

CHAPTER THREE

3 MATERIALS AND METHODS

This chapter lists the materials and equipment used, and then describes the experimental methods used in this thesis. Unless otherwise noted, reagents were used as received.

3.1 REAGENTS AND MATERIALS

Acrylic acid, 99%, AR grade, Aldrich.

Ammonium bicarbonate, 99%, LR grade, Sigma.

Ammonium peroxydisulfate, research grade, Sigma.

Ammonium ferrous persulfate, research grade, Sigma.

Benzene, GR grade, ACS, Merck.

Bovine serum albumin, 98%, M_w 66,700 Da, Sigma.

Calcium chloride $6H_2O$, 98%, AR grade, B.D.H. Laboratory Supplies.

Chloroform, AR grade, Prolab Scientific.

Cyclohexane, laboratory reagent, Unilab Chemicals.

Cytochrome C, M_w 12,400 Da, Sigma.

Dichloromethane, AR grade, Selby Biolab.

n,n-Dimethylacetamide, ReagentPlus, 99%, Sigma-Aldrich.

1-Dodecanol, 98+%, A.C.S. reagent, Aldrich.

Ethanol, AR grade, Scharlau Chemie.

Ethyl acetate, AR grade, Ajax Chemicals.

Gelatine, AR grade, Scientific Supplies.

Glycerine, laboratory reagent, Unilab Chemicals.

Hydrochloric acid, AR grade, Rhone-Poulenc Chemicals.
Isopropanol, AR grade, Ajax Chemicals.
Polycaprolatone, research grade, Aldrich.
Polyethylene glycol, average M_n ca. 4,600 Da, Aldrich.
Poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol), M_n 14,000, Aldrich.
Polyethylene oxidel, average M_w 4,000,000 Da, Aldrich.
Polystyrene, M_w 280,000, Aldrich.
Polysulfone, average M_n ca. 22,000 kDa, Aldrich.
Polyvinyl alcohol, laboratory reagent, B.D.H. Laboratory Supplies.
Polyvinylpyrrolidone, M_w 10,000 Da, Acros Organics.
2-Propanol, HiPerSolve grade, B.D.H. Laboratory Supplies.
Sodium chloride, LR grade, Dominion Salt (N.I.).
Sodium dodecyl sulphate, crystal research grade, Serva.
Sodium hydroxide, AR grade, Scientific Supplies.
Terephthaloyl dichloride, laboratory reagent, Merck.
Tetrahydrofuran, A.C.S. reagent, 99+%, Sigma-Aldrich.
Vitamin B₁₂, M_w 1347 Da, Sigma.
Xylene, AR grade, Ajax Chemicals.
Dialysis membranes, molecular weight cut-off 14,000.
Whatman 4 Filters, Whatman.
H₂O: Milli Q ultra-pure distilled-deionised water (18.2 M Ω).

3.2 EQUIPMENT

Fourier transform infrared spectrometer - Digilab Scimitar Series FT-IR.
Freeze drier - FreeZone 2.5L Freeze Dry System, Labconco, Kansas, USA
High shear homogeniser - L4RT, Silverson.
Laser diffraction particle size analyser - Malvern Mastersizer S, Malvern Instruments, Malvern, UK.
Plasma reactor - built and modified at Waikato University (Figure 3.1).
Selecta Centronic Centrifuge, Alphatech Systems.
Sigma 6-15 Laboratory Centrifuge, SIGMA Laborzentrifugen GmbH.
Surface area and pore analyzer - Nova 1000, Quantachrome, USA.
Ultrospec 2000 UV/visible Spectrophotometer, Amersham Pharmacia Biotech.
Ultrasonic bath - Soniclean 250HT, 650 W, Transtek Systems.
Waterbath shaker - Selecta Unitronic OR, Alphatech Systems.

