Smart Microspheres for Stimuli Responsive Drug Delivery

By

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To my amazing parents, Vinay and Deepa, and
To my global fellow brothers and sisters deprived of education

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LIST OF TABLES

Page

Table

TABLE OF CONTENTS

Page

Chapter 1

REVIEW ON MICROSPHERES

1.Introduction

In recent years, interest in controlled and sustained release of drugs has increased as the pharmaceutical companies seek improved methods of delivering therapeutic dosages of medicines. Benefits to patients would be enhanced if dosages could be sustained for extended periods. Tunable, sustained delivery platforms are therefore necessary to achieve optimal and functional delivery of drugs. Microspheres are one of the widely researched candidates in such delivery platforms. This chapter deals with their advantages, methods of fabrication, characterization techniques, and reviews recent controlled drug release applications using microspheres made from biodegradable and stimuli responsive polymers.

2. Definition of microspheres

Microspheres are defined as spherical microscopic particles that range from 1- 1000 um in diameter [1, 2]. They are homogeneous structures made up of a continuous phase of one or more miscible polymers in which particulate drug is dispersed throughout the matrix unlike microcapsules, which have an inner core surrounded by a distinct outer shell[5]. A wide range of core materials have been encapsulated in microspheres, including adhesives, agrochemicals, live cells, active enzymes, flavors, fragrances,

pharmaceuticals, and ink[3]. Other than drug encapsulation, microspheres have been used as fillers and bulking agents and even for embolization therapy.[4]

3.Utility of microspheres

The idea of controlled release from polymers dates back to the 1960s through the employment of silicone rubber and polyethylene polymer microcapsules as delivery systems[1]. However, the requirement of eventual surgical removal because of non-degradability of these systems limited their applicability and urged the need to prepare systems which would be eventually eliminated from the body. Many new delivery systems like liposomes, hydrogels, etc. were designed thereafter and investigated but none emerged as perfect system due to issues with immunogenicity, stability, site and rate of administration, dosage, control over release rates, pharmacokinetics and pharmacodynamics.

In terms of release kinetics, delivery of most drugs, whether by oral administration or through injection, follows what is known as "first-order kinetics" characterized by initial high blood levels of the drugs, followed by exponential fall in blood concentration. This is problematic because once blood concentrations fall below certain levels, no therapeutic effect will be achieved. Furthermore, some drugs are toxic at high blood level concentrations. It is difficult to achieve a balance between effective levels and toxic levels when blood concentrations fall off so rapidly. Ideal delivery of drugs would follow "zero-order kinetics", wherein blood levels of drugs would remain constant throughout the delivery period.

Consequently, scientists have been searching for methods to deliver drugs with zero-order kinetics. An unmet challenge has been to select the best controlled release technology. Microspheres, with many advantages described below, are on the forefront of this

selection. Advantages of microspheres as controlled drug delivery devices are: a decrease in single dosage size, a continuous drug release, decrease in systemic side effects, reduced possibility of dose dumping, reduced frequency of administration, and, therefore, increased patient compliance. Non-invasive placement and localized release of desired amount of therapeutic agents, circumvents the deleterious side effects of systemic administration[6]. This enables administration of larger, effective dosages. Furthermore, the size and size distribution of microspheres can be controlled to achieve a better predicted response. Moreover, microspheres can be manufactured with biodegradable materials or stimuli responsive materials which eliminate the need for device recovery. Because of their ability to act as a device for controlled release drug delivery, microspheres have been used to encapsulate many types of drugs, including small molecules, proteins, and nucleic acids and are easily administered through a syringe needle. They can provide sustained drug release for long periods of time.

On the other hand, disadvantages of microspheres may include difficulty of large scale manufacturing, high cost of manufacturing, and inactivation of drug during fabrication and poor control of drug release rates. Nutropin Depot, which comprised of Genentech's recombinant human growth hormone (rhGH) encapsulated within poly(D, L-Lactide-co-glycolide) (PLG), was pulled from the market because of high cost of production and manufacturing. Microspheres as fillers and bulking agents tend to migrate away from the injection site, which can cause a loss of therapeutic effects and may also cause embolism, which can eventually lead to organ damage.

4.Materials that can be used for microsphere fabrication

4.1 Natural Polymers

Natural polymers are of interest because of their biocompatibility, relative abundance and commercial availability, and ease of processing. Natural polymers include alginate, proteins, collagens (gelatin), fibrins, albumin, gluten, elastin, fibroin, hyaluronic acid, cellulose, starch, chitosan (chitin), pectin (pectinic acid), galactan, curdlan, gellan, levan, emulsan, dextran, pullulan, heparin, silk, chondroitin 6-sulfate, polyhydroxyalkanoates, etc. [7]

4.2 Synthetic Polymers

Synthetic polymers are largely divided into two categories: biodegradable and non-biodegradable. Biodegradable synthetic polymers include poly(α-hydroxyester)s, polyanhydrides, and polyorthoesters[8]. Among these, the poly(α-hydroxyester)s such as polylactide (PLA), polyglycolide (PGA), and its copolymers are most extensively used. These polymers, which are biocompatible, biodegradable, and bioresorbable, are approved by the Food and Drug Administration (FDA) and are also advantageous due to their controlled degradation behavior.

Some non-biodegradable polymers include polyvinylalcohol (PVA), polyhydroxyethymethacrylate (PHEMA), and poly(N-isopropylacrylamide) (PNIPAAm). Synthetic polymers have an advantage of large scale availability and tunable mechanical and chemical properties.

5.Methods of preparing microspheres

For preparation of microspheres, certain requirements must be met while choosing an appropriate encapsulation process[10]. These are:

1) The biological activity and chemical stability of the incorporated drugs should be maintained as much as possible during the process of encapsulation.
2) The encapsulation efficiency and the yield of the microparticles should be high enough for mass production.
3) The size range of thee microparticles produced should be reasonable (<250 um) so that they can be administered using the syringe needle via the parenteral pathway.
4) The drug release profile should be reproducible without significant initial burst release.
5) The employed process should produce stable, non-aggregating microparticles, thus making it easy to prepare uniform suspension of microparticles.

There are various techniques available for microencapsulation of drugs including the emulsion-solvent evaporation/extraction methods (single emulsion and double emulsion methods including solid-oil-water, water-oil-oil and solid-oil-oil methods), spray drying and spray freeze drying, ultrasonic atomization, electrospraying, microfluidic methods, pore closing method, thermoreversible gel method, microfabrication method and *in situ* polymerization. Each method has its own advantages and disadvantages. The choice of particular technique depends on attributes of the polymer and the drug, the site of drug action, and the duration of therapy[11, 12, 13]. However, emulsion solvent removal is the oldest and most widely used method to accomplish encapsulation. Unlike spray drying

method where temperature-sensitive compounds are degraded and control of the particle size is difficult, the emulsion solvent removal method does not require elevated temperatures. The emulsion solvent removal method does not require phase separation inducing agents, unlike phase separation and coacervation methods where residual solvents and coacervating agents are typically found in the microspheres. The emulsion solvent removal method thus allows for the creation of microparticles that have a more optimized release of the encapsulated material. This chapter deals with this traditional encapsulation method, including single emulsion and double emulsion techniques, and two modern methods involving "open-closed" pores and a thermoreversible gel for preparation of microspheres.

5.1 Single Emulsion Method

The single emulsion method is primarily used for encapsulating hydrophobic drugs through an oil-in-water (o/w) emulsification process. The premise for this method is the emulsification of a polymeric solution in an aqueous continuous phase. The polymer is dissolved in a water-immiscible, volatile organic solvent like dichloromethane (DCM) and the drug is also dissolved or suspended in polymer "oil" solution. The resulting mixture is then emulsified in large volume of water containing a surfactant like polyvinyl alcohol (PVA). The organic solvent in the emulsion is removed by either evaporation or extraction in large amount of water, resulting in the formation of compact microparticles. A schematic diagram of the method is shown in Fig. 1.

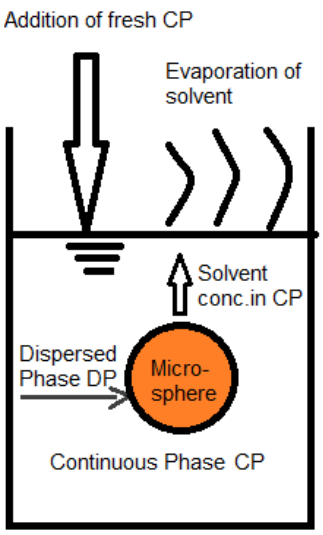

Fig 1: Schematic of microsphere formation using the single emulsion (O/W) method. The polymer and the drug are dissolved in an organic solvent to form a Dispersed Phase (DP). The resulting mixture is then emulsified in large volume of water containing a surfactant like polyvinyl alcohol (PVA) which forms a continuous phase (CP). The organic solvent in the emulsion is removed by evaporation resulting in the formation of compact microspheres.

Many methods have been utilized to achieve the dispersion of oil phase in continuous aqueous phase. The most common method is the use of a propeller style blade attached to variable speed motor. As the speed of the motor is increased, the size of the dispersed droplets decreases as a result of the high shear induced by the propeller. Homogenization is also used to prepare emulsions. A homogenizer equipped with a rotor and stator type blade is attached to a variable high speed motor. Since high shear is used to produce the emulsion, the resultant product has size much smaller than the emulsions produced by conventional agitation. Other methods used include a microfluidizer, sonicator, and potentiometric dispersion[14].

The rate of solvent removal, the concentration and type of surfactant used, type and volume of organic solvent, polymer molecular weight and emulsification stirring speed are reported to affect the physiochemical characteristics, encapsulation efficiency, and

release of drugs from microspheres[15-18]. This method, however, is only applicable for hydrophobic drugs because hydrophilic drugs may diffuse out or partition from the dispersed oil phase into the aqueous phase, leading to poor encapsulation efficiencies[19, 20].

5.2 Double Emulsion Method

Double emulsion or water-in-oil-in-water (W/O/W) methods are largely used for encapsulating water soluble drugs[10]. This method has advantages of relative simplicity, convenience in controlling process parameters, and only requiring inexpensive instrumentation[21]. In W/O/W method, aqueous drug solution is dispersed in a polymer-dissolved organic solution, e.g., PLGA in dichloromethane (DCM) or ethyl acetate (EtAc) to form a primary W/O emulsion. Then this primary emulsion is further dispersed in large volume of water containing an emulsifier, such as poly (vinyl alcohol) (PVA), to form a W/O/W double emulsion. Hardened microspheres are formed by removing organic solvent from the polymer phase by solvent extraction or solvent evaporation.

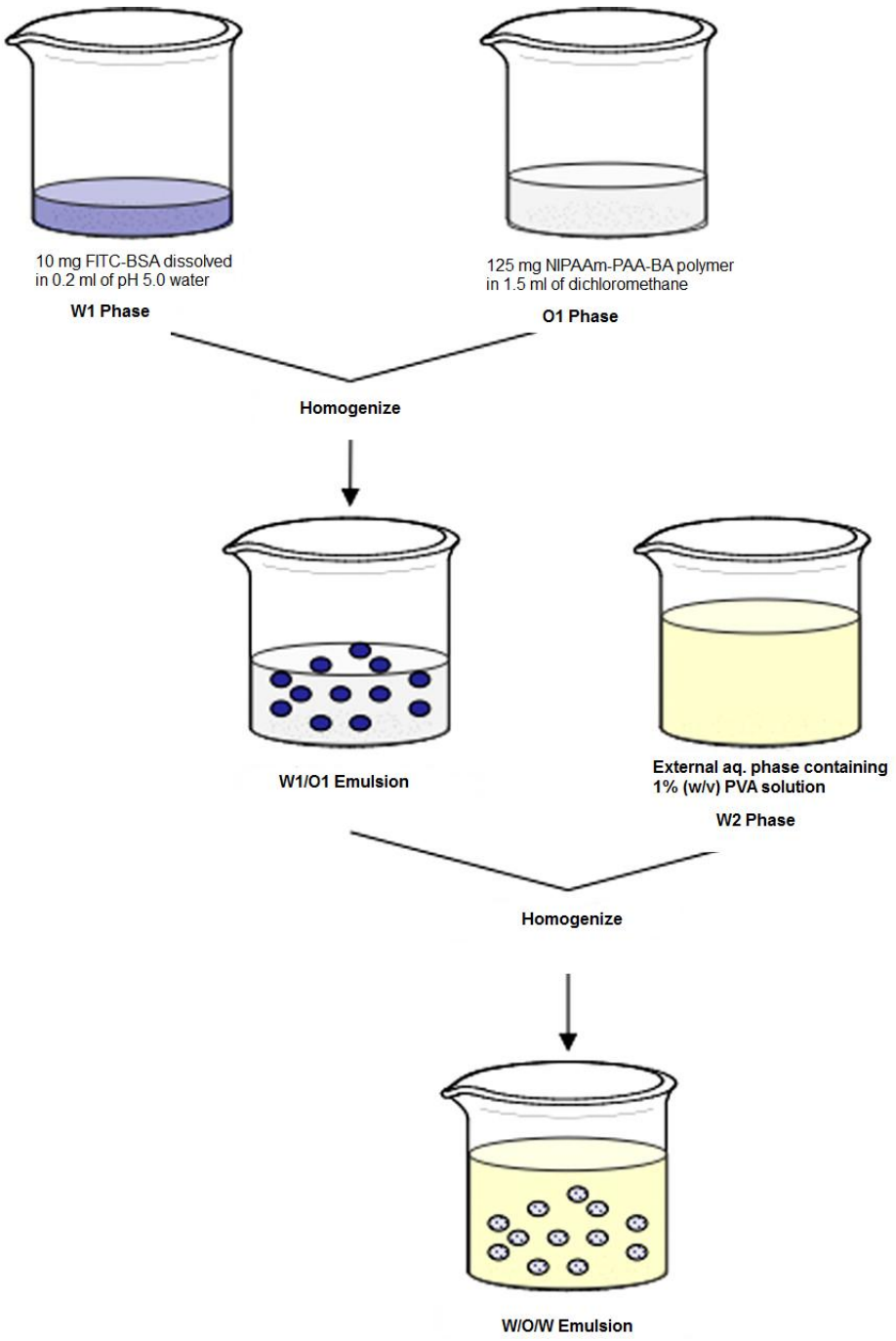

Fig. 2: Schematic of microsphere formation using the double emulsion (W/O/W) method

The properties of W/O/W microparticles (such as loading capacity, encapsulation efficiency, release kinetics, and particle size) can be difficult to control and depend on protein (type and concentration), polymer (composition, MW, and concentration),

volume ratio between drug and polymer solutions, emulsification method (time and intensity), and surfactant (type and concentration)[22].

5.3 "Open – closed" pores method and Thermoreversible gel method

Although double emulsion method is considered as the most convenient for water soluble proteins in terms of protein stability and encapsulation efficiency, there are two main disadvantages of this method. First, it requires use of hydrophobic and halogenated solvents (i.e. dichloromethane) that are toxic and not easily removed completely[23]. Second, inadequate protein release is often observed that is characterized by an initial burst release phase followed by slow or incomplete release that does not match the polymer degradation or removal rate[24]. Additionally, acidic degradation products, such as lactic and glycolic acid are often produced during microsphere degradation and biomacromolecule release[25]. To improve these release modalities and stability of the protein during the release phase, new methods being developed include the pore-closing method and thermoreversible gel method.

In pore closing method, Kim et al. created a novel technique to close microsphere pores for rhGH encapsulation[26]. In the first step, PLGA porous microspheres are created. Then, a blend of Pluronic F127 and PLGA is dissolved in DCM and used to form an O/W emulsion in a PVA solution. After microspheres are hardened and freeze-dried, the rhGH is loaded by dipping these microspheres in rhGH solution. The pores of the protein loaded porous microspheres were then closed by treating with water-miscible solvents under non-aqueous and vapor conditions. The resultant microspheres with closed pores showed a sustained release profile over extender period of time.

