Encapsulating probiotics with an interpolymer complex in supercritical carbon dioxide

F.S. Moolman[a*], P.W. Labuschagne[a], M.S. Thantsa[b], T.L. van der Merwe[a], H. Rolfes[c] and T.E. Cloete[b]

Traditional encapsulation methods in fortified foods and drug delivery applications present difficulties for 'actives', such as probiotics, sensitive to exposure to water, solvents, heat or oxygen, where 'active' refers to a material, chemical or organism that has some potential benefit when consumed. In this paper we present a novel encapsulation technology, based on interpolymer complex formation in supercritical carbon dioxide, which avoids such exposure during the encapsulation process. The method was used to encapsulate indomethacin and *Bifidobacterium longum* in a poly(vinyl pyrrolidone)–poly(vinyl acetate–co-crotonic acid) interpolymer complex. Polymer complexation was confirmed by Fourier Transform infrared and moisture absorption studies. Polymer plasticization and release of encapsulated probiotics were studied with scanning electron microscopy. It was shown that the encapsulation matrix is stable at low pH, but disintegrates at higher pH, triggering release of the encapsulated material. The technology could find application in encapsulation of sensitive actives in the food and pharmaceutical industry.

Introduction

Encapsulation of pharmaceutical actives in polymers allows for improvement of shelf-life and stability. However, the encapsulation process usually includes the use of a solvent, which could damage the active agent. Supercritical fluids are attractive alternative solvents. 'Supercritical fluid' (SCF) describes the state of a material above its critical point, which is the highest temperature and pressure at which its vapour/liquid equilibrium can exist. Above these conditions, the liquid–gas phase transition disappears and properties such as diffusion coefficient and density change continuously with changes in pressure or temperature.

Supercritical carbon dioxide ($scCO_2$) is an environmentally benign solvent with low reactivity and low critical parameters (T_c = 31.1°C and P_c = 73.8 bar[1]). This makes it attractive for pharmaceutical applications. Typical uses of $scCO_2$ in biotechnology include micronization of drugs, encapsulation of sensitive actives for controlled drug release and impregnation of biomaterial scaffolds with pharmaceutical actives.[2–5]

Although very few polymers are soluble in $scCO_2$, it is well known that high-pressure carbon dioxide can dissolve in polymers and depress the glass transition temperature (T_g)[‡] and/or melting point, leading to reduced viscosity and improved processability (plasticization). However, this plasticization still does not allow low temperature processing of most polymers,[6,7] which makes it unsuitable for most pharmaceutical applications. Table 1 lists a number of approaches to overcome this problem as well as their limitations.

It is well known that physical networks can be formed by interpolymer complexation.[18] Interpolymer complexes form through any of four types of attractive interactions: electrostatic attraction, hydrogen bonding, hydrophobic interaction, and Van der Waals forces. They are non-covalently connected, cross-linked networks with complex blend properties deviating from simple linear mixing rules.

We present a novel encapsulation method[20] in which two hydrophilic, low molecular mass polymers (individually soluble in or plasticizable by $scCO_2$) interact to form an interpolymer complex. This is less soluble than the individual polymers in both $scCO_2$ and water and has improved barrier properties compared to the two individual starting polymers, useful in controlled release applications.[21]

The selection of the interpolymer complex system is a critical aspect of the technology. In pharmaceutics and, especially, for oral delivery, the polymer system (and the resulting product) must meet at least the following criteria:

- Both polymers and all other components must be approved for pharmaceutical applications by the US Food and Drug Administration (FDA).
- Both polymers must be processable in $scCO_2$.
- Both polymers must be soluble or at least swellable in water.
- The polymer complex should preferably be less soluble in an acidic environment as protection against the harsh gastric environment, and more soluble in the more alkaline environment found in the small bowel.
- The polymer complex should provide sufficient environmental protection against oxygen and moisture in dry formulations to yield acceptable shelf-life for the final product.
- The polymer complex should have minimal burst release; that is, the release should not be too rapid, as this could lead to toxic systemic concentrations of the encapsulated active.
- The components must be compatible with production in a controlled, aseptic environment compliant with current good manufacturing practice.

One of the potential uses of this encapsulation technology is for probiotics. These are defined as 'mono- or mixed cultures of live micro-organisms which, when applied to animal or humans, beneficially affect the host by improving the properties of the indigenous microflora'[22] or, alternatively, 'a live microbial food ingredient that is beneficial to health'.[23] Probiotic supplementation, either separately or with food, has several potential health benefits, including prevention of diarrhoea, metabolism of lactose and relief of lactose maldigestion, reduction of plasma cholesterol concentration, enhancement or modulation of immune system response, enhanced calcium absorption and improved synthesis of specific vitamins.[24,25]

The most commonly used probiotic bacteria are those belonging to the genera *Lactobacillus* and *Bifidobacterium*.[26] *Bifidobacterium*

[‡]The glass transition temperature (T_g) is the temperature at which polymers undergo a transition from being rigid and brittle below T_g, to being more rubbery and capable of plastic deformation above T.

[a]Polymers, Ceramics and Composites, MSM, CSIR, P.O. Box 395, Pretoria 0001, South Africa.
[b]Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa.
[c]Department of Chemical Engineering, University of Pretoria.
*Author for correspondence. E-mail: smoolman@csir.co.za

Table 1. Approaches used to overcome the low affinity between $scCO_2$ and most polymers.