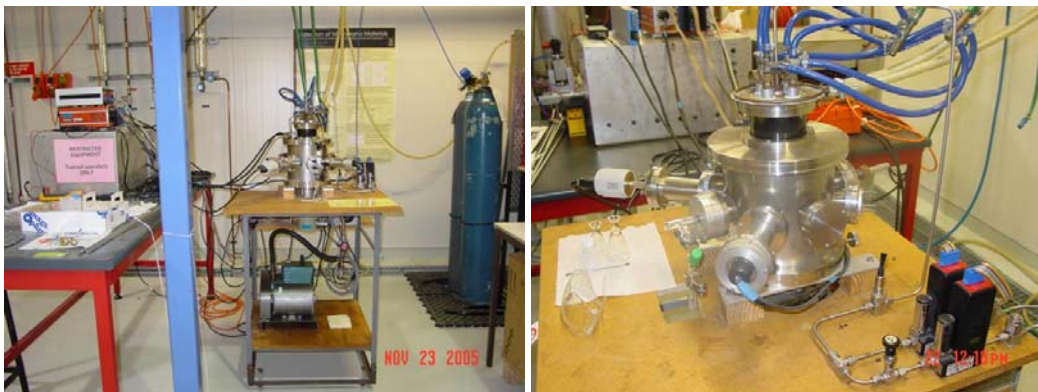


Figure 3.1 Plasma reactor

3.3 PREPARING MICROCAPSULES

3.3.1 Interfacial polymerisation

The general method to prepare hollow polyamide microcapsules involved reacting terephthaloyl dichloride (TDC) with ethylene diamine and diethylene triamine using interfacial polymerisation for different reaction times. Polymerisation was done in an aqueous phase containing sodium dodecyl sulphate (SDS) as a surfactant, with organic solvent mixtures of benzene/xylene or chloroform/cyclohexane to make an oil-in-water emulsion. The temperature was controlled at 10°C during emulsification and interfacial polymerisation. Microcapsules were separated by centrifugation and washed with distilled-deionised water and ethanol, and then freeze dried.

Procedure:

1. Sonicate a 10-ml aliquot of benzene/xylene (2:1) or chloroform/cyclohexane (3:1) containing 0.1M TDC in an ultrasonic bath.
2. Add this organic phase to 160 ml of water phase containing 1% (w/v) SDS in a 250-ml beaker and emulsify the mixture using a homogenizer at 800 rpm for 10 min at 10°C (ice bath).
3. Add 10 ml ethylene diamine, 5 ml diethylene triamine and 20 ml 1.18M sodium carbonate and stir mixture at 200 rpm for 60, 120 or 180 min.
4. Separate the microcapsules by centrifugation (800 rpm, 10 min).
5. Wash the microcapsules twice with 30% ethanol and three times with distilled-deionised water.
6. Freeze microcapsules and store in desiccators at room temperature.

3.3.2 Solvent evaporation

Known weights of polymer were dissolved in 10 ml of dichloromethane (DCM) and then emulsified in a 250-ml beaker containing 100 ml of 0.1–0.5% (w/v) polyvinyl alcohol (PVA) solution at 500–800 rpm for 2 minutes using a homogeniser to give an oil-in-water emulsion. The emulsion was then either warmed to 40°C or poured into 200 ml of 2% (v/v) isopropanol solution, and placed in fume cupboard with a magnetic stirrer at 200 rpm overnight to allow the solvent to evaporate. The resultant microcapsules were separated by centrifuging and washed three times with distilled-deionised water and freeze-dried.

3.3.3 Phase inversion

Known weights of polymer were dissolved in 10 ml of *n,n*-dimethylacetamide (DMAa) and then added drop-wise with a syringe nozzle into a coagulation solution (20-40% ethanol) at room temperature. The microcapsules were kept in the coagulation solution for 3 hours and then rinsed three times with distilled-deionised water.

To obtain microcapsules with a porous surface, the prepared microcapsules were immersed in DMAa solvent for various times to dissolve the dense outer-most layer on the microcapsule surface. The porous microcapsules were immediately rinsed with 30% ethanol and kept in the coagulation solution overnight.

3.4 MORPHOLOGICAL ANALYSES

3.4.1 Particle size analysis

Microcapsule size distribution was measured using a Malvern S laser diffraction particle size analyser. Microcapsules were suspended in distilled water. Analyses were done in triplicate.

3.4.2 Porosity

Porosity of freeze-dried microcapsules was characterised by determining their specific surface area and pore sizes in a B.E.T. analyser with nitrogen gas at 77K.

Approximately 0.1 g (weighed to four decimal places) was added to the glass cell, the glass cell was evacuated and flushed with nitrogen until the steady state (maximum) value, detected automatically by the analyzer, was reached.