Thermoreversible-gel method[27] employs the sol–gel transition of Pluronic F127 solution to encapsulate proteins into microspheres. For example, when F127 solution of more than 20% concentration is warmed up to 10 °C, the liquid changes to gel. This fact is utilized to dissolve protein in 25% F127 solution at 4 °C in the first step, and then the solution is heated to 37 °C for gelation. This gel is then homogenized in a PLA solution in acetone dispersed at 37 °C using a homogenizer and then is cooled down to 4 °C to trigger the gel to sol transition. Acetone diffuses to the aqueous solution, and a large volume of water is added into the system to completely extract acetone. Finally, solidified microspheres (TG-MS) are collected and washed. Confocal microscopy images indicated that the protein was distributed in the core of the polymer matrix. Due to reduced protein association on particle surface, the TG-MS showed a higher encapsulation efficiency (93% vs. 72%) and lower initial burst release (about 30% vs. 50%) as compared with microspheres prepared by W/O/W methods. The protein in the microspheres showed a sustained release up to 70 days[27].

Compared with traditional microencapsulation methods, the microparticles made by these methods have desirable properties related to particle size, encapsulation efficiency, and release kinetics. However, since they are newly developed methods, more studies need to be done to examine various parameters important to making protein formulations[21]. Other studies that can be done include process parameter optimization, formulation, encapsulation of other proteins, and bio- activity studies.

6.Parameters considered in characterization of microspheres

Characterization of microspheres involves examination of several parameters such as microsphere size and size-distribution, drug loading amount (or capacity), encapsulation efficiency, type of release profile, sphere porosity, and intactness of drug encapsulated. Below the major parameters are discussed.

6.1 Particle size

Microsphere size, usually determined by Scanning Electron Microscopy (SEM), can be affected by the polymer concentration, temperature, viscosity, stirring rate and the amount of emulsifier employed.

In the W/O/W method, increasing the polymer concentration often results in increased sphere size[28]. Increase in the processing temperature results in the increase in the mean particle size with a broad size distribution[29]. Stirring rate and emulsifier type and concentration have also been shown to affect the microsphere size. As the stirring rate increased, the particle size was seen to decrease as increased stirring results in formation of finer emulsions [30, 31, 32, 33]. In general, microspheres made by mechanical stirring or vortex mixing are larger than those made by sonication[34, 35]. The presence of poly-vinyl alcohol (PVA) as an emulsifier in the external aqueous bath stabilizes double emulsion droplets against coalescence, leading to smaller microspheres[32, 34].

The volume of aqueous, organic and polymer phases also affects the particle size. Because the increased volume of the internal phase generates greater resistance to mechanical breakdown during emulsification, the particle size increases accordingly[36].

In O/W method, the emulsification power in generating secondary emulsion has a profound influence on the particle size and protein encapsulation efficiency. The higher

the emulsification intensity, smaller particles with higher encapsulation efficiency are obtained[37].

6.2 Drug Loading Capacity and Encapsulation Efficiency

Loading capacity (LC) is defined as:

$$\text{Loading Capacity} = \frac{\text{Weight of the encapsulated protein}}{\text{Weight of the microspheres}} \text{ X } 100\%$$

Loading of microspheres can be divided into two basic strategies. The drug can be added during the synthesis of microspheres or can be absorbed into the microspheres after their production. When spheres are produced using solvent evaporation, the drug can be included in the polymer solution and subsequently, the microspheres can be formed normally. When microspheres are synthesized by suspension, emulsion, or dispersion polymerization, the drug can be added to the monomer mixture before the reaction or soaked in after production. Average drug loading capacity in micro-spheres based on data from various microspheres was found to be 6.7 ± 4.6 % [21].

The Encapsulation Efficiency (EE) of microspheres is defined as:

$$\text{Encapsulation Efficiency} = \frac{\text{Weight of the encapsulated protein}}{\text{Weight of the total protein used for encapsulation}} \text{ X } 100\%$$

W/O/W method has been used to prepare microspheres with high EE. In general, it has been found that inclusion of a drug protecting agent and the balance of osmotic pressure

between internal and external aqueous phases are the key to obtaining high EE. Poor encapsulation efficiency becomes a limiting factor for scale-up production of microparticulate formulations. Many factors affect the encapsulation efficiency. In W/O/W method, increasing the concentration of polymer in the organic phase has been shown to increase the encapsulation efficiency[38]. In the case of proteins, a study on encapsulation of two BSAs with different solubility showed that the higher water solubility of BSA resulted in lower EE due to the higher tendency to diffuse into the external aqueous phase during microsphere formation[27]. The higher drug loading generates a higher concentration gradient between emulsion droplets and the continuous aqueous phase, resulting in the higher protein flux to the external water phase and lower EE[31]. Another factor affecting the EE is emulsification method[35]. Incorporation of surfactant usually reduces the protein EE[30]. The osmotic pressure between the inner and outer water phases also affects the EE. When salt is added into the external phase, a dense polymer film is generated around the microspheres to balance the osmotic pressure generated in the inner aqueous phase, and an increase in EE ocurrs[21].

In O/W method, the higher drug loading results in lower EE similar to W/O/W methods, due to more loss of the drug into the continuous phase[39]. EE, along with particle size, was also seen to be affected by the emulsification power used in generating the secondary emulsion. Higher emulsification intensity leads to smaller particles with higher EE [37].

6.3 Type of release profile

The drug release profiles from microspheres can be broadly categorized into 4 different categories as shown in following figure[21].

Since the time to reach steady state is often not clear, burst release is usually regarded as release in first 24 h. As seen from the figure, type D shows the most desired type of profile with low initial burst release followed by steady state release until most of the loaded drug is released, assuming that a constant concentration over time is the ideal pharamocokinetic profile for the drug's effect.

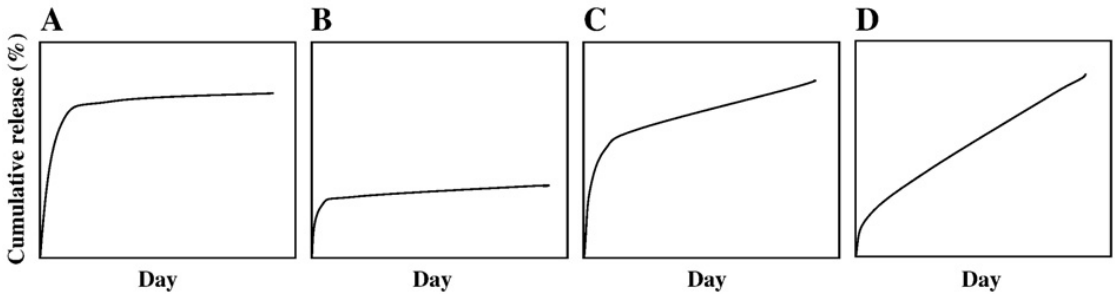

Fig 3: Categories of in vitro release profiles. The categories, based on magnitude of initial burst release, extent of drug release and the steady state release kinetics following the burst release, are: (A) High initial burst release (>30%) followed by little additional release. (B) Low initial burst release (≤30%) followed by little additional release. (C) High initial burst release (>30%) followed by steady state release. (D) Low initial burst release (≤30%) followed by steady state release.

The factors affecting the drug release rate revolve around the structure of matrix where the drug is contained and the chemical properties associated with both polymer and drug. Slower release can be achieved by using a polymer with slow degradation kinetics, but polymer degradation is not the only mechanism for release. The drug release can also be diffusion-controlled if the drug can travel through the pores formed during

microsphere hardening. Thus, the release profile is highly dependent on the pore status of microsphere[40]. Other factors that affect the release profiles include the polymer molecular weight, drug distribution, polymer blending, crystallinity, size distribution of the microspheres and the effect of the drug itself on polymeric microsphere, and these are well reviewed in a paper by Freiberg et al.[1].

7. Mechanisms of drug release from microspheres

7.1 Degradation

The drug release kinetics are affected by the type of polymer used in microsphere fabrication and the way in which the polymer degrades. Depending on the rate of hydrolysis of their functional groups, polymers can be broadly classified into two types: bulk eroding and surface eroding. Microspheres formed from bulk-eroding polymers like PLGA, degrade throughout the microsphere matrix and the resulting monomers, oligomers and the drug diffuse out of the sphere into the surrounding medium (Fig. 4 A). Such microspheres show either a biphasic or triphasic release profile characterized by an initial burst release. Burst release is thought to occur as a result of the drug located at or near the surface of sphere or in pores/voids connected to the surface. After the burst phase, a network of pores is formed in the microsphere, as the drug, monomers and oligomers diffuse out of the sphere into the surrounding medium. Subsequently, this network of water-filled pores increases in size until the sphere falls apart. The third phase of release may be seen if the drug that is remaining in the sphere that reaches this critical state, is released completely as a result of the collapse.

In contrast, surface eroding polymers like polyanhydrides are composed of relatively hydrophobic monomers linked by labile bonds that can degrade quickly into oligomers and monomers at the polymer/water interface via hydrolysis. Thus, penetration of water into polymer bulk is resisted. Drug is released at the microsphere surface as the polymer breaks down around it and also typically to a lesser extent by diffusion through the polymer phase (Fig. 4 B). In such microspheres, largest rate of drug release is expected at the beginning of degradation and as time goes, since the surface area of sphere decreases, the release rate is expected to decrease[41].

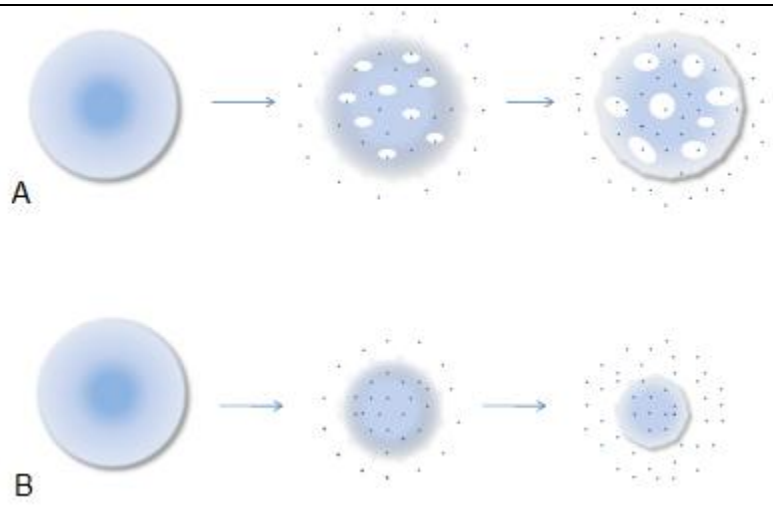

Fig 4: Polymer erosion and drug release from microspheres fabricated from (A) bulk-eroding polymers (B) surface eroding polymers

Thus, the release of encapsulated drugs can be controlled by polymer degradation under physiological conditions. The factors influencing this type of drug release mechanism are found to be varying copolymer ratio, crystallinity, polymer molecular weight, microsphere size, type and amount of excipient used and the interaction of drug itself with the polymeric microsphere [41, 42].

7.2 Stimuli responsive release

Control over drug delivery can also be achieved by employing stimuli – triggered release. Much interest has been focused on polymer systems that show a phase transition in response to external stimuli such as temperature, pH, ionic strength, and electric potential[43]. In particular, stimuli-sensitive polymer systems have the potential for application in modulated drug-delivery because these polymers not only respond to external stimuli but also control the release rate of drugs. Such stimuli-sensitive polymer systems exploit either one or more properties of the polymer in being thermo responsive, pH responsive, electro-responsive, magnetically responsive, ultrasonically responsive, light responsive, glucose responsive, enzyme responsive, and inflammation responsive[44,45].

In particular, microspheres with pH and thermo-responsive properties have been a topic of much interest. Among the thermo-responsive polymers, poly (*N*-isopropylacrylamide) (PNIPAAm) has attracted the most attention because of its sharp phase transition or lower critical solution temperature (LCST) around 32 °C in aqueous solution, which is close to the human body temperature[46]. Below the LCST, the polymer is hydrated, adopts an extended coil conformation and is water soluble. When the temperature of the polymer in the gel state is raised above LCST, it dehydrates and becomes hydrophobic and insoluble by adopting a globular conformation as shown in Fig. 5.

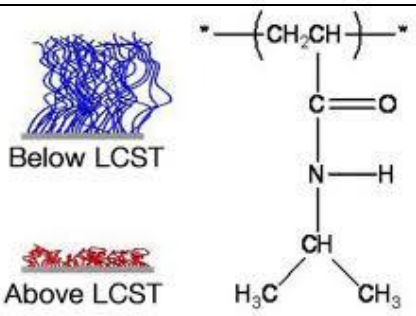

Fig. 5: Coil to globule transformation of pNIPAAm above its LCST

This thermo-responsiveness of pNIPAAm has been exploited in making various microspheres [47, 48, 49]. Chemical modifications are usually performed on NIPAAm to introduce functional groups that can increase the LCST towards body temperature and to improve the mechanical properties or to interact with certain drugs[50].

Added control over drug delivery can be achieved by employing pH-triggered release. By the incorporation of pH-sensitive groups, microspheres can be targeted to various biological environments or to specific organs. For example, a poly(β-amino ester) was used which was stable in the pH range of 7.0–7.4 but readily solvable below pH 6.5 to attain very slow release at pH 7.4 but full and immediate release at pH 5.1, suggesting a very good material for endosomal targeted release[51]. In other studies, acrylic Eudragit® (poly (methacrylic acid-co-methylmethacrylate)) is employed for pH-sensitive release. When a chitosan microcore was coated with Eudragit®, controlled release occurred only after the pH-sensitive coating was dissolved [52]. Even though chitosan dissolves rapidly at pH 7.4, no drug was released from the microspheres until the pH of shell degradation was attained. Such an outer shell provides usefulness in pH-targeted release. In addition to only temperature or only pH sensitivity, researchers have also shown that controlled

release is possible for acrylamide-based microsphere systems which are both pH- and temperature-sensitive[43, 53.]

Another interesting stimuli responsive microsphere example includes magnetically and thermo-responsive microspheres prepared by encapsulating silica-coated superparamagnetic magnetite nanoparticle clusters with a cross-linked pNIPAAm shell[55]. The microspheres exhibited a temperature-dependent swelling ratio; the hydrodynamic diameter decreased from 750 nm to 500 nm when the temperature increased from 20°C to 60°C. Additionally, the microspheres had greater magnetic responsivity at temperatures higher than the volume phase transition temperature due to the decrease in size at higher temperatures.

8.Applications of microspheres

The global market for microspheres in 2010 was estimated to be $2 billion and growing at a 5–year compound annual growth rate (CAGR) of 11.6%, reaching global sales of $3.5 billion by 2015[56]. Microspheres are used as controlled drug delivery systems for a variety of applications including chemotherapy, cardiovascular disease, hormone therapy, therapeutic protein delivery, and vaccine development[57]. Below are discussed some applications of microspheres.

Fig 6: Possible applications of microspheres

8.1 Vaccine delivery

Vaccine containing microspheres have been made from a variety of materials including hydroxypropyl cellulose/ PLGA[58], PCL[59], PLA[60], chitosan[61] and collagen[62].

In development of microsphere vaccines, loss of antigen bioactivity due to contact with organic solvents and hydrophobic polymers used in microsphere preparations is a major challenge. Researchers are trying to enhance vaccine stability by focusing on different approaches including use of adjuvants to protect the protein antigens or by choosing different microsphere materials[41]. For example, Puri et al. reported a novel approach in which ovalbumin (OVA), a nontoxic biodegradable protein was used to encapsulate muramyl dipeptide (MDP) [63]. In this case, ovalbumin which was the microsphere forming material, acted as the delivery matrix as well as antigen while the encapsulant, MDP,

acted as the adjuvant. A single shot injection in mice resulted in an immune response for 3 months, demonstrating that sustained release of adjuvant, rather than antigen, can aid in the development of a single-shot vaccine. This would help in eliminating the need and risk associated with reuse of disposable syringes and needles.

Microspheres for delivery of DNA vaccines have been made from poly (ortho-ester) and tested in mice[64]. The polymer can prevent the DNA from degrading, and the release takes place inside the cell where the weakly acidic environment degrades the polymer without compromising the biological activity of DNA. Diameter of spheres was adjusted to 5 um, a size believed to be preferentially taken up by cells.