Approach	Elaboration	Limitations
Polymer design[8]	Incorporation of 'CO_2-philic' functional groups in new polymers	Requires FDA approval for new polymers
Surfactants[9–11]	Addition of CO_2-soluble surfactants	Requires FDA approval for surfactants
Cosolvents[12–14]	Addition of a cosolvent such as methanol or ethanol to increase the solvation power of $scCO_2$	Reintroduces requirement for use of a solvent. Many actives are sensitive to solvents
Mixtures of SCFs	Use of a second supercritical fluid to enhance polymer processability low critical temperature & pressure, low cost, etc.)	No obvious second supercritical fluid available with desired combination of properties (low/no toxicity,
Gas anti-solvent (GAS) technique[15]	Use $scCO_2$ as an anti-solvent to extract the solvent from a sprayed polymer solution and thus precipitate the polymer	Reintroduces requirement for use of a solvent.
Use low molar mass and low polarity polymers[16]	These polymers are more processable in $scCO_2$.	These polymers generally have low mechanical integrity and/or barrier properties
Use fats/waxes for encapsulation[17]	Fats, waxes and oils are generally soluble in $scCO_2$	Limited flexibility with regard to properties

species predominate in breast-fed infants, but not in formula-fed infants.[27] While *L. acidophilus* is microaerophilic, *Bifidobacteria* are strict anaerobes and thus exhibit limited survival in environments where they are exposed to oxygen. Additionally, most bacteria are sensitive to temperature and solvents. Water or moisture should be avoided during encapsulation and storage to prevent the bacteria from 'awakening' from their dormant state, as they then consume all the available food and subsequently decline rapidly in numbers.

The basic polymer system used in this study consisted of poly(vinyl pyrrolidone) (PVP) and poly(vinyl acetate–co-crotonic acid) (PVAc–CA) (Fig. 1). These two polymers are both plasticizable in $scCO_2$, most probably through interactions of their carbonyl groups with CO_2. Carbon dioxide can act as a weak Lewis acid and interact with Lewis bases, such as carbonyl groups, and can also form weak hydrogen bonds and complex with amines.[28]

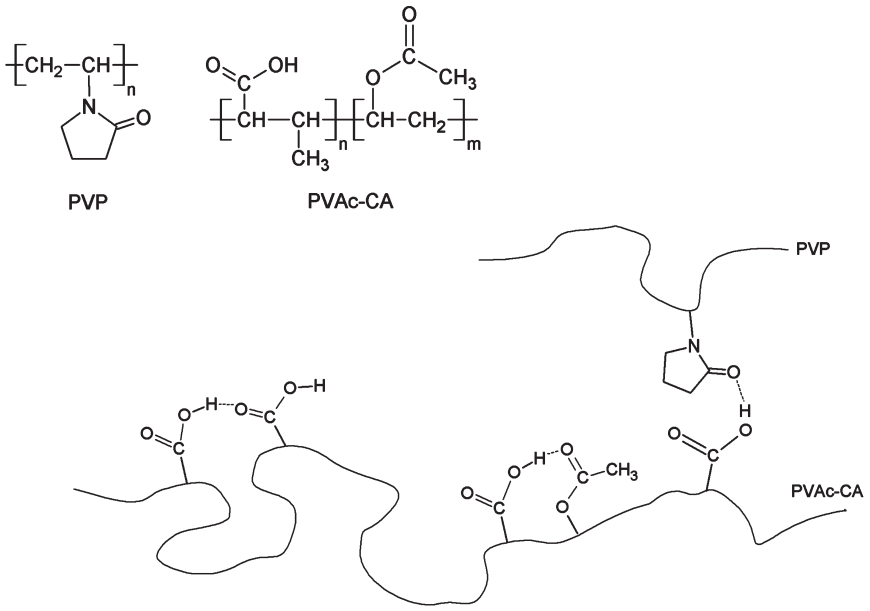

Fig. 1. Poly(vinyl pyrrolidone) (PVP) and poly(vinyl acetate–co-crotonic acid) (PVAc–CA) repeat units and a schematic representation of some possible hydrogen-bonding interactions.

PVP and PVAc–CA form an interpolymer complex through hydrogen bonding between the carboxylic acid groups of the PVAc–CA and the carbonyl groups of the PVP. Figure 1 shows a schematic representation of this interaction, as well as some intrapolymeric hydrogen bonding in PVAc–CA. While PVP is soluble in water, PVAc–CA and the complex of the two polymers are only swellable in water. The swellability of PVAc–CA is pH-dependent and swelling increases with increase in pH, due to the carboxylic acid group ionization at higher pH. This imparts protection to the encapsulated material in lower pH environments and results in release of the encapsulated material in a higher pH setting. Additives such as polyethylene glycol, a viscosity modifier, and glyceryl monostearate, which creates a moisture and oxygen barrier, can optionally be added to the system.

