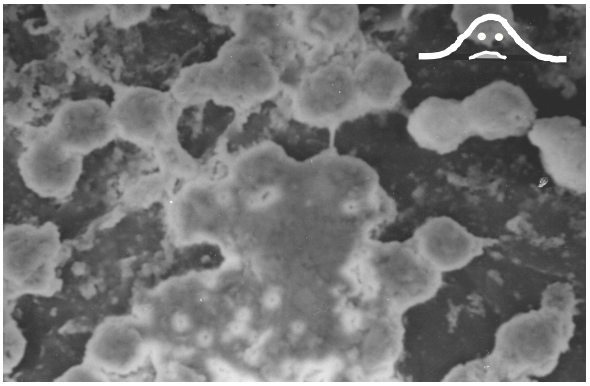

Fig. 10 Macrophages seeded onto UHMWPE/Ag composite containing traces of organometallic and ligand residues. The cell membranes are damaged, demonstrating an unfavorable response.

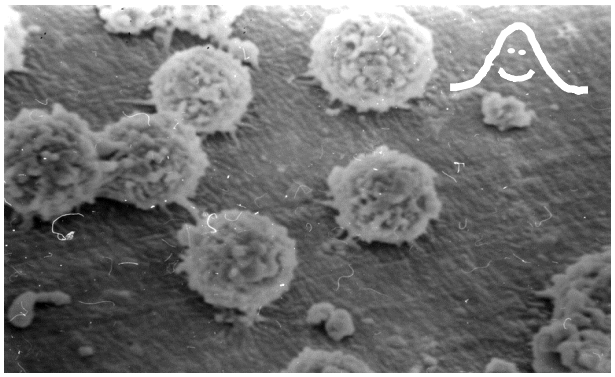

Fig. 11 After a $scCO_2$ extraction step, all soluble residues are substantially removed. The cells have rounded intact membranes, a favorable response.

fluorescent signal. Our results demonstrate that the supercritically extracted composites (Fig. 11) show a very low level of toxic response; identical to that of pure UHMWPE, an accepted biomaterial [16]. The major advantage of $scCO_2$ is that it enables residue free modification of UHMWPE, a factor of major importance if the biological response to this biomaterial is to remain unaffected.

CONCLUSIONS

$ScCO_2$ is a versatile and unique solvent. We have demonstrated that design of surfactants and complexes specific to $scCO_2$ allows solubilization of biologically important molecules and metallic precursor species. In the latter case, we have used the unique combination of gas- and liquid-like properties of $scCO_2$ to modify polymeric biomaterials without affecting macrophage response.

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