8.2 Protein delivery

Peptides such as LHRH have been encapsulated in microspheres for sustained release. Futo et al. used a relatively large molecular weight (11000 to 27000) lactic acid polymer or its salt to make microspheres via single or double emulsion[65]. Ducrey et al. incorporated LHRH in the form of a water insoluble peptide salt (the LHRH agonist triptorelin pamoate) to provide slow release microparticles made up of PLGA type via the emulsion method[66]. The encapsulation of hormones such as calcitonin has also been achieved by Woo et. al[67]. Sustained release of stabilized, non-aggregated, biologically active erythropoietin (EPO) has also been achieved from microparticles prepared by solvent extraction in non emulsion processes[68]. Encapsulation of growth hormones using single or double emulsion methods has also been successfully achieved[69]. Bone morphogenic protein 2 (BMP-2) releasing and vascular endothelial growth factor releasing degradable microspheres have also been prepared[70].

8.3 Nucleotide delivery

DNA encapsulation in microparticles for oral administration was achieved using W/O/W emulsion to make biodegradable polymers under reduced shear[71-75]. Biodegradable microspheres that stimulate the production of cytokines in a host cell and contain a single stranded ribonucleic acid (ss-RNA) material, a stabilizing agent, and a biologically active macromolecule, have also been produced[72].

8.4 Therapeutic and diagnostic magnetic microspheres

Magnetic targeting can be used to diagnose tumors and deliver chemotherapeutic drugs and also therapeutic radio isotopes[79]. The advantage of this method over external beam therapy is that the dose can be increased, resulting in improved tumor cell eradication, without harm to nearby normal tissue[80]. Similar to chemotherapeutic drugs, many other drugs including peptides and proteins can be absorbed or encapsulated into magnetic microspheres. A very recent development in the field of magnetic targeting is the use of magnetically enhanced gene therapy. Advantages of such an approach are targeted gene transfection at rapid speed and high efficiencies[81]. The magnetic component in microspheres can also be used for purposes other than targeting. This is possible by filling an additional magnetic component into capsules or tablets. The speed of travel through the stomach and intestines can then be slowed down at specific positions by an external magnet, thus changing the timing and/or extent of drug absorption in stomach or intestines[82].

8.5 Microspheres for embolization

Chemo-embolisation is an endovascular therapy, which involves the selective arterial embolisation of a tumor together with simultaneous or subsequent local delivery the chemotherapeutic agent. The theoretical advantage is that such embolisations will not only provide vascular occlusion but will bring about sustained therapeutic levels of chemotherapeutics in the areas of the tumor. For trans arterial chemo-embolization, microspheres made from material such as PVA can release anticancer drugs like doxorubicin or cisplastin[83-86]. Microspheres can be accurately injected into tumor feeding arteries, thus blocking the circulation to the tumor. Then, doxorubicin released can attack tumor cells from inside the tumor, thus creating a "double" treatment.

For uterine artery embolization, ibuprofen loaded microspheres are being investigated to combat inflammation and pain frequently experienced after intervention[83]. This strategy is more effective than traditional application of pain killers as fresh blood is no longer available to reach the embolized uterine fibroid. Such microspheres have been made from PVA and in vivo tested in a sheep model[87].

8.6 Diagnostic and therapeutic radioactive microspheres

Various cells, cell lines, tissues and organs can be imaged using radio labeled microspheres. The particle size range of microspheres is an important factor in determining the imaging of particular sites. Diagnostic studies include dynamic and static imaging and in vivo function tests. Information about bio distribution and pharmacokinetics of drugs in organs can be obtained via dynamic imaging. When done

over a period of time, dynamic studies performed with a gamma camera provide clues to functioning of an organ. Therapeutic radioactive microspheres are radio labeled or encapsulate therapeutic alpha or beta emitter group. They have been used in local applications for treatments of rheumatoid arthritis, liver tumors and cystic brain tumors. Various diagnostic and therapeutic applications of radioactive microspheres include ^{90}Y-glass microspheres, ^{186}Re/^{188}Re-glass microspheres for radioembolisation of liver and spleen; ^{90}Y-resin microspheres, ^{169}Er.citrate microspheres for radiosynovectomy of arthritis joints; ^{90}Y-labeled poly(lactic acid) microspheres and ^{211}At-microspheres for local radiotherapy; polystyrene microspheres labeled with γ- emitters ^{141}Ce, ^{57}Co, ^{114m}In, ^{85}Sr, ^{51}Cr for blood flow measurements; ^{3}H, ^{14}C-labelled microspheres and ^{141}Ce-polystyrene microspheres for investigating bio distribution and pharmacokinetics of drug loaded microspheres; ^{99m}Tc-impregnated carbon particles, ^{99m}Tc-macro aggregated human serum albumin microspheres for lung scintigraphy and radioembolization; and ^{99m}Tc-macro aggregated human serum albumin microspheres for liver and spleen imaging[79, 80, 88].

8.7 Surface modified microspheres

Different approaches have been utilized to change the surface properties of carriers to protect them against phagocytic clearance and to alter their body distribution patterns. For example, the adsorption of the poloxamer on the surface of the polystyrene, polyester or poly methyl methacrylate microspheres makes them more hydrophilic and hence can decrease their uptake. Protein microspheres covalently modified by PEG derivatives show decreased immunogenicity and clearance. The most studied surface modifiers are

antibodies and their fragments, proteins, mono-, oligo- and polysaccharides, chelating compounds (EDTA, DTPA or Desferroxamine) and synthetic polymers[41].

9.Future Scope

Polymeric microspheres have gained in popularity because they can be easily produced with well-defined physical parameters and in each desired size range. Additionally, the straightforward control over dimensions of the injected particles makes them predictable and easier to inject. Also, microspheres pose a great opportunity to be used as reservoirs for drugs and carriers of bioactive molecules on their surface.

In spite of these advantages and their proved clinical applications, many challenges remain to be addressed in the design and production of particulate formulations. Many address purely formulational issues, and even though these are not new issues, they can prove arduous for every new application. These include such factors as macromolecule encapsulation, minimizing burst release, extending the duration of release, increasing macromolecule loading, encapsulating small hydrophilic molecules, and many others.

Particles that were environmentally responsive would be useful in a wide range of applications, as they would be designed to release their payloads only in environments or physiologic states with specific characteristics (pH, temperature, reactive oxygen species, etc.). While many such already exist, there is plenty of room for innovation and improvement, particularly in the development of novel stimuli-responsive polymers, which is the topic of discussion of next two chapters.

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Chapter 2

TEMPERATURE AND PH RESPONSIVE MICROSPHERES FOR SUSTAINED PROTEIN RELEASE IN ISCHEMIC ENVIRONMENTS

1. Introduction

There is a great demand for the development of novel therapies for ischemic cardiovascular disease, a leading cause of morbidity and mortality worldwide[1]. Similarly, chronic wounds and fibrosis associated diseases are amongst the most common disorders for which no effective treatment is available.[2]

Thus, controlled release of therapeutic proteins targeting ischemic sites such as chronic wounds and ischemic myocardium is a potentially high impact area of drug delivery. Therapeutic protein delivery to such ischemic sites has been attempted using various materials such as hydrogels and microspheres[3]. However, these release drugs at a rate dictated by hydrolytic degradation mechanisms rather than any environmental cues. Besides, other problems associated with the current delivery systems include burst release of the drug, resulting in a bolus delivery. Bolus delivery of proteins can potentially cause undesirable systemic effects, and the local concentration is not sustained for an ideal time-frame due to short protein half-lives that lead to rapid degradation and/or distribution throughout the body[5]. Therefore, a new platform to deliver drugs or proteins to ischemic sites in an optimized, sustained pattern would be of high impact.

Utilization of materials that are responsive to environmental stimuli such as temperature and pH presents one potentially viable route for controlling release to the diseased ischemic sites. Such "smart", stimuli-responsive polymers act by exhibiting large and sharp physical–chemical changes in response to small physical or chemical stimuli. Stimuli responsive microspheres have earlier been reported, including pH responsive[6-9], thermoresponsive[10-12] and magnetic microspheres[13]. However, most of these reported systems are found to be responsive to only one specific stimulus[14] which is why they find limited application. We hypothesized that dual responsiveness may enable better control over release than single stimulus and thought to prepare a novel "intelligent" temperature and pH responsive microsphere delivery system with a potential for sustained protein release in ischemic environment. These microspheres are made up of "smart" random copolymer consisting of temperature responsive N-isopropylacrylamide (NIPAAm) and pH responsive Propylacrylic acid (PAA) along with Butyl acrylate (BA) used to tune the gelling temperature of the polymer. This polymer was selected from a range of random copolymers prepared with a target in mind to exhibit sharp transitions as determined by the Lower Critical Solution Temperature (LCST) in the ischemic pH range of 5.5−7. Previously, researchers have pursued in situ gelling hydrogels with similar polymer compositions for application to ischemic sites [16]. However, inability of these hydrogel systems to gel quickly and form mechanical robust gels limits their initial retention at the ischemic site. Hence, we sought to pre-fabricate a polymer into "micro-gels" that would still enable injectable delivery but not require a rapid phase change upon initial tissue delivery. To this end, we present such "smart" microspheres retaining their "gel" form in acidic pH values of ischemic sites upon

injection, and undergoing gradual dissolution as they release their payload and the ischemic site returns to physiological pH. Microspheres were prepared from this polymer using a water–in–oil-in-water double emulsion method and characterized for their morphology, loading capacity, encapsulation efficiency and release profile.

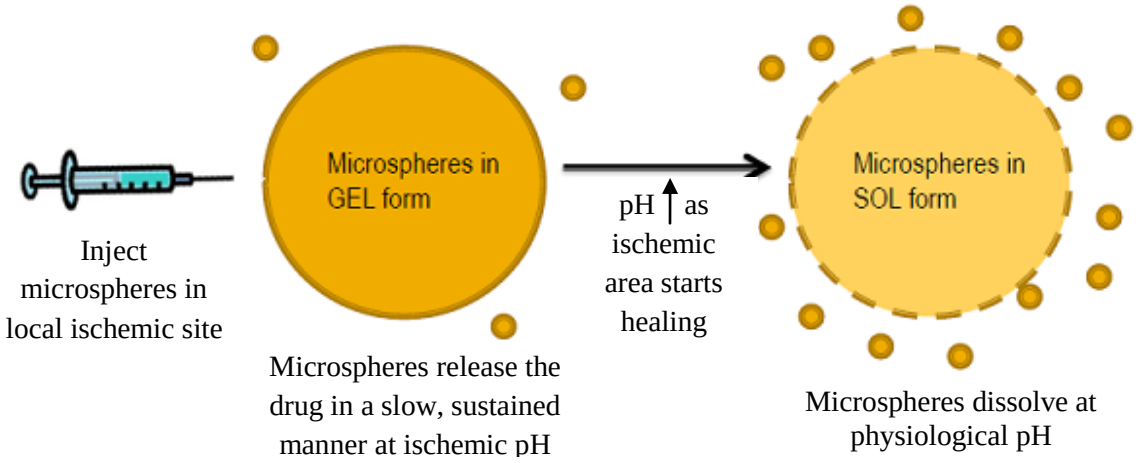

Fig. 1: Overview of the project

2. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and used as received unless otherwise described. N-isopropylacrylamide (NIPAAm) was recrystallized using hexane. Propylacrylic acid (PAA) was synthesized as shown previously[15]. Butyl acrylate (BA) was purified by distillation. 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) was used as the RAFT chain transfer agent. 2,2′-Azobisisobutyronitrile (AIBN) used as the free radical initiator, was recrystallized using methanol. Diamethylformamyde (DMF) was used as a solvent and anhydrous diethyl ether was used to precipitate the polymer. For polymer purification, dialysis tubing

(Fisherbrand) of molecular weight cut off 6000-8000 was used. Deuterated Dimethylsulphoxide (DMSO) was used to dissolve the polymer for ^{1}H NMR. Fluorescein isothiocyanate conjugated- bovine serum albumin (FITC-BSA) was used as a model protein for encapsulation in microspheres. Dichloromethane (DCM) was used to form the oil phase in microsphere preparation. 87-90% hydrolyzed Poly(vinyl alcohol) of average molecular weight 30,000-70,000 was used to prepare 1% w/v solution in deionized water. The homogenizer used for the emulsification steps was an Ultra-Turrax TP 18-10 (Janke and Kunkel KG, IKA-WERK). For changing the pH of Phosphate Buffer Saline (PBS), 0.1 N NaOH and 0.1 N HCl was used.

3. Methods

3.1 Polymer synthesis

A random copolymer series composed of N-Isopropyl acrylamide (NIPAAm), Propyl acrylic acid (PAA) and Butyl acrylate (BA) was synthesized using RAFT polymerization[16]. The NIPAAm:PAA:BA molar feed ratio was varied as follows, with PAA held constant: 90:10:0 %, 87.5:10:2.5 %, 85:10:5 %, and 77.5:10:12.5 %, 75:10:15 %, 72.5:10:17.5 % in order to tune the polymer to have optimal pH- and temperature-dependent LCST behavior. Chain transfer agent 2-(Dodecyl thiocarbonothioylthio)-2-methyl propionic acid (DMP) to initiator 2,2′-Azobis(2-methylpropionitrile) (AIBN) ratio was 10:1, degree of polymerization was 200, and monomer to solvent weight ratio was 50:50. After 30 min of initial nitrogen purging, the reaction was carried out at 60 ^{0}C for 24 hours or until a viscous product was obtained. The polymer was then precipitated

twice in cold diethyl ether to isolate it from the solvent Dimethylformamide (DMF). The polymers were then further purified by dissolving in ice cold PBS (1X) at a pH of 8.0-8.5 and then dialyzing in deionized water at pH 8.0. After dialysis, the purified polymer was lyophilized forming final product with a fluffy white appearance.

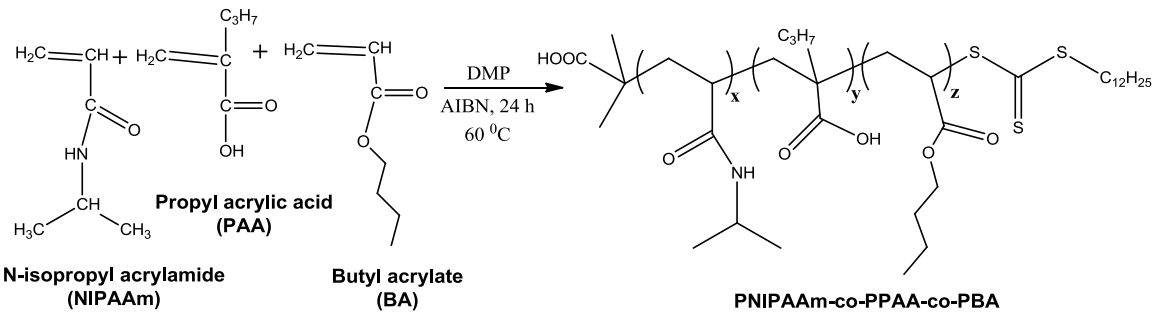

Fig. 2: NIPAAm-co-PAA-co-BA synthesis by RAFT polymerization

3.2 Polymer Characterization

^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) (NMR, Bruker 400 MHz Spectrometer equipped with 9.4 T Oxford magnet) in $CDCl_3$ and deuterated DMSO was used to determine composition and verify the formation of the polymer. The polymer was analyzed by gel permeation chromatography (GPC, Shimadzu Corp., Kyoto, Japan) with an inline Wyatt miniDAWN TREOS light scattering detector (Wyatt Technology Corp., Santa Barabara, CA) for molecular weight and polydispersity and dn/dc. The dn/dc, which is refractive index increment of polymer, was calculated using serial dilutions of each polymer, and it was then used to calculate absolute number average molecular weight Mn of the polymer based on refractive index and multiple angle light scattering detector data. For GPC characterization, 10 mg of polymer was dissolved in 1

ml DMF, syringe filtered by 0.45 um filter and injected using the mobile phase Dimethylformamide (DMF) containing 0.1 M LiBr. Lower critical solution temperature (LCST) LCST was determined from the absorbance of polymers at 500 nm as a function of temperature using the UV-Vis spectrophotometer (Varian) equipped with a Peltier temperature controller. The LCST behavior of each polymer was investigated at different pHs in ischemic to physiological range (pH 5.5, 6.2, 6.5, 6.8, 7.0, and 7.5) The temperature was ramped from 2 ^{0}C to 80 ^{0}C at a heating rate of 2°C every 5 min. Each polymer's LCST was defined as the temperature where the absorbance reached 10% of its maximum saturation value based on a convention previously described[17].