Materials and methods

CSIR process

A simplified flow diagram of the PGSS (Particles from Gas-saturated Solution) system used for particle production is shown in Fig. 2. Carbon dioxide from the CO_2 cylinder passes into a condenser and the liquid is then pressurized using a gear pump and back-pressure regulator in a circulating setup. The pressurized CO_2 is heated to above 31.1°C and transferred into the reactor, which is preloaded with a blend of the two polymers and the active. The polymer system is plasticized by the $scCO_2$, and it is subsequently stirred to form an interpolymer complex with suspended active. The slurry is then released into a product chamber through a capillary nozzle, with pressure in the reactor being maintained through automatic switching of the gear pump.

As the plasticized interpolymer complex leaves the capillary, it is atomized to form droplets that rapidly solidify into particles due to the escaping carbon dioxide and the consequent polymer formation. The pressure in the product chamber can be adjusted to assist in controlling particle morphology. The free-flowing dried powder product thus obtained consists of interpolymer complex matrix particles with active encapsulated therein.

The encapsulation process

All equipment was wiped with 70% ethanol in water (NCP alcohols) using a paper towel, and allowed to dry before contact with the materials. Two grams of PVP (Kollidon 12PF, mass-average molar mass 2000–3000 g/mol, BASF) were dried for 5 h at 80°C and 60 mbar (absolute) in a vacuum oven (Model VO65, Vismara) and immediately placed in a desiccator to prevent moisture absorption.

A sealed packet of *B. longum* (Bb-46, Chr. Hansen) or *B. lactis* (Bb-12,

Chr. Hansen) was removed from storage at –12°C and allowed to warm to room temperature while sealed. Two grams of the bacteria were ground to a powder and passed through a 150-μm sieve using a coffee grinder (Model CG100, Kenwood). Six grams of PVAc–CA (Vinnapas C305, mass-average molar mass 45 000 g/mol, Wacker) were added to the bacteria and the dried PVP, without or with additives, such as glyceryl monostearate (Croda Chemicals). The blend was then ground and mixed for 1 min and immediately transferred to the pre-heated 1-litre reaction chamber. The chamber was sealed, flushed and pressurized with sterile filtered CO_2 (99.995% purity, Air Products) up to a pressure of 300 bar, with the temperature controlled at 40°C. The material was left to equilibrate for 2 h with intermittent stirring, after which the plasticized product was sprayed through a 500-μm capillary, with length 50 mm, into a 10-litre expansion chamber pressure-controlled at 15 bar (gauge). The following experimental procedures were performed on the product:

Particle size distribution analysis

Particle size distribution analysis was carried out using a Malvern Mastersizer 2000, multiple-angle, laser light-scattering analyser (Malvern Instruments, U.K.). Particles were dispersed in water and sonicated before measurement at 25°C.

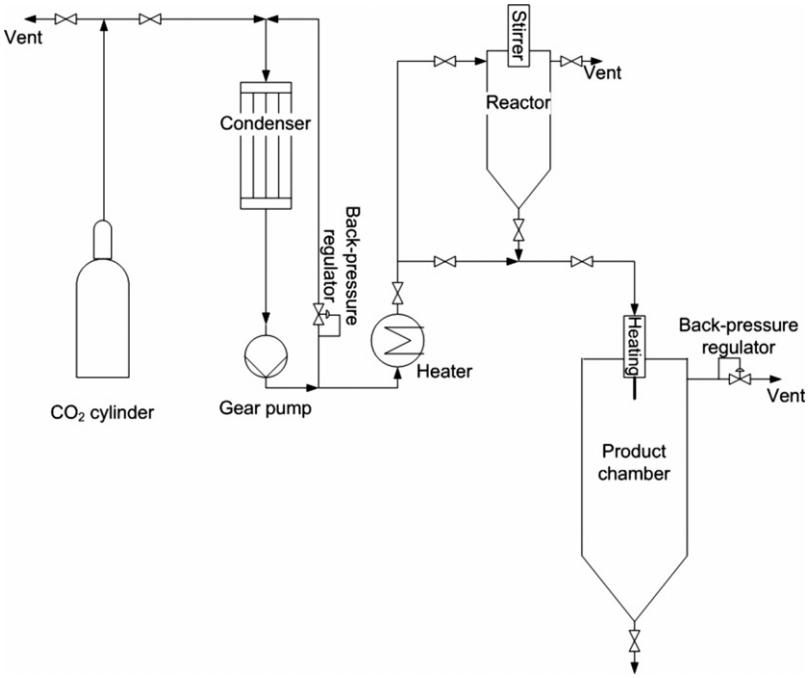

Fig. 2. Flow diagram of PGSS system used to produce encapsulated probiotics.

Fourier Transform infrared (FTIR)

The polymer systems were ground together with potassium bromide (FTIR grade, Aldrich) and the mixture compressed to form discs. Eight scans of each sample were taken in the range 400–3500 cm^{-1} using a Perkin-Elmer 1710 FTIR spectrophotometer.

Moisture absorption

Moisture absorption was determined through storing open samples in a Labcon Humidity Chamber Model FSIE-RH 40. Samples were tested for moisture absorption through measuring mass increase over a period of 72-h exposure to a relative humidity of 60% at a temperature of 30°C.

Controlled release studies

Indomethacin (TEVA Pharmaceutical Industries) was encapsulated using the process described above for the bacteria. Subsequently, twelve 6-mm tablets were pressed from the product powder using a Manesty F3 tablet press (Manesty Machinery). The dissolution study was then carried out using a Hanson SR2-8 dissolution bath with 1-litre containers. Eight tablets were placed in buffer solution at pH 6.85 and four tablets in buffer solution at pH 1.2. The dissolution bath paddles rotated at 75 rpm and the bath temperature was maintained at 37°C. Indomethacin concentration was determined using a Helios Alpha UV Analyzer by measuring absorbance at 320 nm after calibration with standard solutions.