Data were presented as sample density, specific surface (surface area per unit weight) and radius of pores. Pore size distribution was calculated from pore volume and radius and porosity was calculated from the measured specific pore volume and microcapsule density. Analyses were done in triplicate.

3.4.3 Optical microscope

Samples of wet and dry microcapsules during processing were observed using an Olympus optical microscope with 10, 20, 50, and 100 times magnification.

3.4.4 Scanning Electronic Microscope (SEM)

Surface morphology and internal structure of microcapsules was observed using a Hitachi S-4100 field emission scanning electron microscope. Freeze-dried microcapsules were placed onto metallic stubs using special double-sized adhesive tape. Samples were sputter coated with carbon layers under vacuum for 1 minute and then examined at 20kV.

3.4.5 Cross-sectioning technique

Cross-sections of the microcapsules were obtained by imbedding microcapsules in an aqueous gelatine gel containing glycerine as a plasticiser. After vacuum drying overnight, the hardened gel was cut in very thin slices with a scalpel blade.

Trials to determine the best embedding media were performed with 10%, 20% and 30% w/v gelatine gel containing 2, 5, 7 or 10% glycerine. The best thin slices could be cut by embedding microcapsules in 20% gelatine gel containing 7% glycerine.

3.5 SURFACE MODIFICATION

3.5.1 Plasma-induced grafting

Freeze-dried microcapsules were placed in the reaction chamber of a modified DC discharge plasma reactor and the whole system was evacuated to 1 Pa. Instrument-grade argon gas was introduced into the chamber at $\sim 30 \text{ cm}^3/\text{sec}$ and the microcapsules were treated with plasma for known times.

The acrylic acid monomer solution was deaerated using three freeze-pump-thaw cycles (see Section 3.5.4). Activated microcapsules were then immersed in 10-30% (v/v) acrylic acid monomer solution. Graft co-polymerisation was done under nitrogen at 50°C for 2 to 6 hours. After the grafting reaction, microcapsules were rinsed with distilled-deionised water several times and dialysed against distilled-deionised water overnight, and then freeze-dried.

Detailed plasma process is described in an operating manual in Department of Engineering at The University of Waikato.

3.5.2 Determining grafting yield

- Titration

Poly(acrylic acid) (PAA) content was determined by back titration of total carboxylic acid group capacity following Boehm's method (1966). Analyses were done in triplicate. Grafted microcapsules were equilibrated by immersing them in a standardised 0.05 N NaHCO_3 solution on a Minishaker at room temperature for 18 hours. Excess NaHCO_3 was then titrated with standardised 0.025 N HCl.

- FT-IR

The Fourier transform infrared spectra of ungrafted and PAA-grafted microcapsules were determined by Digilab Scimitar Series FT-IR using a KBr disc method.

3.5.3 Microcapsule functional processing

A schematic (Figure 3.2) shows microcapsule functional processing using interfacial polymerisation and plasma-induced grafting. The procedures are described in Section 3.3.1 and 3.5.1.