3.3 Microsphere fabrication

FITC-BSA encapsulated microspheres were fabricated from polymer R61 because it demonstrated the most ideal LCST behavior. Microspheres were fabricated using a commonly-utilized water-in-oil-in-water (W1/O/W2) emulsion method[18], as shown in Fig. 3. In short, 10 mg of FITC-BSA was dissolved in 0.2 ml of deionized water at pH 5.0 (internal W1 phase). 125 mg R61 polymer was dissolved in 1.5 ml dichlromethane (O phase). The W1 phase was then added to this O phase and emulsified for 30 seconds using Ultraturrax TP 18-10 type at 20,000 rpm. This primary emulsion was then added to 30 ml of external aqueous phase which consisted of deionized water with 1% w/v PVA (W2 phase) and emulsified again for 30 seconds.

For organic solvent evaporation and microsphere solidification, the W1/O/W2 emulsion was then kept on a shaker for 3 hours. The resultant microspheres were washed twice with deionized water at pH 5 and lyophilized for 24 hours.

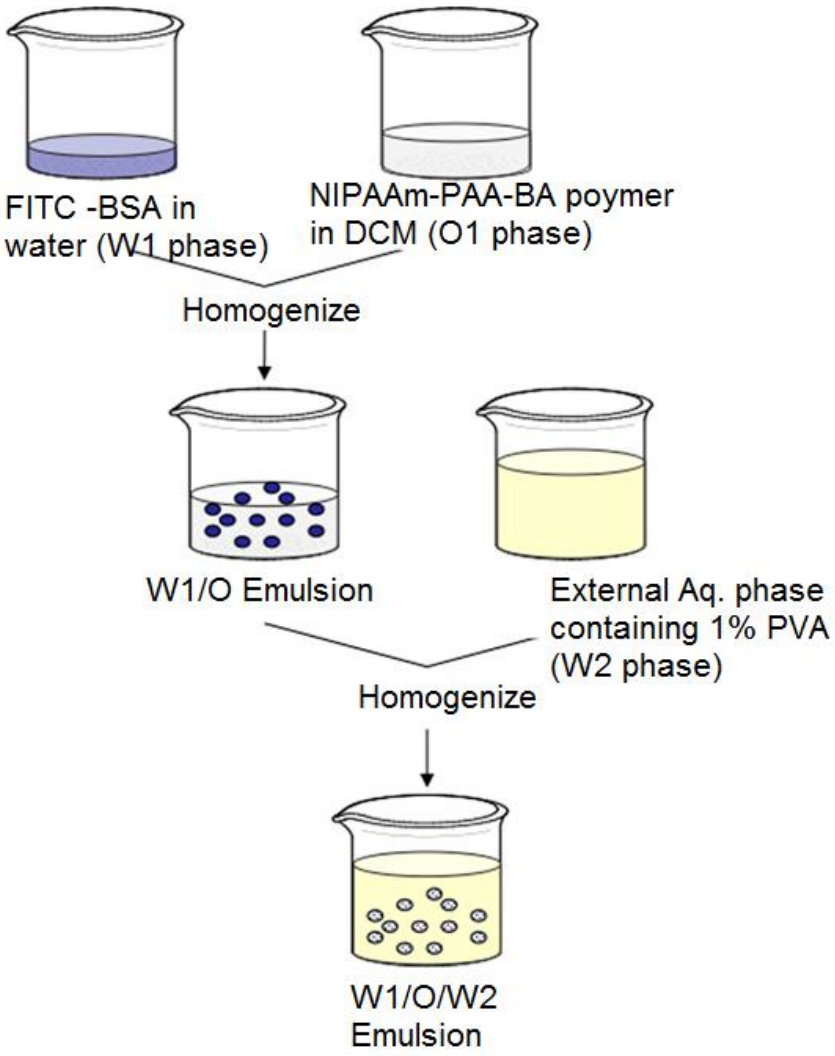

Fig 3: Schematic representation of Water-in-Oil-in-Water (W1/O/W2) method for microsphere fabrication

3.4 Microsphere characterization

Microspheres prepared from R61 polymer were characterized for size and morphology by scanning electron microscopy (SEM) and both optical and fluorescence microscopy. For SEM, the microspheres were suspended in a water drop and placed on a double sided sticky carbon tape attached to an aluminium stub. After drying, the samples were sputter coated with gold for 40 seconds. For optical and fluorescent microscopy, a water drop with microspheres suspended in it was placed on a glass slide and imaged after covering with a glass cover slip.

The loading capacity and encapsulation efficiency of the microspheres was calculated as follows:

$$\text{Loading Capacity} = \frac{\text{Weight of the encapsulated protein}}{\text{Weight of the microspheres}} \text{ X } 100\%$$

$$\text{Encapsulation Efficiency} = \frac{\text{Weight of the encapsulated protein}}{\text{Weight of the total protein used for encapsulation}} \text{ X } 100\%$$

3.5 Release study

In vitro protein release was quantified by measuring the fluorescence of the FITC-BSA released from the microspheres. Microsphere suspensions of 1 mg solids per mL of PBS of pH 5.5, 6.2, 6.5, 6.8 and 7.4 were placed in a shaker water bath at 37 ^{0}C. At periodic time points, the microspheres were spun down at 13500 rpm in an eppendorf centrifuge for 3 minutes, and 100 µl of supernatant was aspirated out and used to quantify the amount of FITC-BSA released based on using a FITC-BSA standard curve. Fluorescent measurements were taken using 485 nm excitation and 535 nm emission wavelengths on a plate reader (Tecan Infinite 500). The releasates were gathered and characterized over a period of three weeks in order to determine cumulative percentage release.

An additional release experiment was completed in order to mimic the change from acidic to physiologic pH that would occur during wound healing or recovery of an ischemic tissue. In this experiment, (1 mg/ml) were suspended in pH 6.2 PBS for 11 days, then switched to pH 6.8 PBS for the next 11 days, and finally exchanged into pH 7.4 PBS. The supernatant was collected at periodic time points for analysis as described above.

4. Results and Discussion

The series of polymers prepared by RAFT showed molecular weights close to the target Mn and polydispersity ~1.1 as shown in table 1.

Table 1: Series of random copolymers with increasing BA content

Polymer	NIPAAm:PAA:BA % Feed ratio	NIPAAm:PAA:BA % ratio obtained by NMR	Target Mn	Obtained Mn	PDI
R11	90:10:0	82.42:17.58	22648	25230	1.07
R21	87.5:10:2.5	74.04:18.92:7.03	22723.15	20930	1.09
R31	85:10:5	72.49:19.89:7.61	22798.3	19310	1.08
R41	77.5:10:12.5	66.7 :16.7 :16.6	23023.7	24030	1.05
R51	75:10:15	59.0:16.9: 24.0	23098.9	19870	1.06
R61	72.5:10:17.5	57.2:17.8 :25.0	23174.1	25670	1.09

The low polydispersities observed can be attributed to the well-controlled polymerization kinetics achievable using RAFT. The target molecular weight of the polymers was calculated as follows:

Target Mn = [(%NIPAAm feed)* Molecular Weight of NIPAAm + (% PAA feed)* Molecular Weight of PAA + (% BA feed)* Molecular Weight of BA] * Degree of polymerization

Actual Mn, Mw, and pDI were obtained by GPC, where:

Mn: Number-average molar mass is defined as:

$$M_n = \frac{\sum c_i}{\sum \frac{c_i}{M_i}}$$

Mw: Weight-average molar mass is defined as:

$$M_w = \frac{\sum (c_i \ M_i)}{\sum c_i}$$

And Polydispersity = (Mw/Mn)

To allow calculation of Mn, the dn/dc of the polymers which is the refractive index increment of the solute, had to be first determined. The dn/dc, was found out by injecting each polymer in increasing concentrations into the RI detector with mobile phase of DMF. For example, the data gathered to determine the dn/dc of the R61 polymer is shown in Fig. 4. Using this dn/dc value, refractive index and light scattering detector data the polymer absolute molecular weight was obtained. The example refractive index and light scattering detector GPC traces for R61 are shown Fig. 5.

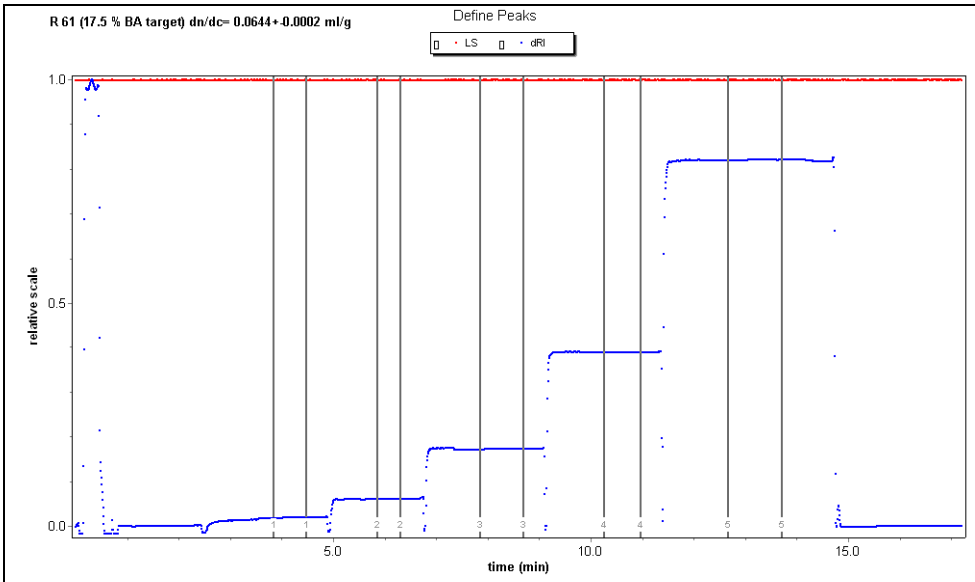

Fig. 4: Dn/dc of polymer R61 obtained by injecting a series of samples with increasing polymer concentrations into the GPC refractive index detector and recording the data.

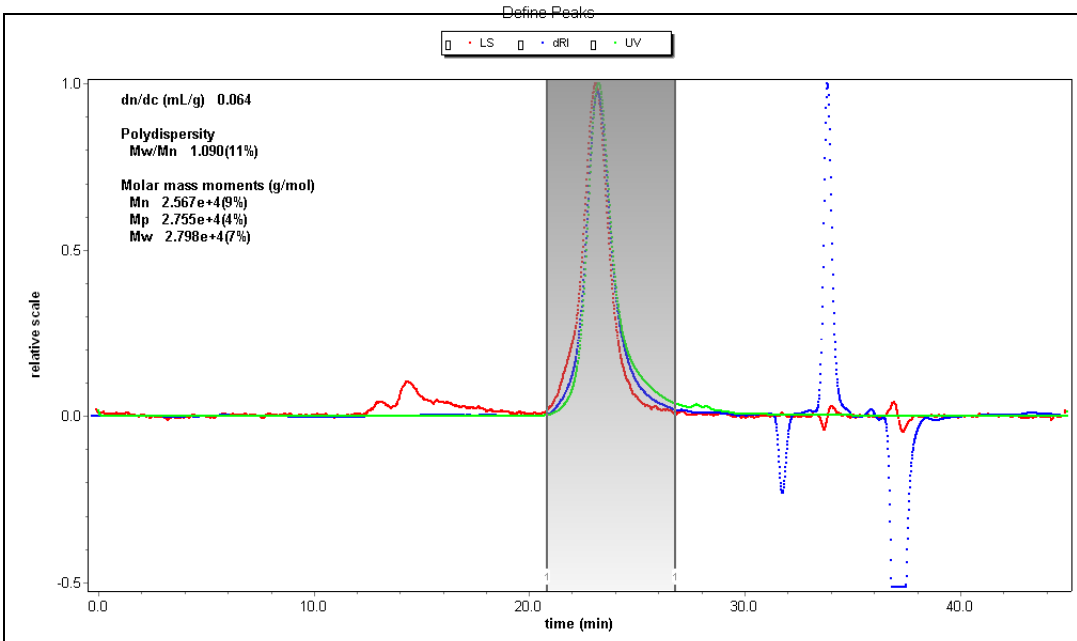

Fig. 5: Mn and Mw of the polymer R61 obtained by GPC using measured dn/dc

The molecular composition of all the polymers was confirmed by ^{1}H NMR and it is shown in Fig. 6.

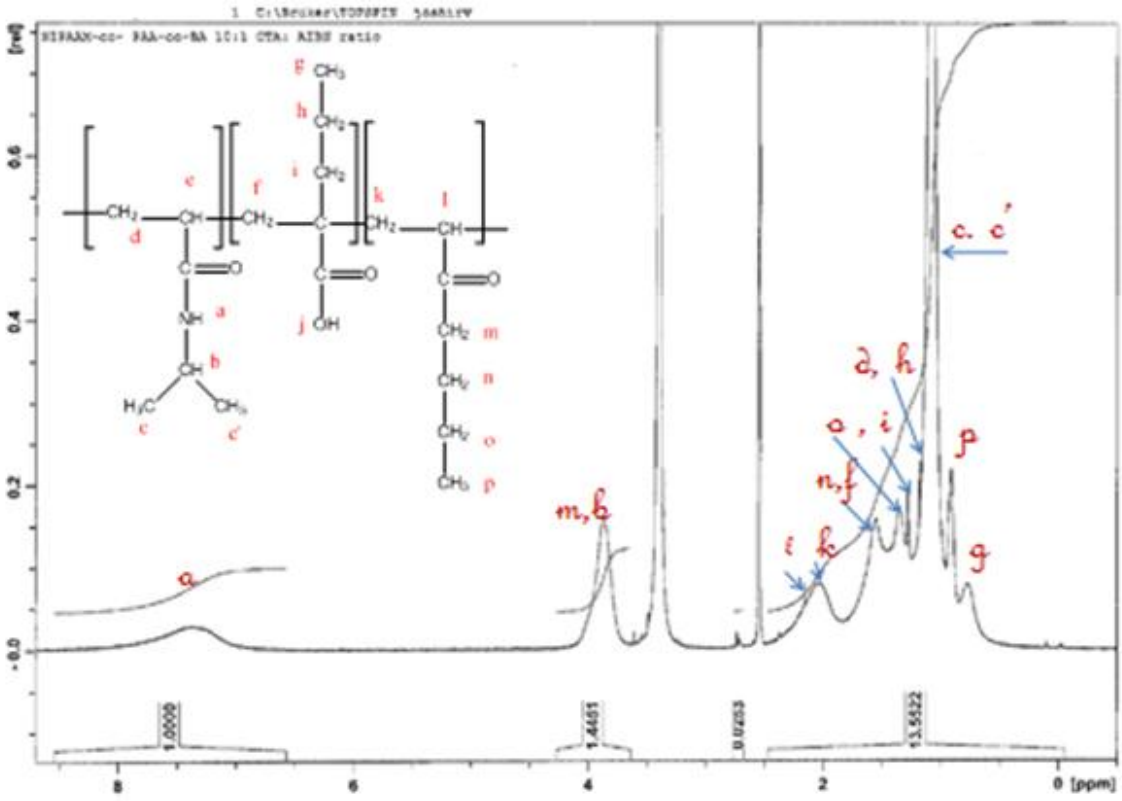

Fig. 6: Composition of R61 polymer confirmed by ^{1}H NMR

The ^{1}H NMR data for each polymer was also used to calculate the experimental NIPAAm:PAA:BA ratio that is given in table 1. Calculation of PAA and BA percentages in the copolymers was initially difficult due to the fact that the peak from the proton on the -CONHC[H] group of NIPAAm overlapped with the -COOC[H2]CH2CH3 on BA. To solve this issue, the broad peak around 6.5-8.5 ppm shown by the proton in N[H] group of NIPAAm was used, and the following calculations were made:

Integrate the following peaks (AUC, area under the curve):

AUC_A: 6.5-8.5 ppm - N[H] group on NIPAAm

AUC_B: 3.7-4.5 ppm - sum of NHC[H] and -COOC[H2]CH2CH3 proton peaks

AUC_C: 0.7-2.3 ppm - sum of remaining hydrocarbon hydrogens

Then, the composition can be calculated with the following equations, where N = NIPAAm, P = PAA, B = BA:

$N = AUC\_A$

$N + 2B = AUC\_B$

$9N + 9P + 10B = AUC\_C$

And the percent compositions are determined by the following equations:

$\% NIPAAm = N / (N + P + B)$

$\% PAA = P / (N + P + B)$

$\% BA = B / (N + P + B)$

The next step in polymer characterization was identifying their lower critical solution temperatures (LCSTs). A polymer is in a collapsed state above its LCST, but is soluble below the LCST. For validating the set up for LCST measurement, LCST of pNIPAAm was first measured to be 33^0C. Since the LCST of pNIPAAm was in agreement with the given value in literature (~33^0C)[17], the same experimental setup was used for the remainder of our polymer measurements.