Bacterial enumeration

One gram of *B. longum* (either control or encapsulated) was dissolved in 9 ml Ringer's solution (pH 7). A series of dilutions up to 10^{-10} were prepared from this suspension. A small volume (0.1 ml) of the diluted bacterial solutions was pour plated onto MRS (De Man, Rogosa and Sharpe) agar (from Merck) supplemented with 0.05% cysteine hydrochloride. Each dilution was plated out in triplicate. The plates were incubated anaerobically in anaerobic jars, with Anaerocult A gaspaks (Merck), at 37°C for 72 h. To confirm anaerobic conditions inside the jars, Anaerocult C test strips were included. The numbers of colonies grown were counted and from these the numbers of viable cells were calculated (cfu/g).

Scanning electron microscopy (SEM)

SEM was used to verify encapsulation of *B. longum* cells by the polymer and release of the cells from the polymer during subsequent solubilisation of the encapsulated powder. Two different samples were examined: freeze-dried (controls) and encapsulated *B. longum.* The samples were suspended in Ringer's solution. The suspensions thus obtained were then filtered through a 0.2-μm Millipore filter membrane. The material remaining on the filter membrane were fixed by exposing it to 2.5% gluteraldehyde for 30 min and then washed for 3 × 15 min in 0.15 M × phosphate buffer. The fixed samples were then dehydrated using increasing concentrations of ethanol as follows: 50% (1 × 15 min), 70% (1 × 15 min), 90% (1 × 15 min) and 100% (3 × 15 min). The samples were then dried in a critical point dryer for 24 h, mounted on stainless steel studs and subsequently coated with gold plasma. The samples were then examined using a JEOL 840 scanning electron microscope.

Testing in simulated gastric juice (SGJ) and simulated intestinal fluid (SIF)

A comprehensive discussion of the test method and results for SGJ and SIF will be presented elsewhere. Briefly, SGJ (pH 2) was prepared according to Lian *et al.*[29] and SIF (pH 6.8) was prepared according to the *US Pharmacopeia*.[30] Free and encapsulated bacteria were then exposed to the SGJ for two hours, and subsequently to SIF for 8–24 h.

Determination of the glass transition temperature (T_g) using differential scanning calorimetry (DSC)

A Perkin-Elmer DSC-7 was used for T_g determination, with a sample size of 14.2 mg in purging nitrogen through the temperature range 25–100°C at a rate of 20°C/min.

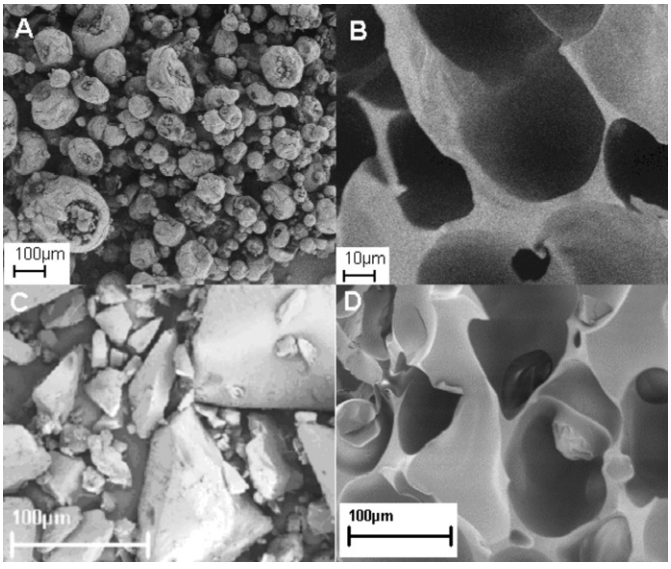

Fig. 3. Comparison of PVP before (**A**) and after (**B**), and PVAc–CA before (**C**) and after (**D**) exposure to scCO2.

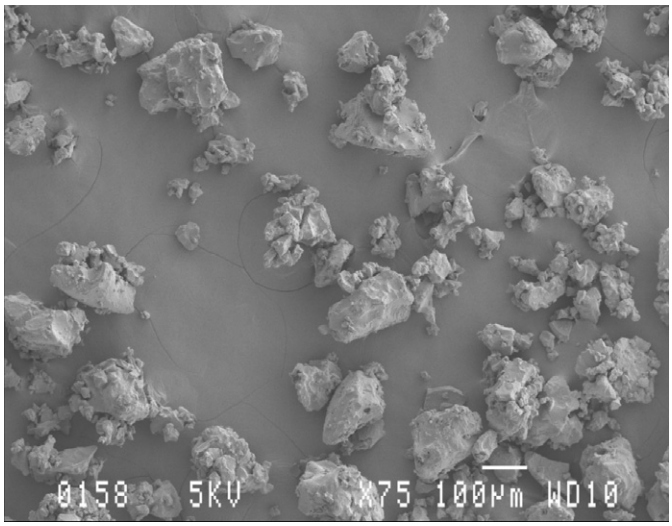

Fig. 4. PVP:PVAc–CA powder containing encapsulated *B. lactis*, produced according to the $scCO_2$ encapsulation process.