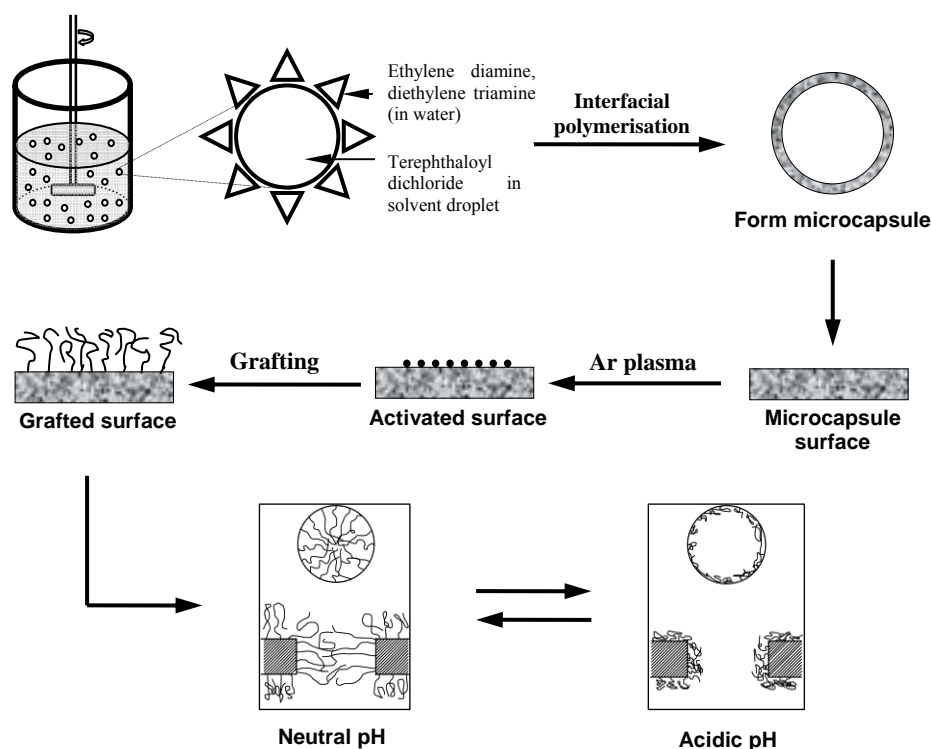


Figure 3.2 Schematic of microcapsule functional processing

3.5.4 Freeze-pump-thaw method for degassing liquids

This is the most effective method for solvent degassing compared with other methods such as sonication and inert gas purging.

1. Place solution in a Schlenk flask and close the stopcock. Use less than 50% of flask volume as overfilled flasks can shatter during the process.
2. Hook the flask to a Schlenk line and freeze the solution, preferably with liquid nitrogen. Maintain vacuum throughout this procedure.
3. When the solution is frozen, change the stopcock to vacuum and remove the atmosphere (2-3 min) while the flask remains immersed in liquid nitrogen. Close the stopcock to seal the flask.
4. Thaw the solution until it just melts, using a tepid water bath. Gas bubbles will evolve from the solution. Try not to disturb the liquid.

5. Replace the water bath with the cooling bath and refreeze the solution.
6. Repeat steps 3 to 5 until no more gas evolves as the solution thaws. A minimum of three cycles should be used.
7. Backfill the flask with an inert gas and seal. The degassed solution is ready to use and can usually be kept for 1-2 days in a sealed Schlenk flask.

3.5.5 Free radical grafting polymerisation

Microcapsules were put into a 100-ml flask containing 30 ml 10% (w/v) ammonium peroxydisulfate (APS) solution, which had been degassed using three freeze-pump-thaw cycles. The reaction was carried out in a shaking water-bath at 80°C with nitrogen gas purge for 2 hours (Figure 3.3). Microcapsules were then removed from the flask and immediately immersed in distilled-deionised water and ultrasonicated for 5 minutes three times. Microcapsules were then put into 30 ml 5–20% (v/v) acrylic acid monomer solution that had been degassed using three freeze-pump-thaw cycles, and 1 ml of 15 mM ammonium ferrous sulphate was added. The grafting polymerisation was carried out at 50°C under nitrogen for 2–6 hours. After grafting, microcapsules were washed three times with distilled-deionised water using ultrasonication for 5 minutes and then were dialysed against distilled-deionised water overnight (Section 3.5.6).



Figure 3.3 Free radical grafting polymerisation

The possible reaction mechanisms for APS activation and free radical grafting polymerisation process are given in Figure 3.4.