LCSTs of the synthesized copolymers are given in Table 2. Our goal was to identify a polymer that remains collapsed in ischemic pH (pH 5.9 to 7.2) at physiological temperature (37 ^{0}C) so that controlled sustained release can be achieved by diffusion from the microparticles. To do so, a polymer exhibiting an LCST below 37 ^{0}C at or

below pH 7.0 was desired. Based on this criteria, polymer R61 showed the most desirable LCST and hence was selected for further microsphere fabrication (Fig. 7 and 8). All other polymers had higher LCSTs making them dissolve and rapidly release their payloads at 37 ^{0}C (Table 2).

Gel formation occurs only when the temperature is above the LCST and the pH is below the pK_a of the PAA monomer. It has been observed that p(NIPAAm-*co*-PAA) alone does not form a gel at pH 6, likely due to insufficient hydrophobic aggregation above the pK_a (~6) of the polymer[16]. Clinical applications in ischemic wounds and ischemic myocardium might require the formation of a gel above pH 6. In order to shift up the pK_a and facilitate gelation at higher pH values, increased hydrophobic content is needed in the polymer. This requirement is met by the incorporation of butyl acylate (BA) into the polymer feed. Indeed, the LCST of the polymer could be decreased to below 37 ^{0}C as desired by increasing the BA percent in feed ratio. This is observed in the LCST data obtained for polymers, as shown in Table 2. The lowering of the LCST can be explained by longer alkyl chain on butyl acrylate making it more hydrophobic and enhancing the ability of the polymer to gel at lower temperature.

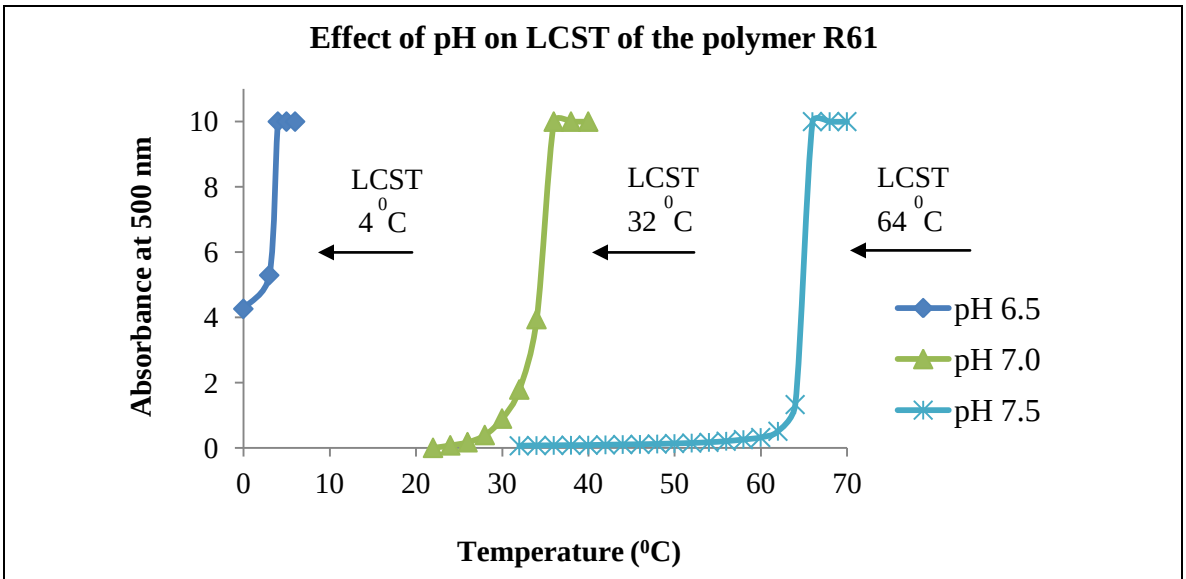

Fig. 7: LCST behavior of the R61 polymer as a function of pH. LCST of 32 ^{0}C at pH 7 indicated the polymer's tendency to remain collapsed at physiological temperature (37 ^{0}C) and slightly acidic pH. Furthermore, the LCST of 64 ^{0}C at pH 7.4 confirmed this polymer's solubility at physiologic pH and temperature.

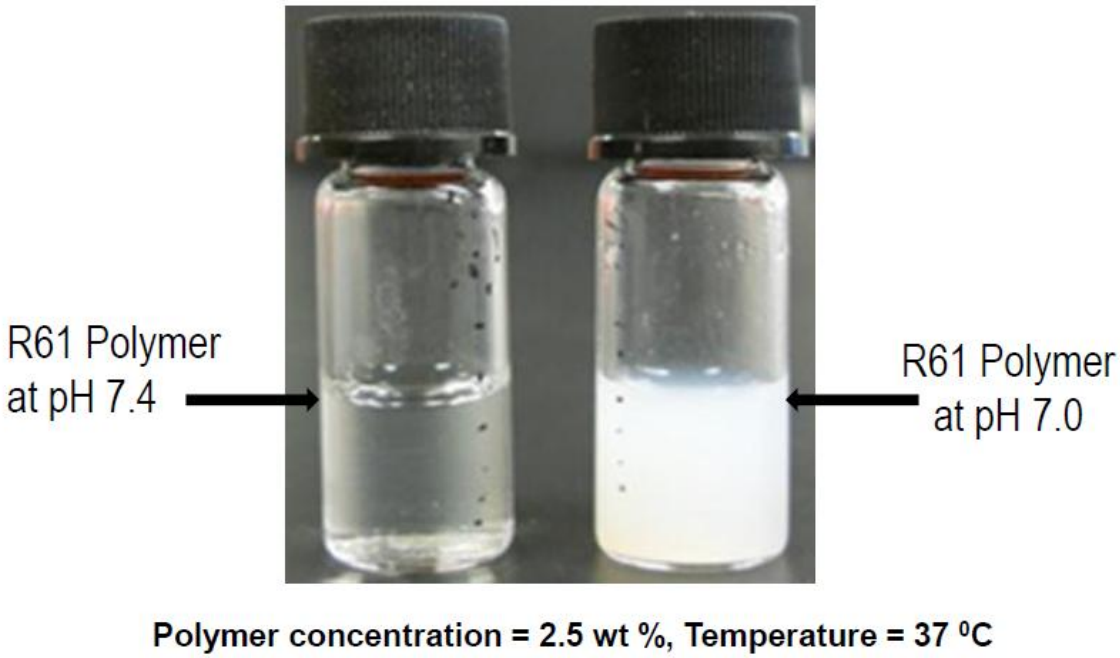

Fig. 8: Visual demonstration of LCST transition of the polymer R61. The polymer was dissolved in PBS at pH 7.4 in the concentration of 2.5 mg/ml but became cloudy when dissolved in pH 7.0 PBS at the same concentration.

Table 2: LCST of N-P-B polymers at different pHs			
Polymer	**LCST in °C at pH 6.5**	**LCST in °C at pH 7.0**	**LCST in °C at pH 7.5**
R31	59	71	101
R41	28	74	>84
R51	<4	47.5	76
R61	**<4**	**32**	**64**

After studying the LCST of the polymers, and determining that R61 showed the most desired LCST behavior, the next step was microsphere fabrication. It was decided to use conventional W/O/W method for microsphere fabrication because of its simplicity and suitability for encapsulating hydrophilic drugs (like proteins). FITC–BSA was used as a model protein for encapsulation in microspheres because it enabled the measurement of released protein based on fluorescence. The microspheres showed a loading capacity of 19.54%, and an encapsulation efficiency of 89.02%. The encapsulation of FITC-BSA within the microspheres was confirmed using optical and fluorescent microscopy as shown in Fig. 9.

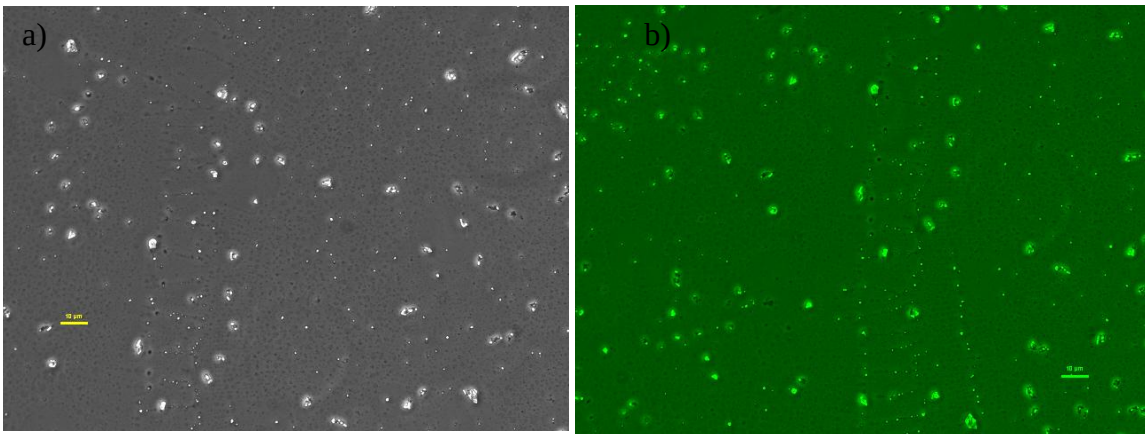

Fig. 9: a) Optical and b) fluorescent microscopic images of R61 polymeric microspheres. Scale bar refers to 20 um.

Microspheres prepared from R61 polymer were then characterized for their size and morphology using Scanning Electron Microscopy as shown in Fig. 10. Microspheres showed a smooth, spherical morphology and their average size was found to be 6.18 $\pm$ 13.84 um.

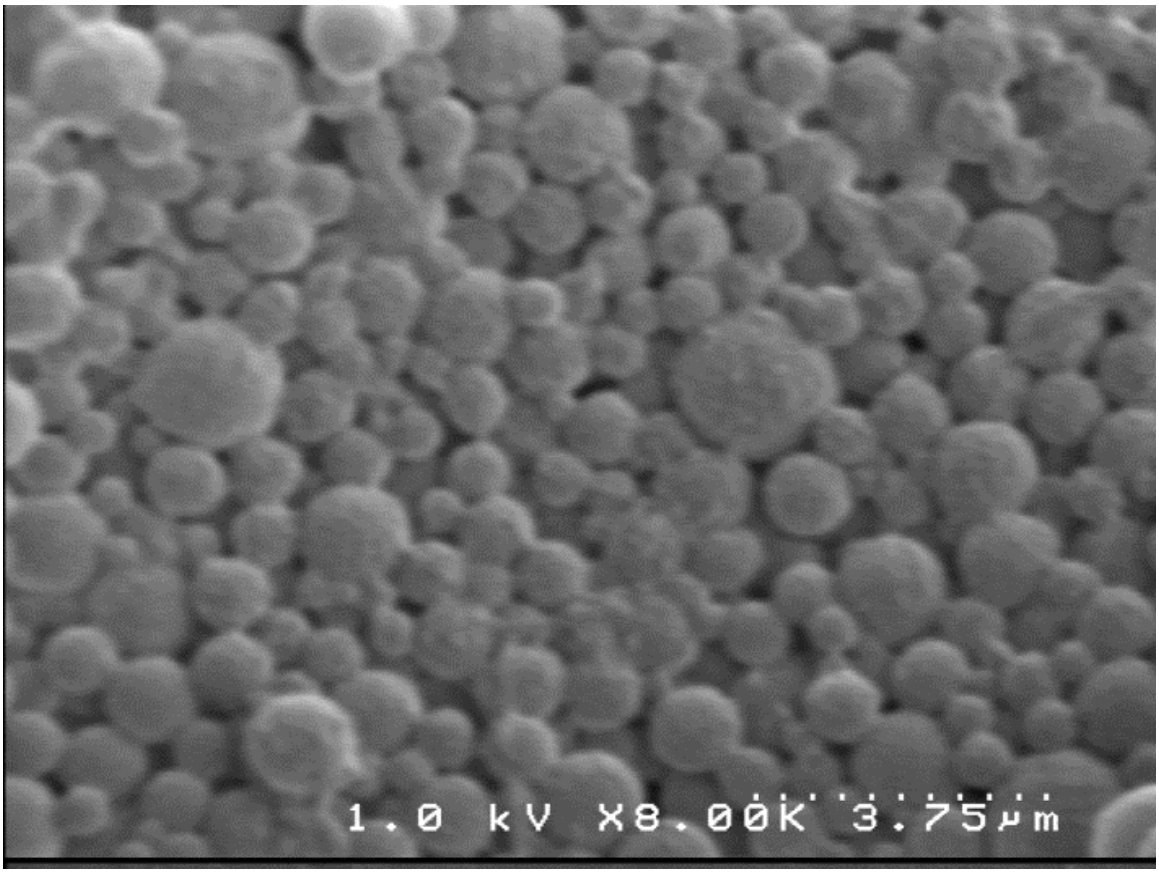

Fig. 10: Scanning electron micrograph of R61 microspheres. a), b) and c) are imaged at 3.5 kv, 6.0 kv and 8.0 kv respectively to show larger magnification of microspheres.

The polydispersity of the microsphere size may be due to the W/O/W method used, and in future, modified methods could be tested to see if they can yield more uniform microspheres.

In vitro pH dependent release experiments of microspheres are shown in Fig. 11. It indicates that pH 7.4 media triggered the more rapid release as microspheres were more quickly solubilized under this condition. At pH 5.5 and 6.5, which mimic ischemic tissue environments, the release was much slower and persisted over the span of one month. These data indicate that the microspheres retain their collapsed particle integrity. This likely allows the drug to be more slowly released by a diffusion-based mechanism dictated by the diffusivity of the protein through the polymer microsphere matrix and was confirmed using the Weibull distribution analysis of the release curves as shown in Fig. 12.