Results

Plasticization and particle formation

Figure 3 shows SEM micrographs of PVP and PVAc–CA before and after exposure to $scCO_2$ at 40°C and 300 bar. It is clear that plasticization occurred for both polymers, since the polymer particles have changed into a monolithic foam structure, which could have formed only through an intermediate plasticized state. These results suggest T_g and/or melting point depression, since T_g for the specific PVAc–CA grade (Vinnapas C305) is 48°C, with softening point 93°C,[31] whereas T_g for the specific PVP grade (Kollidon 12PF) was determined as 80°C. These polymers were, thus, plasticized below their T_g values in $scCO_2$. Figure 4 shows the interpolymer complex powder obtained from the process, with particle size distribution for two different systems indicated in Fig. 5. Through the addition of a suitable viscosity modifier (in this case glyceryl monostearate), particle size can be reduced by more than an order of magnitude.

Complex formation — FTIR, moisture absorption and DSC

The FTIR analyses indicated a shift in wavenumber for the carbonyl (C=O) stretching band for PVP (from 1664 to 1682 cm^{-1}) caused by interpolymer complex formation when compared

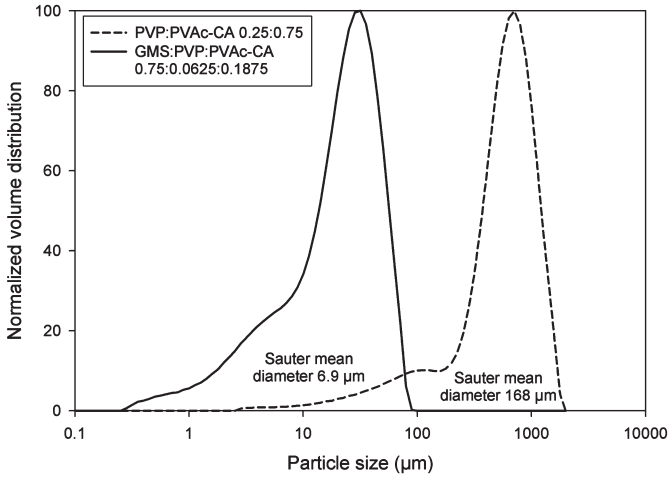

Fig. 5. Particle size distributions for the PVP:PVAc–CA system and for the same system containing 75% GMS (glyceryl monostearate).

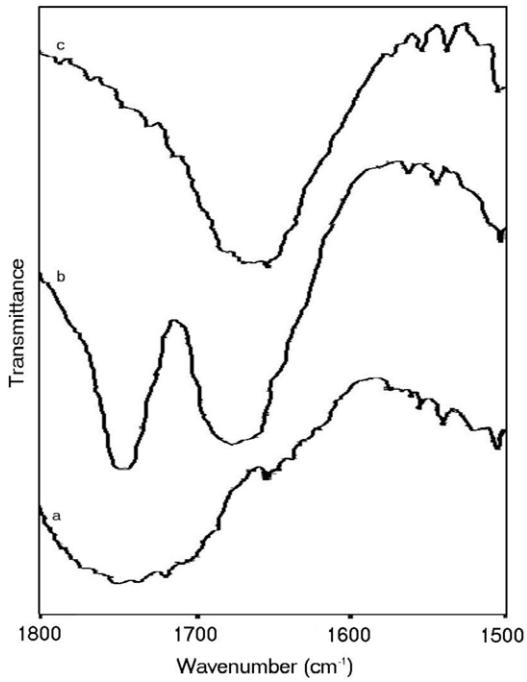

Fig. 6. Compound FTIR spectra of pure PVAc–CA (**a**), $scCO_2$-processed blend (**b**) and pure PVP (**c**).

with the pure polymer (Fig. 6). For the $scCO_2$-processed blend of PVP:PVAc–CA, there was also a substantial narrowing of the broad PVAc–CA absorption band consisting of an acetate absorption band overlapping with two carbonyl stretching modes of the free and self-associated carboxylic acid groups.

Table 2 compares moisture absorption of three different dry-blended and $scCO_2$-processed polymer blends. There is an apparent reduction in moisture absorption for the $scCO_2$-processed blends compared to the physical blends, supporting the formation of an interpolymer complex. The reduction in moisture absorption is also illustrated by Fig. 7, which shows a physical blend of PVP:PVAc–CA compared to the $scCO_2$-processed blend after 24 h exposure at 30°C to 60% RH. It is evident from this photograph that the physical blend absorbs more moisture than the interpolymer complex. The dramatic difference in appearance is probably due to a) increased plasticization of the physical blend due to the higher moisture content; as well as b) reduced mobility of the polymers in the $scCO_2$-processed blend as a result of the interpolymer complex interactions.

Release studies

Figure 8 shows the release of indomethacin from tablets pressed from powder produced as described earlier. In the acidic environment (pH 1.2), almost no release of indomethacin (<5%) occurred over a period of 24 hours, whereas in the more alkaline setting (pH 6.8), about 85% of the encapsulated indomethacin was released in 24 hours. It is also evident from the release curve

Table 2. Comparison of moisture absorption of dry-blended and $scCO_2$-processed polymer systems.