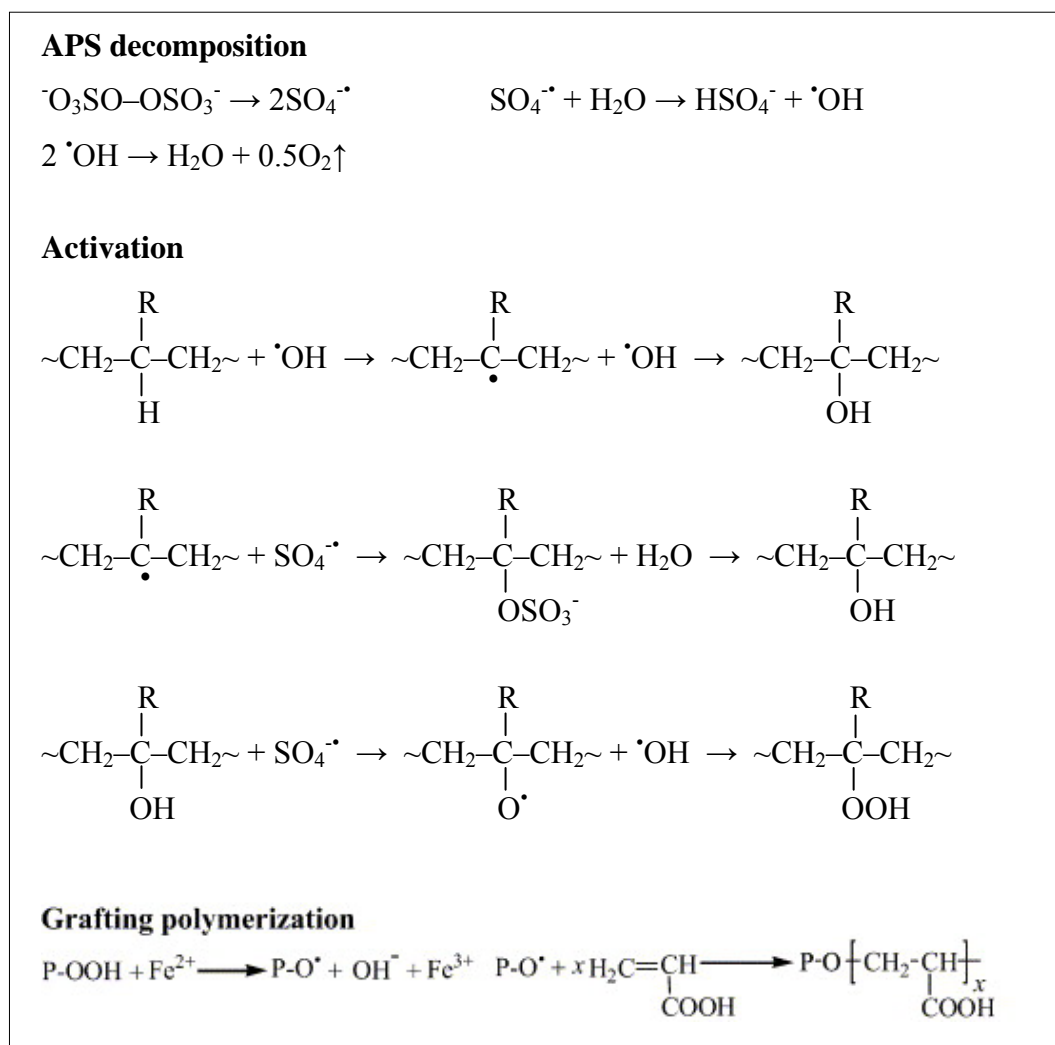


Figure 3.4 Scheme of the chemical grafting process

3.5.6 Dialysing grafted microcapsules

To remove residues of monomers and homo-polymers, grafted microcapsules were dialysed against 2 litres distilled-deionised water for 24 hours (Figure 3.5). The dialysis water was continuously circulated using a peristaltic pump. The water was refreshed at least three times during dialysis.



Figure 3.5 Dialysing grafted microcapsules

3.6 MODEL DRUG RELEASE STUDY

The effect of pH on release of a model drug from the microcapsules was determined to study microcapsule structure. Vitamin B₁₂ (molecular weight 1347 Da), cytochrome *c* (molecular weight 12,400 Da) and bovine serum albumin (molecular weight 66,700 Da) were chosen to model *in vitro* drug release. Microcapsules were shaken (mini-shaker at <100 rpm) with known concentrations of the model drug at pH 2 overnight, and then adjusted to pH 7. The microcapsules were gently blotted with filter papers and then transferred to 10 ml of release medium and the pH switched between 2 and 7. The amount of model drug released was measured by taking 1 ml of the release medium at known times and monitoring absorption at 361 nm and 409 nm (Amersham Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer). Samples were then returned to the container to maintain the same volume. Standard calibration curves were done for each model drug and percentage of released contents was calculated based on mass balance. Each experiment was done in triplicate unless otherwise noted.

3.7 STATISTICAL SIGNIFICANCE

Statistical significance of differences between different experimental groups was evaluated by the t-test.