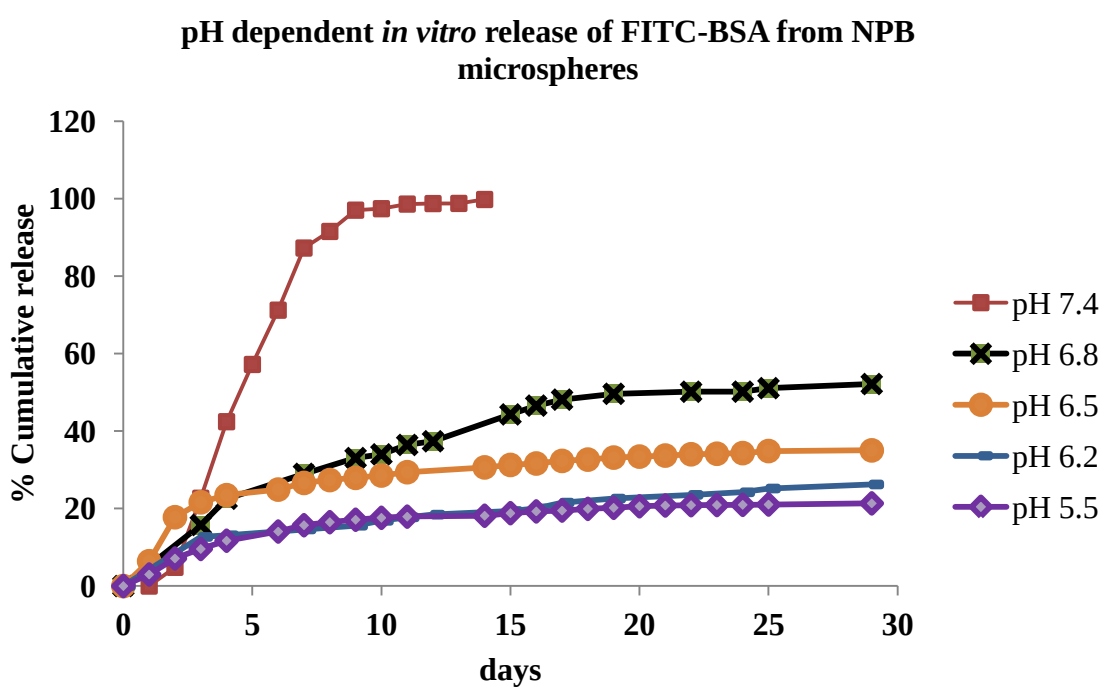

Fig. 11: In vitro release profile of FITC –BSA from R61 polymeric microspheres at different pHs

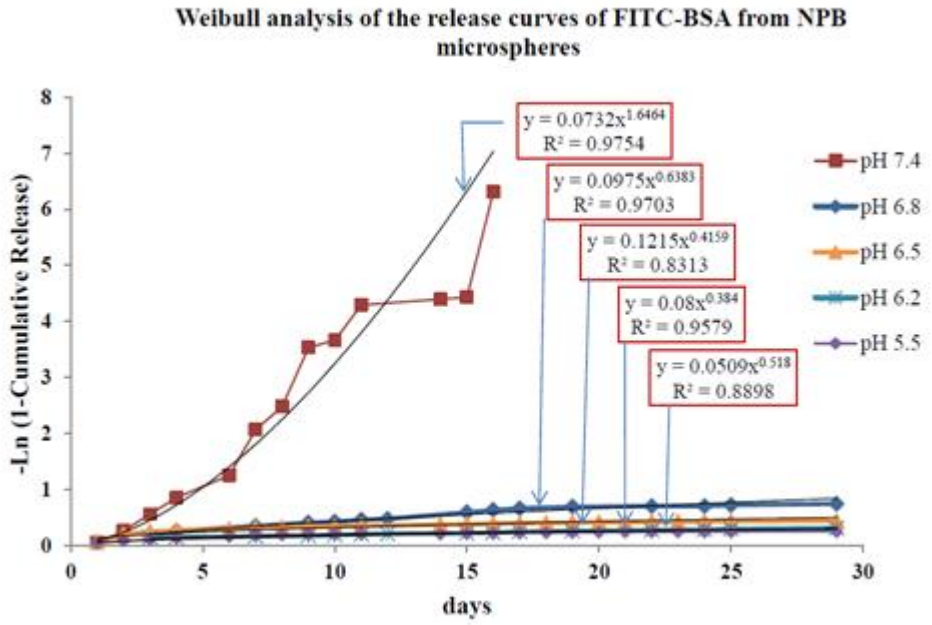

Fig. 12: Modelling FITC-BSA release curves from NPB microspheres using Weibull analysis. The trendlines show the best fits plotted using Weibull.

The Weibull function has been previously used to evaluate the drug release mechanisms of drug eluting matrices that efficiently release their payload (cumulative release exceeding 60%) [19, 20]. The release of FITC BSA from microspheres was fit to the Weibull empirical model in Eq. (1):

$$\frac{M_t}{M_\infty} = 1 - \exp\left(-a \cdot t^b\right)$$

where M_t is the mass of FITC-BSA released at time t, M_∞ is the total mass of FITC-BSA, a is a constant based on the system, and b is a constant based on the release kinetics. Previous reports suggest that values of $b < 0.75$ indicate that Fickian diffusion is

the dominant release mechanism [19, 20]. The values of a and b parameters obtained from the best-fits for respective pHs are given in table 3. These values indicate that diffusion based release is dominant in ischemic pH range 5.5-7.0, but at pH 7.4, combined release mechanism is dominant indicating the release due to dissolution of the microspheres at pH 7.4 in addition to diffusion.

Table 3: Parameters a and b obtained from Weibull best fits of the release data

pH	a	b	R^2
5.5	0.0509	**0.52**	0.89
6.2	0.08	**0.38**	0.96
6.5	0.1215	**0.42**	0.83
6.8	0.0975	**0.64**	0.97
7.4	0.0732	**1.65**	0.98

In order to assess the morphological change of the microspheres based on pH change from acidic to physiologic conditions, the microspheres used in the release experiments were taken from the solutions of pH 5.5, 6.5 and 7.4 and prepared for scanning electron microscopic imaging. As illustrated in Fig.13 a), b) and c), the microspheres remain intact at pH 5.5 but start solubilizing with increasing pH (6.5) and are completely dissolved at pH 7.4.

Overall, the release study and the SEM images of microspheres in different pHs demonstrated that the microspheres achieved a sustained release of encapsulated FITC-BSA in the ischemic pH range, but are dissolved at physiological pH 7.4. This propery is deemed to be potentially desirable for growth factor release in ischemic tissues. Based on this release profile there would be a small intial burst release followed by a constant

(zero order-like) release to the ischemic, low pH tissue. As the tissue heals and physiologic pH is restored, the microsphere would fully dissolve and be cleared from the site of activity.

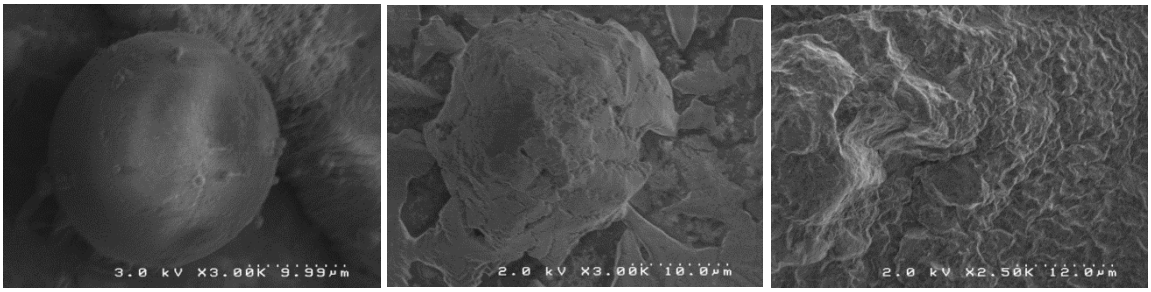

Fig. 13: SEM images of microspheres in PBS at different pHs a) pH 5.5 b) pH 6.5 c) pH 7.4

We next developed a model system to mimic the concept of environmental pH change from acidic to normal, physiologic pH. In this system, he pH-dependent release behavior was characterized by subjecting the R61 polymeric microspheres to staged, increasing pH conditions. Microspheres were subjected to pH 6.2 buffer for 11 days, followed by pH 6.8 for the next 11 days, and finally transferred to pH 7.4. The cumulative release profile for FITC–BSA is shown in Fig. 14 for this experiment. Percentage cumulative release per day was found out from the slopes of release curves during each of the pH zones and it was 0.71 % during pH 6.2 zone, 1.75 % in pH 6.8 zone and 27.05 % in pH 7.4 zone. It was evident that the rate of release was highly dependent on the environment, and that the microspheres rapidly dissolved quickly at pH 7.4. This type of profile indicates that the targeted behavior for sustained release as the damaged tissue heals followed by particle dissolution once normoxia has been reached.

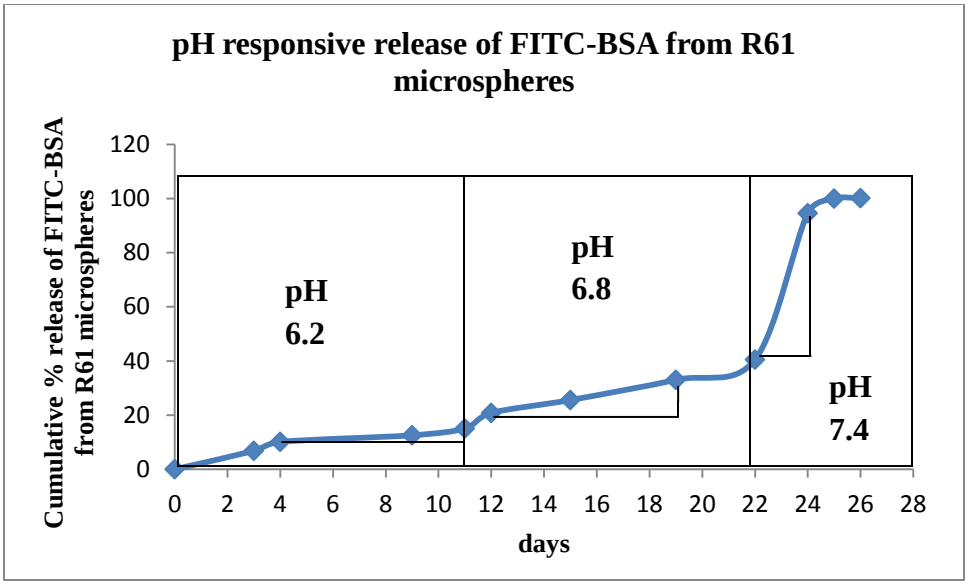

Fig. 14: The release profile of FITC-BSA from polymeric microspheres under changing pH conditions. 1 mg/ml of the microspheres subjected to PBS of pH 6.2, 6.8 and 7.4 for the given time span demonstrated a pH-dependent rate of release. Slope of the release curve in each zone was used to find cumulative % release per day during each of the zones.

5. Conclusion

This study illustrates the ability of pH- and temperature-responsive, "intelligent" microspheres fabricated from poly(NIPAAm-co-PAA-co-BA) to release encapsulated protein in a sustained manner to ischemic (acidic) environments and be subsequently cleared upon restoration of physiologic pH. Microspheres were successfully formed using a water-in-oil-in-water emulsion method and we have shown that at 37 ^{0}C, this injectable system solubilizes at pH 7.4 but forms microgel spheres in conditions with slight acidity (pH 5-7). This novel, injectalbe delivery system shows potential of therapeutic protein delivery to ischemic environments in a spatio-temporally controlled manner.

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Chapter 3

REACTIVE OXYGEN SPECIES (ROS) SENSITIVE MICROSPHERES FOR SUSTAINED RELEASE OF CURCUMIN

1. Introduction

In rheumatoid arthritis (RA), there is a large influx of monocytes, neutrophils and macrophages into the joint spaces due to enhanced vascularization[1, 2]. These cells become activated by pro-inflammatory cytokines, which are known to cause them to produce a respiratory burst which is accompanied by the generation of reactive oxygen species (ROS). Generally, ROS include superoxide ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and a variety of their reaction products[3] which are considered to be responsible for the decrease in synovial fluid viscosity due to the depolymerization and degradation of hyaluronic acid (HA)[2]. Amongst the ROS, H_2O_2 itself is not a radical, but it can give rise to highly reactive hydroxyl radicals in the presence of transition metals (Fenton reaction)[4]. It has been suggested that H_2O_2 causes inhibition of proteoglycan and HA biosynthesis, degradation of HA, and chemical modification of proteins necessary for stabilization of proteoglycan aggregates in human articular cartilage[5,6]. H_2O_2 is believed to penetrate the cell membrane, inducing degradation of DNA and suppression of ATP production[2].

Although the production of oxygen radicals during inflammation can be limited by oxygen concentration[7], there is considerable evidence that the oxygen scavenging activity in rheumatoid synovial fluid is low and is not sufficient to control damage in the joint

mediated by ROS[8-11]. We proposed to enhance the scavenging activity using "on demand", smart delivery of curcumin, a dietary polyphenol known chemically as diferuloylmethane. Curcumin is derived from the rhizome of turmeric (*Curcuma longa* Linn.), an East Indian plant, and it may be ideal because it has multiple, positive biological activities in addition to its scavenging effect on free radicals, superoxide anion, singlet oxygen and hydroxyl radical[12]. Curcumin is known for its antioxidant, anti-inflammatory, anti-cancer, and anti-proliferative activities[13-16] and is believed to be a potent agent against many ailments such as cough, anorexia, diabetes, hepatic disorders, Alzheimer's disease, and rheumatism[17-19]. The mechanism of the anti- inflammatory effect of curcumin is mediated by reduction of inflammatory cytokines including interleukin-8 (IL-8), monocyte inflammatory protein-1α (MIP-1 α), monocyte chemotactic protein-1 (MCP-1), interleukin-1β (IL-1 β) and tumor necrosis factor- α (TNF- α) from monocytes and macrophages[20].

Despite its promising biological effects, clinical application of curcumin is limited because of its poor water solubility, instability, and short half-life in vivo metabolism[21-23]. Furthermore, although curcumin is an edible spice, it is not orally bioavailable (i.e., it does not get absorbed into systemic circulation from the digestive tract). To overcome the limitations due to these three rate limiting factors, various attempts have been made that include synthesis of curcumin analogues[24], the use of adjuvants, as well as the development of improved delivery platforms such as liposomes and nanoparticles[25-28]. Here, we propose a novel delivery technology for "cell-demanded", local release of curcumin. This new approach utilizes environmentally-responsive (ROS sensitive) smart polymers for ROS-dependent delivery of anti-inflammatory drugs like

curcumin. Towards this end, we have fabricated ROS-sensitive microspheres from poly(propylene) sulfide (PPS) using an oil-in-water emulsion method. PPS is converted from its hydrophobic form into a more hydrophilic poly(sulfoxide) and poly(sulfone) form upon exposure to ROS. This ROS sensitive "switch" can cause a phase change (water insoluble to soluble) that leads to release of encapsulated curcumin from the microspheres only when ROS is present at the local site (i.e., "cell-demanded" release)[29, 30].

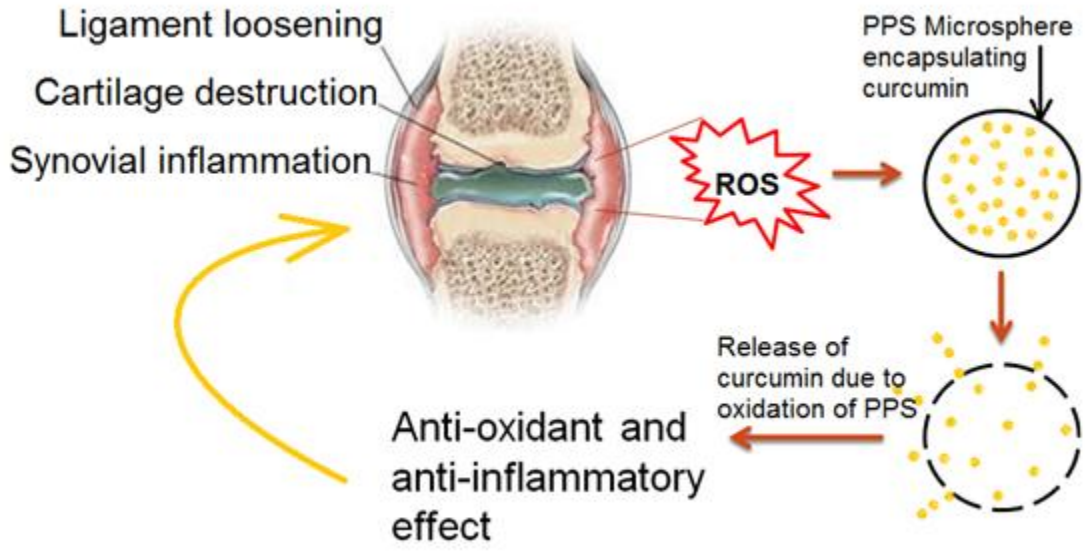


Fig. 1: ROS-dependent, "on-demand" anti-inflammatory drug delivery

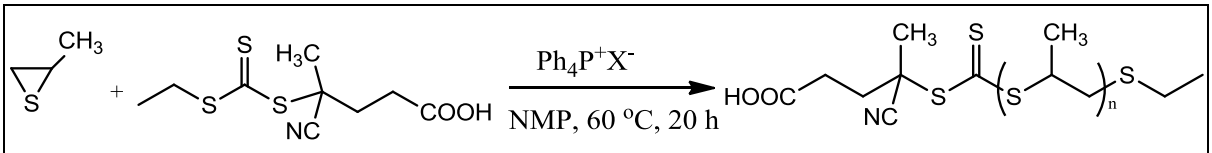
2. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received unless otherwise described. Poly(propylene sulfide) (PPS) was synthesized as described in methods section 3.1. Curcumin (molecular weight (MW) 368.38; ≥94% curcuminoid content) was used. 87-90% hydrolyzed Poly(vinyl alcohol) of average molecular weight 30,000-70,000 was used to prepare 2% w/v solution in deionized water. Tween – 20 was used in a microsphere washing step to get rid of curcumin crystals. Phosphate buffer saline (PBS) containing 0.1% w/v N-acetyl- L-cysteine (NAC), and 0.01% w/v butylated hydroxytoluene (BHT) was used as the release buffer. Six-well Transwell inserts used for release study were purchased from Corning (Lowell, Massachusetts). 200 proof, absolute, anhydrous, ACS grade ethyl alcohol was purchased from Parmaco-Apper, CT, USA. Dimethyl sulfoxide was used for calculating drug loading capacity and encapsulation efficiency.