System	Moisture absorption dry blend (%)	Moisture absorption $scCO_2$ processed (%)
PVP:PVAc–CA	8.2	5.7
PVP–VAc[†]:PVAc–CA	4.2	2.1
PEO–PPO–PEO[‡]:PVAc–CA	0.5	0.4

[†]PVP–VAc is poly(vinyl pyrrolidone–co-vinyl acetate) (Kollidon VA64, BASF).
[‡]PEO–PPO–PEO is ethylene oxide–propylene oxide–ethylene oxide triblock copolymer (Pluronic 68NF, BASF).

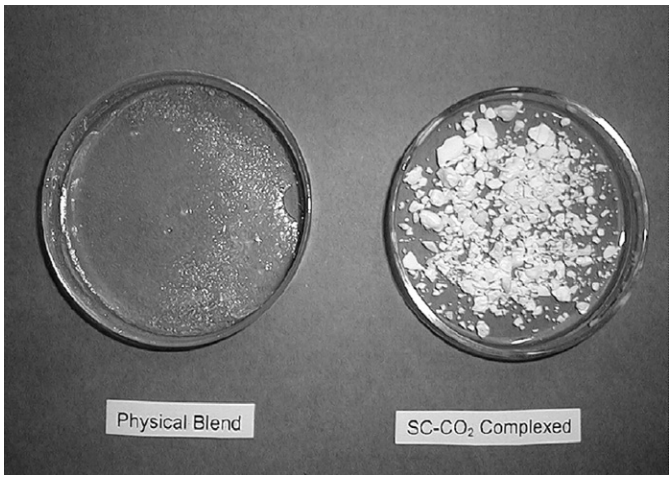

Figure 7. Moisture absorption of physical PVP:PVAc–CA blend compared to interpolymer complex after 24 hours' exposure to 60% RH at 30°C.

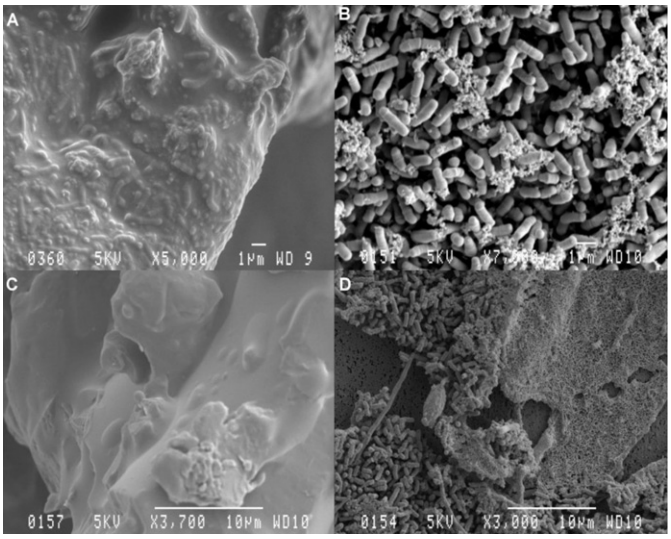

Fig. 9. SEM images of *B. longum*: **A**, freeze-dried powder (control); **B**, control suspended in water; **C**, encapsulated powder; **D**, encapsulated powder suspended in water.

in acidic environment that there was very limited burst release, indicating good encapsulation.

Free (non-encapsulated) *B. longum* was exposed to $scCO_2$ at 40°C and 300 bar for two hours, and the number of live bacteria was 2×10^{11} cfu/g for both the exposed and non-exposed bacteria. After 6 weeks of storage at room temperature, the counts dropped to 5×10^8 cfu/g for both exposed and non-exposed bacteria. Thus, exposure to the supercritical conditions described in this paper does not appear to cause immediate or latent damage to the bacteria.

From four SGJ and SIF trials, the average improvement in survival for encapsulated versus free *B. longum* was (1.81 ± 0.56) log(cfu/g) with $P < 0.05$. Figure 9 shows SEM micrographs of free (A) and encapsulated *B. longum* (C) in the dry form. Micrograph B shows the free bacteria after suspension and micrograph D illustrates release of encapsulated *B. longum* from the PVP:PVAc–CA polymer system after suspension in alkaline medium (pH 6.8).

Discussion and conclusions

The formation of hydrogen bonds causes an increase in wavenumber for acceptor carbonyl IR absorption peaks[32] (where acceptor refers to the role of the carbonyl in the hydrogen-bonding interactions). The shift in the PVP carbonyl stretching band towards higher wavenumber could be due to hydrogen bonding with the carboxylic acid groups of the PVAc–CA associated with interpolymer complex formation. This surmise was supported by the narrowing of the broad absorption band of PVAc–CA consisting of an acetate band overlapping with two carbonyl stretching modes of the free and self-associated carboxylic acid groups. The observed narrowing indicates stronger interaction/coupling of these groups, probably through hydrogen bonding with the carbonyl groups of the PVP. These changes in FTIR spectra point towards the formation of an interpolymer complex.

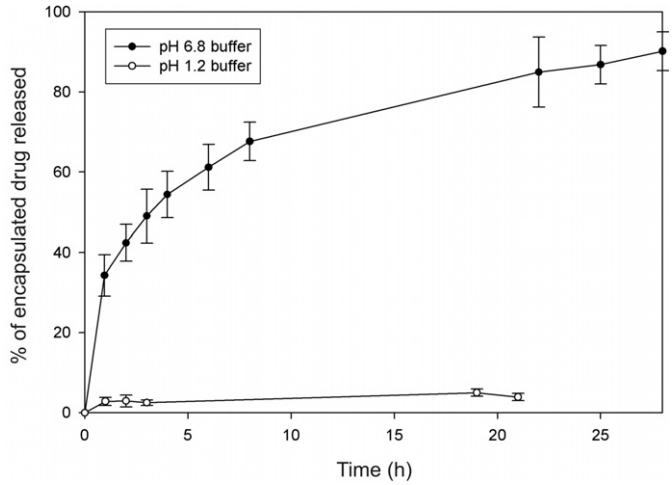

Fig. 8. Release of indomethacin from PVP:PVAc–CA interpolymer complex system at different pH values.

This conclusion was further supported by moisture absorption tests, in which there was a marked reduction in moisture absorption by the $scCO_2$-processed polymers compared with physical blends, probably because of reduced capacity for water uptake due to stronger interpolymer interactions. Thus, we have shown for the first time that interpolymer complexes can be formed in $scCO_2$ environments.

It was also shown that, through careful selection of polymers, some interpolymer complexes are plasticizable in $scCO_2$. These interpolymer complexes can be formed into dry powder products with different properties through control over the expansion conditions, namely, expansion chamber pressure, nozzle choice and the addition of viscosity modifiers. Thus a solvent is not required to facilitate H-bonding-based, interpolymer complex formation–increased chain mobility is sufficient.

The controlled release tests on encapsulated indomethacin illustrated that the polymer system exhibits limited swelling and release in acidic environments, while achieving effective release in more alkaline environments. In the latter case, 85% was released in 24 hours, and about 50% was released in the first three hours. This makes the encapsulation process suitable for oral drug delivery systems, as the normal residence time in the gastrointestinal tract is generally longer than 24 hours, with small intestine (the main site of drug absorption and the location of first appearance of bifidobacteria[27]) transit around 3 hours.[33] These experiments also showed that there is minimal burst release resulting from this encapsulation method.

The differential pH responsiveness should provide protection to sensitive actives encapsulated in the PVP:PVAc–CA system on transit through the aggressive gastric environment. This was confirmed by SGJ and SIF testing, in which encapsulated *B. longum* exhibited more than an order of magnitude improved survival compared to free bacteria controls. Additionally, the exposure tests of free bacteria to the supercritical conditions

showed no damage to the bacteria.

Polymer–drug complex formation can sometimes occur and consequently influence release behaviour. However, the release of indomethacin and *B. longum* from the interpolymer complex matrix did not seem to be significantly influenced by such interactions, if such occurred, since 85% of the indomethacin was released in 24 hours, and *B. longum* released from the interpolymer complex was higher than unencapsulated controls.

The use of $scCO_2$ as a 'benign' solvent for food and pharmaceutical processing has considerable potential. The widespread application of $scCO_2$ in these industries is, however, hampered by limited polymer solubility. The CSIR's interpolymer complex-based $scCO_2$ encapsulation technology (patent pending) is a novel solution to this problem, with significant potential for use in food and pharmaceutical applications, including the fortification of dry food products with encapsulated probiotics and other sensitive actives, and encapsulation of drugs and vaccines for oral delivery.

Further investigation is required to determine the shelf-life of the encapsulated probiotics and drugs for oral use and the *in vivo* success of their release into the intestinal tract.

We thank Ellipsoid Technology (Pty) Ltd for sponsoring the research.