3. Methods

3.1 Synthesis and characterization of poly(Propylene sulfide) (PPS)

The method for the synthesis of poly(propylene sulfide) is given in scheme 1.



Scheme 1: Synthesis of poly(propylene sulfide)

Propylene sulfide (3.16 mL, 40.4 mmol), ECT (52 mg, 0.20 mmol), TPPC (tetraphenylphosphonium chloride – $Ph_4P^+X^-$) (14.9 mg, 0.040 mmol), and NMP (N-methyl pyrolidone) (10 mL) were placed in a dry glass ampoule equipped with a magnetic stirring bar, and the solution was degassed by three freeze-evacuate-thaw cycles. The reaction mixture was stirred at 60 ºC for 24 h. A characteristic pale red or yellow color remained during the polymerization. The polymer obtained was purified by precipitation into a large excess of hexane and dried at 60 ºC under vacuum to yield a red/yellow polymer oil (Scheme 1).

The polymers were characterized for molecular weight and polydispersity by gel permeation chromatography (GPC). Offline dn/dc was measured using standard dilutions of the polymer, and dn/dc was then used to calculate Mn of the polymer. For GPC, 10 mg of polymer were dissolved in 1 ml DMF, syringe filtered using a 0.45 um filter, and injected in mobile phase Dimethylformamide (DMF). After passing through 3 columns in a series, the data gathered from the refractive index and multiple angle light scattering detectors was utilized to calculate polymer absolute molecular weight.

3.2 Microsphere fabrication

PPS microspheres encapsulating curcumin were prepared using a modification of the oil-in-water (O/W) emulsion solvent evaporation method[31]. In brief, 20 mg curcumin and 20 mg PPS were dissolved in a 10:1 mixture of chloroform (1.5 mL) and methanol (0.15 mL) and sonicated using an ultrasonic cleaner (Cole-Pamer, USA) until both polymer and curcumin were completely dissolved to form the oil (O) phase. The 10% methanol was

necessary to aid in dissolution of curcumin in chloroform. The prepared O phase was then emulsified in 2% (w/v) aqueous PVA solution (6 mL) by homogenization using Ultra-Turrax TP 18-10 (Janke and Kunkel KG, IKA-WERK) at 20,000 rpm for 1 min. For solvent removal, the emulsion was then subjected to high vacuum (~635 mm Hg) at 4^0C using a rotary evaporator (Rotavapor RII, BUCHI, Switzerland) for rapid (< 1 hour) and complete removal of the organic solvents. Microspheres were recovered by centrifuging (Allegra X-12 Centrifuge, Beckman Coulter, USA) at 3000 rpm for 5 minutes. The microspheres were then washed twice with 10% (w/v) Tween 20 in deionized water (50 mL) to remove non-encapsulated curcumin crystals. Finally, the microspheres were washed twice with deionized water (50mL) to remove any residual solvents or surfactant and lyophilized (Labconco Freezone 4.5, USA) for 24 hours.

Plain unloaded PPS microspheres were made as a negative control using the same method described above with the exception of not adding any curcumin during the fabrication.

3.3 Microsphere characterization

PPS microspheres encapsulating curcumin were characterized for size and morphology by scanning electron microscopy (SEM). The microspheres were suspended in a water drop and placed on a double sided sticky carbon tape attached to an aluminium stub, air dried, and then sputter coated with gold for 30 seconds. Curcumin encapsulation was confirmed by optical and fluorescent microscopy using a Nikon microscope. A water drop with microspheres suspended in it was placed on a glass slide and imaged after covering with a glass cover slip. To see the morphology of the microspheres after

exposure to hydrogen peroxide, the PPS microspheres were imaged before and after being exposed to different amounts of hydrogen peroxide for 31 days.

Drug loading in the microspheres was determined by fully dissolving microspheres in DMSO (1 mg/ml) overnight, then centrifuging at 16500 rpm for 3 minutes and quantifying the curcumin concentration in the supernatant using fluorescence of curcumin measured at 488 nm excitation wavelength in a plate reader (Tecan, Infinite F 500, USA). Drug loading in microspheres (%, w/w, $n = 3$) was defined as the amount of curcumin in 100 mg of microspheres. Drug encapsulation efficiency was defined as the percent of curcumin added during microsphere fabrication that was encapsulated into microspheres[32].

Size of the microspheres was quantified using Image J 1.45s software (Freeware, NIH, Bethseda, MD) by measuring >100 microspheres.

3.4 Release study

In vitro release profile of curcumin released from PPS microspheres was obtained by exposing the PPS microspheres to 0, 0.5, and 500 mM H_2O_2 for 31 days and quantifying the amount of released curcumin by measuring fluorescence at 485 nm excitation wavelength using a plate reader (Tecan, Infinite F 500, USA). PBS (1X, pH 7.4) containing 0.1% w/v N-acetylcysteine and 0.01% w/v butylated hydroxytoulene was used as the release buffer (control buffer). The additives were used to improve stability of curcumin once it was released to ensure accurate fluorescent quantification of curcumin concentration in supernatents. A solution containing 3 wt % hydrogen peroxide (H_2O_2)

was then added in required quantities to the release buffer to form 0, 0.5, and 500 mM H_2O_2. 1 mg of PPS microspheres encapsulating curcumin were then suspended in 1 ml of release buffer and placed in the top of transwell inserts (pore size of 400 nm). The release buffer alone was put in into six-well plates and the microsphere-containing transwell inserts were placed onto these wells which form the lower release chamber. The six well-plates were sealed with parafilm and placed in water bath with a shaker at 37 ^{0}C, 30 rpm. At regular time intervals, the entire release buffer in the bottom chamber was removed and replaced with a fresh release buffer. The removed release buffer was then 50% diluted with 200-proof absolute alcohol to ensure complete dissolution of curcumin. These samples were then read on a fluorescence plate reader to quantify the curcumin content. Curcumin concentration in the releasates was quantified by generating standard curves of the fluorescence of curcumin determined across a range of serial dilutions in the same release buffer containing 50% ethanol. Similarly, a control experiment was performed to verify that there was no effect of hydrogen peroxide on quantification of free curcumin by measuring its fluorescence before and after treatment for 14 hours. The sample size for all the release experiments was n=2.

4. Results and Discussion

Poly(propylene sulfide) synthesized as described in section 3.1 was analyzed for its molecular weight (Mn) and polydispersity (PDI) using gel permeation chromatography. An experimental molecular weight of 17,720 Da was obtained and was close to the targeted molecular weight of 15,000 Da, and the polymer showed acceptable

polydispersity (1.36). The results from GPC confirmed the formation of a high molecular weight PPS, which is predicted to be more hydrophobic in nature and suitable for forming microspheres to encapsulate the hydrophobic small molecule curcumin. For microsphere fabrication, the O/W emulsion method was used because of its suitability for encapsulating hydrophobic drugs such as curcumin. The loading capacity of microspheres was found to be 10% and the encapsulation efficiency was 40%. The relatively low encapsulation efficiency of the curcumin is likely the result of the drug forming crystals that were not incorporated into the microspheres. This resulted in loss of the drug during the washing steps with the tween-20 solution that were performed to dissolve these free curcumin crystals. Luckily, curcumin is quite inexpensive and available in large quantities, so this was not a limiting factor in these studies. Fig. 2 (a) shows the PPS microspheres with curcumin crystals that were found before the polysorbate tween-20 wash and (b) PPS microspheres without curcumin crystals after the wash. Polysorbate tween-20 (10% v/v) was found to dissolve the curcumin crystals effectively to yield a pure microsphere product.

Plain PPS microspheres not encapsulating any curcumin were also made as a negative control for the release experiments. The unloaded, plain microspheres as seen in Fig. 3 were similar in size but slightly larger than the PPS microspheres encapsulating curcumin.

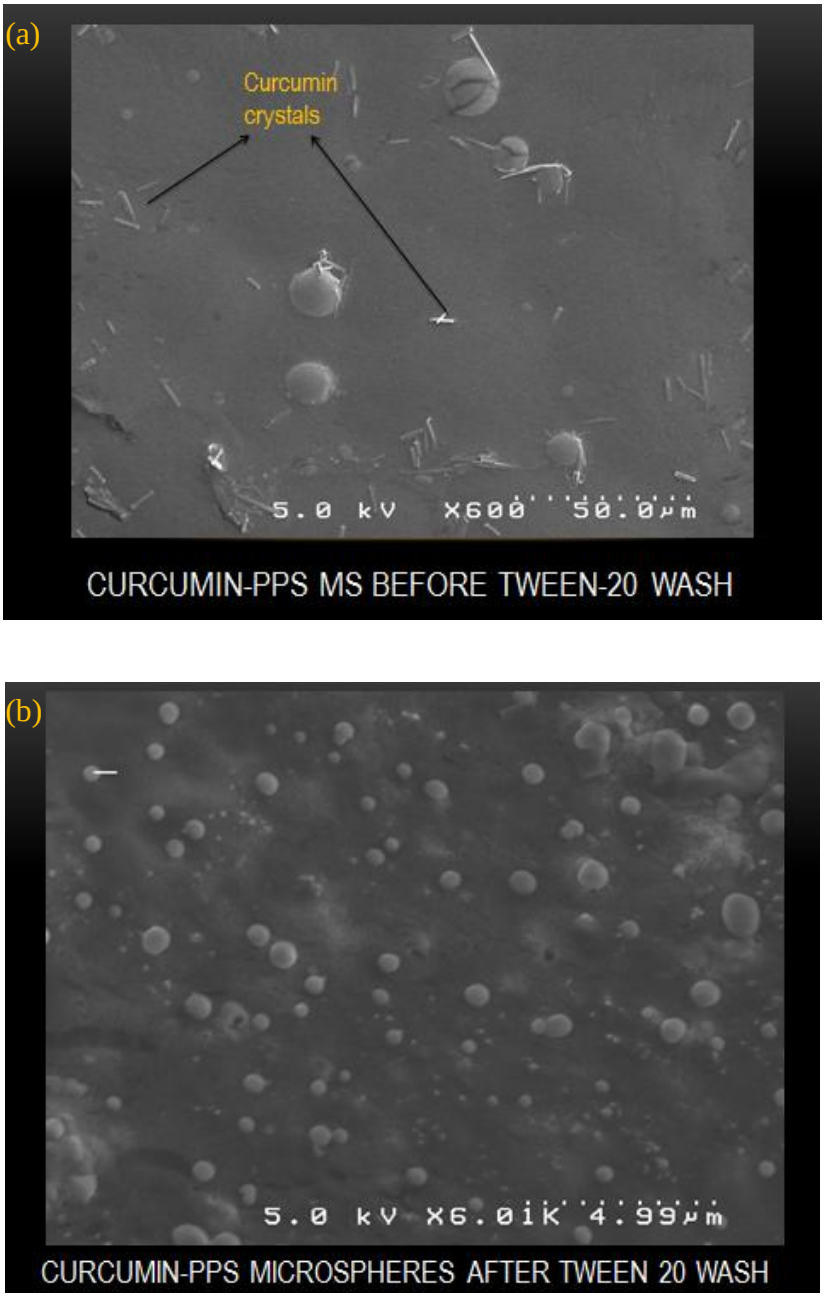


Fig. 2: Scanning electron microscopic (SEM) images of PPS microspheres encapsulating curcumin (a) before and (b) after the wash with tween-20 (10% v/v).

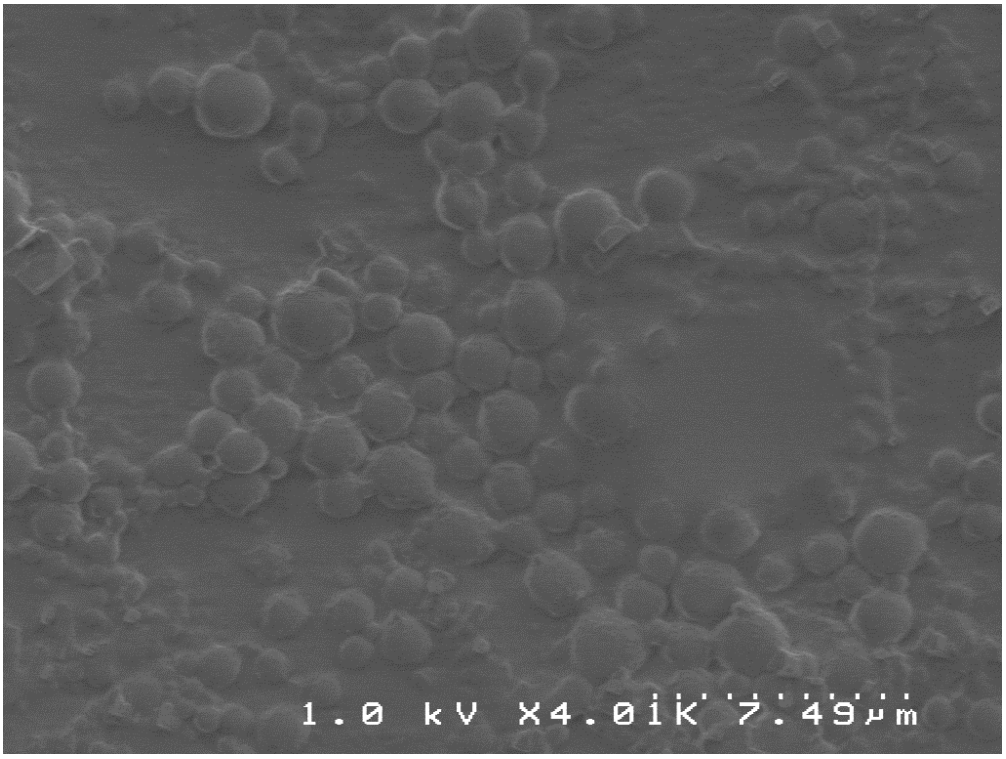


Fig. 3: Unloaded, plain PPS microspheres without encapsulation of curcumin.

The encapsulation of curcumin within the microspheres was confirmed using optical and fluorescent microscopy as shown in Fig. 4 a) and b). The microspheres showed aggregation initially, so were imaged again after sonication to see a clearer picture of individual fluorescent microspheres (Fig. 4 c).

Analysis of particle size done using Image J 1.45 s software showed that the average size of the PPS microspheres encapsulating curcumin was 0.28 $\pm$ 0.13 µm and that they were quite homogeneous in size (Fig. 5).

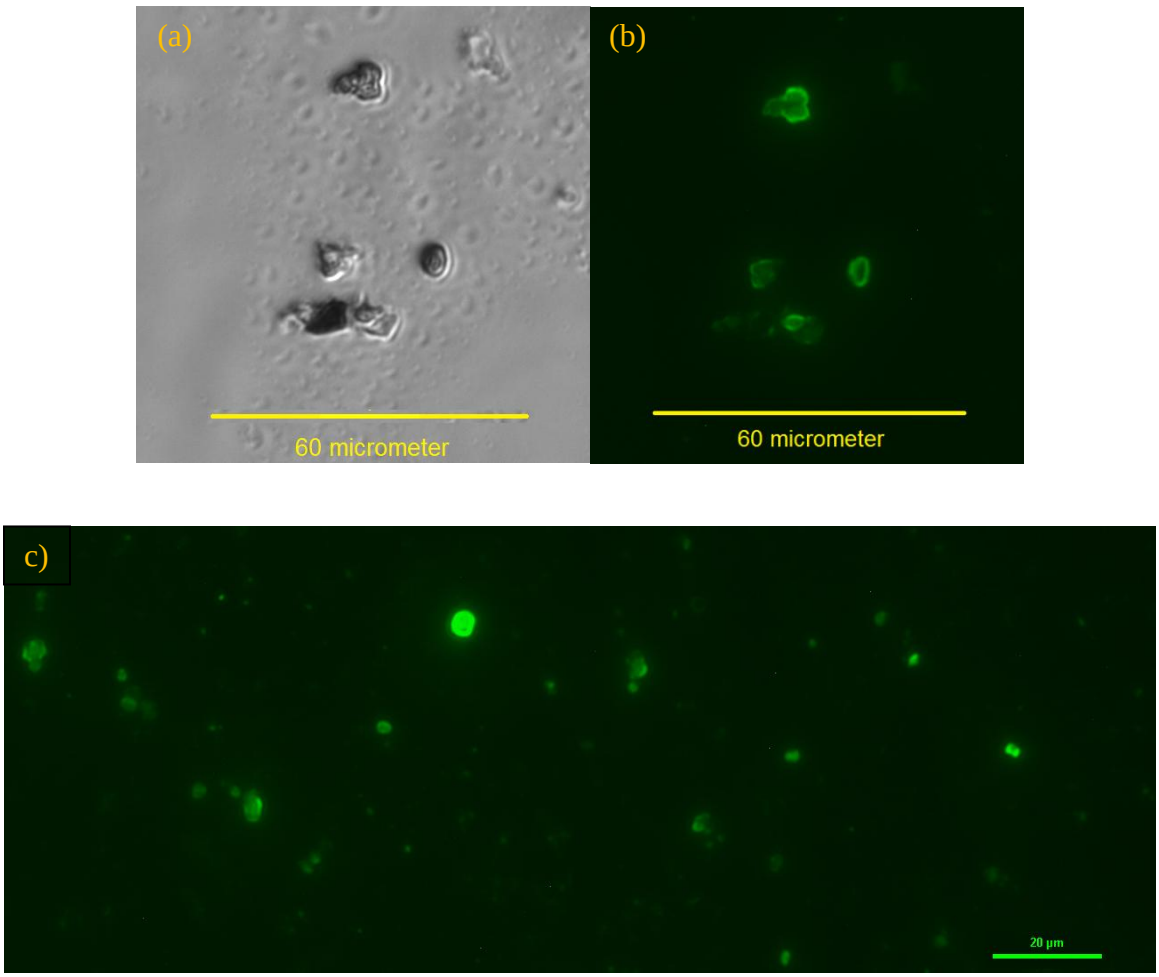


Fig. 4: a) Optical and b) fluorescent microscopic images of PPS microspheres confirming the encapsulation of curcumin. c) Fluorescent microscopy of PPS microspheres after sonication Scale bar in c) is 20 µm.

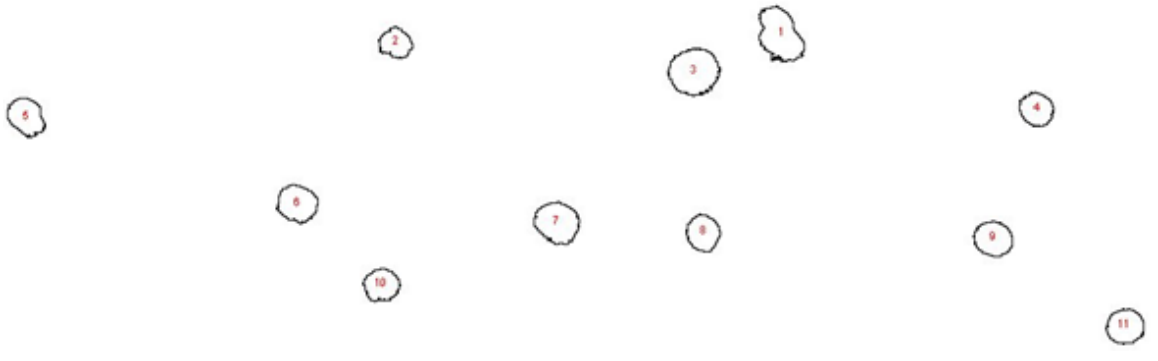


Fig. 5: Analysis of microsphere size by image J software. Sample size was n=104

To examine how the morphology of the microspheres would change in response to exposure to hydrogen peroxide, the microspheres were imaged after 31 days of exposure as shown in Fig. 6. At 0 mM hydrogen peroxide concentration, PPS microspheres seemed to be intact but aggregated, and the aggregation seemed to present in all the releasates imaged (Fig. 6 b, c and d) The deformation in the microsphere morphology at highest concentration of hydrogen peroxide (500 mM) is noticeable (Fig. 6-d). Similar results were confirmed by imaging large PPS microspheres individually as shown in Fig. 7.

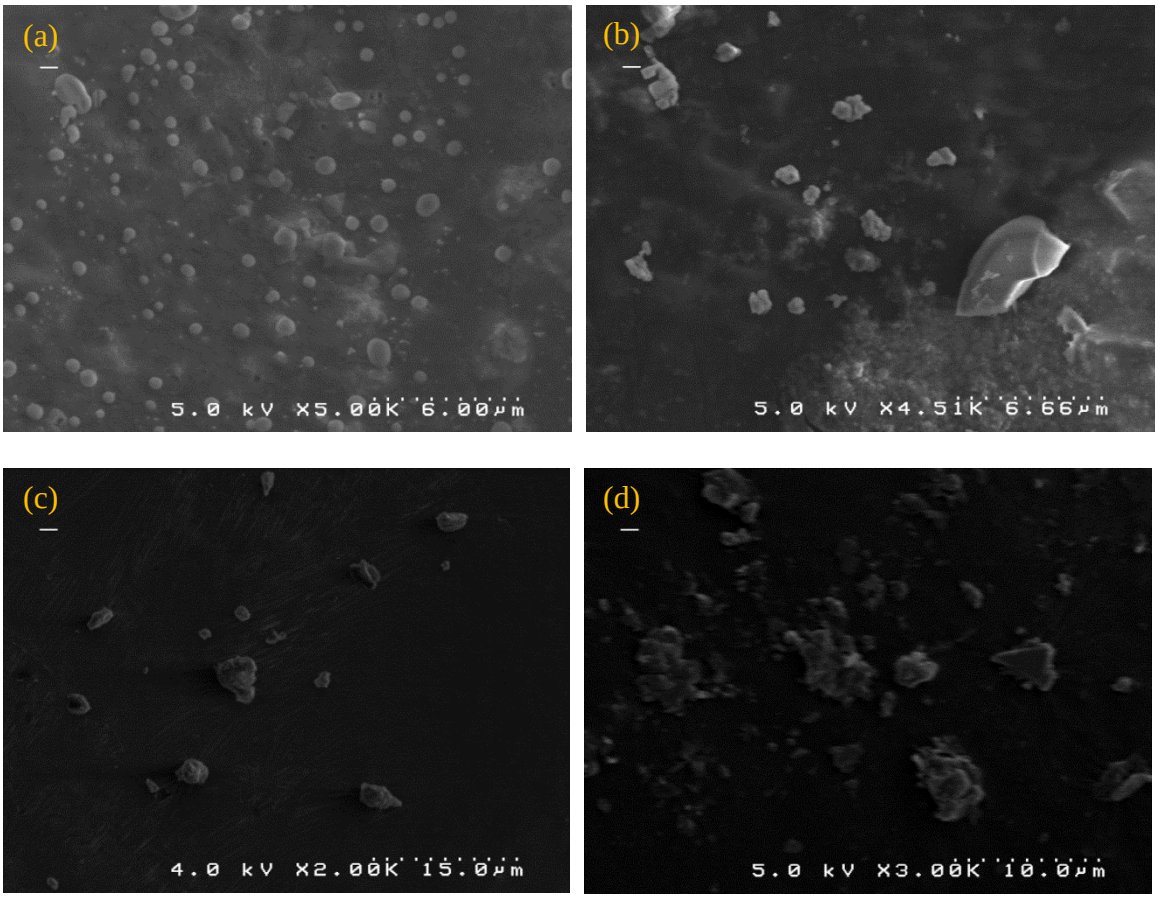


Fig. 6: SEM images of PPS microspheres encapsulating curcumin at different hydrogen peroxide concentrations: (a) 0 mM; before starting the release experiment (b) 0 mM; after 31 days of release experiment (c) 0.5 mM; after 31 days of experiment (d) 500 mM; after 31 days of experiment

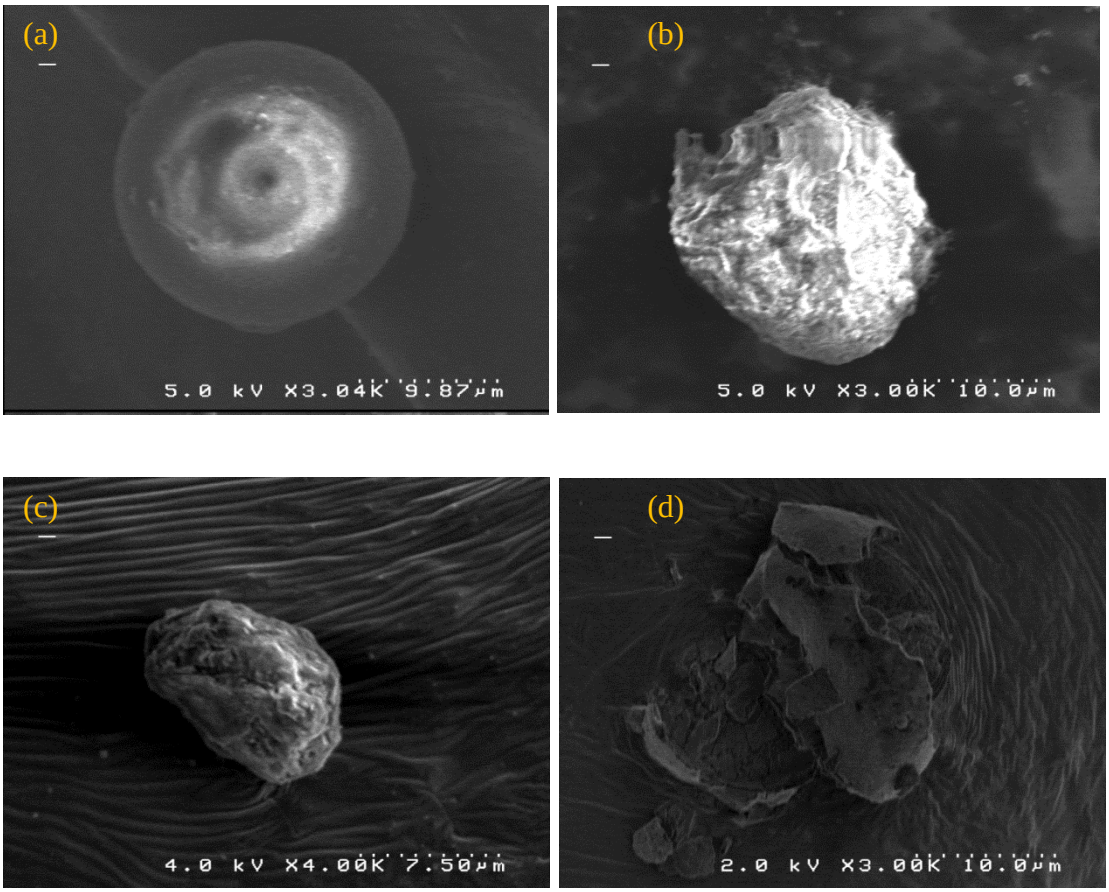


Fig. 7: SEM images of large, individual PPS microspheres encapsulating curcumin at different hydrogen peroxide concentrations: (a) 0 mM; before starting the release experiment (b) 0 mM; after 31 days of release experiment (c) 0.5 mM; after 31 days of experiment (d) 500 mM; after 31 days of experiment

Fig. 8 shows the hydrogen peroxide concentration-dependent in vitro release profile of the curcumin from the PPS microspheres. There was a small (<10%) initial release of curcumin from microspheres in the absence of an ROS trigger, which was followed by no significant release over the 20 days of the experiment. For hydrogen peroxide treatment groups, there was a clear concentration-dependent effect. The highest dose of hydrogen peroxide (0.5 M H_2O_2) resulted in the most rapid "burst release" of curcumin over the first 10 days of the experiment. However, at 0.5 mM H_2O_2, which is estimated to be a

more physiologically-relevant level of hydrogen peroxide[33-36], the release was sustained over the entirety of the experiment. In this group, there was a more rapid early release phase for the first 7 days that was followed by an extended stage that approximates zero order (constant/linear) release kinetics. It is presumed that in longer experiments, this treatment group would continue to release a constant rate until the cumulative curcumin release approached 100%.

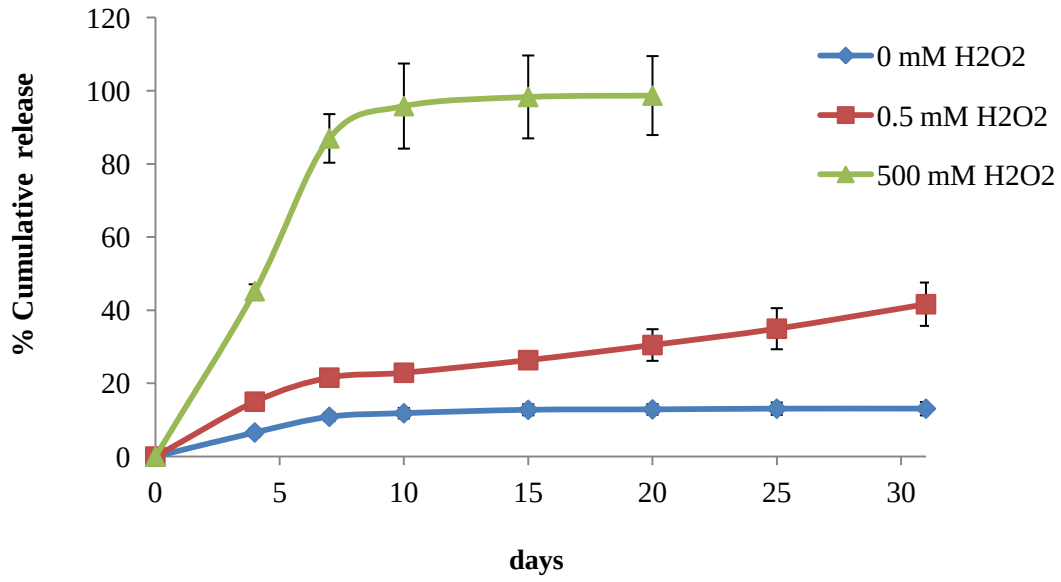


Fig.8: In vitro release profile of curcumin from PPS microspheres exposed to different concentrations of hydrogen peroxide. Positive control was 500 mM hydrogen peroxide, while negative control was 0 mM hydrogen peroxide. The experiment was done in duplicate (n=2).

5. Conclusion

Hydrogen peroxide and other reactive oxygen species are relevant in rheumatoid arthritis and a variety of other pathophysiologic conditions. In this study, microspheres loaded with the antioxidant and anti-inflammatory compound curcumin were successfully prepared from poly (propylene sulfide) (PPS) polymer. These microspheres showed a sustained, controlled release of curcumin that was hydrogen peroxide dose-dependent. These promising release studies motivate further investigation of this delivery technology for treatment of inflammatory diseases such as rheumatoid arthritis.

6. References

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