1. Lide D.R. (Ed.)(2003). *CRC Handbook of Chemistry and Physics, 84th edition.* CRC Press, Boca Raton.
2. Jung J. and Perrut M. (2000). Particle design using supercritical fluids: literature and patent survey. *J. Supercrit. Fluids* **20**, 179–219.
3. Fages J., Lochard H., Letourneau J-J., Sauceau M. and Rodier E. (2004). Particle generation for pharmaceutical applications using supercritical fluid technology. *Powder Technol.* **141**, 219–226.
4. Ginty P.J., Whitaker M.J., Shakesheff K.M. and Howdle S.M. (2005). Drug delivery goes supercritical. *Materials Today* **8**, 42–48.
5. Yeo Sang Do and Kiran E. (2005). Formation of polymer particles with supercritical fluids: a review. *J. Supercrit. Fluids* **34**, 287–308.
6. O'Neill M.L., Cao Q., Fang M. and Johnston K.P. (1998). Solubility of homopolymers and copolymers in carbon dioxide. *Indust. Engineer. Chem. Res.* **37**, 3067–3079.
7. Kirby C.F. and McHugh M.A. (1999). Phase behavior of polymers in supercritical fluid solvents. *Chem. Rev.* **99**, 565–602.
8. Sarbu T., Styranec T. and Beckman E.J. (2000). Non-fluorous polymers with very high solubility in supercritical CO_2 down to low pressures. *Nature* **405**, 165–167.
9. Yazdi A.V., Lepilleur C., Singley E.J., Liu W., Adamsky F.A., Enick R.M. and Beckman E.J. (1996). Highly carbon dioxide soluble surfactants, dispersants and chelating agents. *Fluid Phase Equilibria* **117**, 297–303.
10. Hoefling T.A., Beitle R.R., Enick R.M. and Beckman E.J. (1993). Design and synthesis of highly CO_2 soluble surfactants and chelating agents. *Fluid Phase Equilibria* **82**, 203–212.
11. McClain J.B., Betts D.E., Canelas D.A., Samulski E.T., DeSimone J.M., Londono J.D., Cochran H.D., Wignall G.D., Chillura-Martino D. and Triolo R. (1996). Design of nonionic surfactants for supercritical carbon dioxide. *Science* **274**, 2049–2052.
12. Ekart J., Bennet K.L., Ekart S.M., Gurdial G.S., Liotta C.L. and Eckert C.A. (1993). Cosolvent interactions in supercritical fluid solutions. *AIChE J.* **39**, 235–248.
13. Kazarian S.G., Vincent M.F., West B.L. and Eckert C.A. (1998). Partitioning of solutes and cosolvents between supercritical CO_2 and polymer phases. *J. Supercrit. Fluids* **13**, 107–112.
14. Mishima K., Matsuyama K., Hayashi K., Ishikawa H. and Hirabaru T. (2001). Formation of polymer microcapsules by rapid expansion of supercritical solution with a nonsolvent. *IUPAC Chemrawn XIV Conf.*
15. Subramaniam B., Saim S., Rajewski R.A. and Stella V. (1999). *Methods for particle micronization and nanonization by recrystallization from organic solutions sprayed into a compressed antisolvent.* U.S. Patent no. 5,874,029.
16. Rindfleisch F., DiNoia T.P. and McHugh M.A. (1996). Solubility of polymers and copolymers in supercritical CO_2. *J. Phys. Chem.* **100**, 15581–15587.
17. Benoit J.P., Rolland H., Thies C. and Vande Velde V. (2000). *Method of coating particles and coated spherical particles.* U.S. Patent no. 6,087,003.
18. Tsuchida E. (1994). Formation of polyelectrolyte complexes and their structures. *Pure Appl. Chem.* **A31**, 1–15.
19. Tilly K.D., Foster N.R., Macnaughton S.J. and Tomasko D.L. (1994). Viscosity correlations for binary supercritical fluid mixtures. *Indust. Engineer. Chem. Res.* **33**, 681–688.
20. Moolman F.S., Rolfes H. and Van der Merwe T.L. (2005). *Method of encapsulating an active substance.* U.S. Patent Application no. 20050112205.
21. Rolfes H., Van der Merwe T.L. and Truter P-A. (2001). *Method of making controlled release particles of complexed polymers.* U.S. Patent no. 6,221,399.
22. Havenaar R., Ten Brink B. and Huis in 't Veld J.H.J. (1992). Selection of strains for probiotic use. In *Probiotics, the Scientific Basis,* ed. R. Fuller, chap. 9, pp. 209–224. Chapman & Hall, London.
23. Salminen S., Bouley C.H., Boutro-Ruault M.C., Cummings J.H., Franck A., Gibson G.R., Isolauri E., Moreau M.C., Roberfroid M.B. and Rowland I. (1998). Functional food science and gastrointestinal physiology and function. *Br. J. Nutr.* **80**, S147–S171.
24. Ziemer C.R. and Gibson G.R. (1998). An overview of probiotics, prebiotics and synbiotics in the functional food concept: Perspectives and future strategies. *Dairy J.* **8**, 473–479.
25. Tuohy K.M., Probert H.M., Smejkal C.W. and Gibson G.R. (2003). Using probiotics and prebiotics to improve gut health. *Drug Discovery Today* **8**, 692–700.
26. Bielecka M., Biedrzycka E. and Majkowska A. (2002). Selection of probiotics and prebiotics for synbiotics and confirmation of their *in vivo* effectiveness. *Food Res. Int.* **35**, 125–131.
27. Holzapfel W.H., Haberer P., Snel J., Schillinger U. and Huis in 't Veld J.H.J. (1998). Overview of gut flora and probiotics. *Int. J. Food Microbiol.* **41**, 85–101.
28. Raveendran P., Ikushima Y. and Wallen S.L. (2005). Polar attributes of supercritical carbon dioxide. *Accounts Chem. Res.* **38**, 478–485.
29. Lian W.C., Hsiao H.C. and Chou C.C. (2003). Viability of microencapsulated bifidobacteria in simulated gastric juice and bile solution. *Int. J. Food Microbiol.* **86**, 293–301.
30. United States Pharmacopeial Convention (2005). *United States Pharmacopeia,* 25th edn. Webcom, Toronto.
31. Vinnapas C305 (CAS no. 25609-89-6) Data Sheet Version 2.00/01-11-03/ RG. Wacker Polymer Systems GmbH.
32. Krusic M.K., Dzunuzovic E.D., Trifunovic S. and Filipovic J. (2004). Polyacrylamide and poly(itaconic acid) complexes. *Eur. Polym. J.* **40**, 793–798.
33. Ashford M. (2002). The gastrointestinal tract — physiology and drug absorption. In *Pharmaceutics: The Science of Dosage Form Design,* ed. M.E. Aulton, chap. 16, pp. 217–233. Elsevier Science, Spain.