Université de Montréal

Microgels for oral delivery of therapeutic proteins

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Microgels for oral delivery of therapeutic proteins

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a été évalué(e) par un jury composé des personnes suivantes :

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Professeur Kishore Patel	membre du jury

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SOMMAIRE

Le sujet de cette étude est la libération contrôlée de substances actives de grandes masses moléculaires: protéines et peptides. L'inconvénient commun de ces médicaments est leur administration par injection. Des microgels ont été synthétisés en tant qu'excipients potentiels administrés par voie orale afin de protéger les protéines thérapeutiques contre les enzymes digestives, la dénaturation provoquée par le suc gastrique et pour permettre la libération prolongée de ces grosses molécules peu perméables directement aux muqueuses intestinales.

Ces microgels sont formés d'un réseau de copolymères de poly(acide acrylique) entrecroisés de diméthacrylate d'éthylèneglycol et greffées de copolymères séquencés - poly(oxyde d'éthylène)- poly(oxyde de propylène). Ils sont synthétisés par une copolymérisation entrecroisée radicalaire en une étape relativement simple.(**11**) La formulation est simplement constituée d'un mélange à sec de microgels avec la protéine lyophilisée qui est ensuite directement comprimé. Les microgels répondent au changement du pH de l'environnement: ils restent intact en conditions acides et se gonflent en pH neutre. Ils possèdent des propriétés mucoadhesives et sont non irritants.(**6, 8, 10**) Ces caractéristiques font de ces microgels de excipients potentiels pour la livraison orale des protéines thérapeutiques.

La première section de cette thèse est un examen des différentes formes de libération contrôlée. La section expérimentale décrit la synthèse de trois microgels et leur caractérisation par une variété de méthodes sous forme de particules sèches aussi bien

que dans un milieu aqueux. Dans cette partie, des microgels sont formulés avec de l'albumine lyophilisée de sérum de boeuf utilisée comme protéine modèle. La capacité de chargement de ces microgels a été évaluée. Des profils de libération de microgels en conditions imitant le milieu gastro-intestinale sont comparés entre eux et aux deux excipients généralement utilisés: lactose et Carbopol. Les résultats de l'étude de libération de protéines ont été interprétés par la méthode de Ritger et Peppas.

MOTS CLES: microgels, libération orale contrôlée, protéines thérapeutiques

SUMMARY

The subject of this study is controlled release of proteins and peptides. Common disadvantages of these medications are their administration by injection. Microgels were synthesized as potential excipients for oral delivery of therapeutic proteins for their protection from the digestive enzymes and the denaturation caused by the stomach acid and to provide prolonged release of these large and poorly permeable molecules directly to the intestinal mucosa.

Microgels are composed of copolymers of ethylene glycol dimethacrylate crosslinked network of poly(acrylic acid) with grafted block-copolymer chains of poly(ethylene oxide)-poly(propylene oxide), which are synthesized by a relatively straightforward one-step free-radical crosslinking copolymerization.(**11**) The microgels respond to environmental pH change: they collapse in acidic conditions and swell at neutral pH. They are proven to possess mucoadhesive properties and are non-irritating.(**6**, **8**, **10**) The drug product is formulated by simple blending of dry microgel with lyophilized protein and directly compressed.

The theoretic part of this thesis is a review of the different controlled-release forms, therapeutic proteins and their delivery systems. The experimental part describes the synthesis of three microgels and their characterization by a variety of methods alone as well as in formulations with a model protein, bovine serum albumin, in conditions mimicking the gastro-intestinal tract. The results of protein release study were interpreted by the method of Ritger and Peppas.

KEY WORDS: microgels, therapeutic proteins, controlled oral delivery,

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List of abbreviations:

Abbreviation	Name
AAM	Acrylic Acid monomers
API	Active pharmaceutical ingredient
BSA	Bovine Serum Albumin
Carbopol	Carbopol® 971 NF
DCM	Dichloromethane
DSC	Differential scanning calorimetry
EGDMA	Ethylene Glycol Dimethacrylate
FDA	US Food and Drug Administration
FTIR	Fourier transformed infrared
GI tract	Gastro-intestinal tract
HCl	Hydrochloric Acid
Microgel F127	Pluronic® F127-based microgel
Microgel L92	Pluronic® L92-based microgel
Microgel PPO	Poly(propylene glycol) 3500-based microgel
NaOH	Sodium Hydroxide
NMR	Nuclear magnetic resonance
PAA	Poly(acrylic acid)
PEO	Poly(ethylene oxide)
PPG 3500	Poly(propylene glycol) 3500
PPO	Poly(propylene oxide)
TFA	Trifluoroacetic Acid

THEORETIC PART

1. CONTROLLED DRUG RELEASE

1.1. Introduction

Controlled-release products are formulated to release drug's active ingredient gradually and predictably over a 12-hour to 24-hour period. These formulations compared with the immediate-release drugs might provide potentially greater effectiveness in the treatment of chronic conditions through a more consistent delivery of the medication: reduced side effects, greater convenience and higher levels of patient compliance due to a simplified dosage schedule.

The administration of a conventional or immediate-release dosage form provides rapid absorption initially with drug concentration in patient's plasma reaching peak maximum rapidly followed by a decrease in drug level due to progressive elimination of active pharmaceutical ingredient (urinary or other excretion, metabolism, degradation, *etc*).

With conventional fast release drug products, in order to maintain plasmatic drug concentration within therapeutic range in order to provide effective therapy it was recommended to multiply the drug administrations. This type of medication has demonstrated numerous disadvantages such as fluctuation "peak and valley" of plasmatic concentration of the drug, adverse side effects (if maximum plasma concentration reaches toxic level) or lost of therapeutic effect, (plasma concentration decreasing rapidly, falling lower then the minimum effective concentration if the active pharmaceutical ingredient has a short half-life)

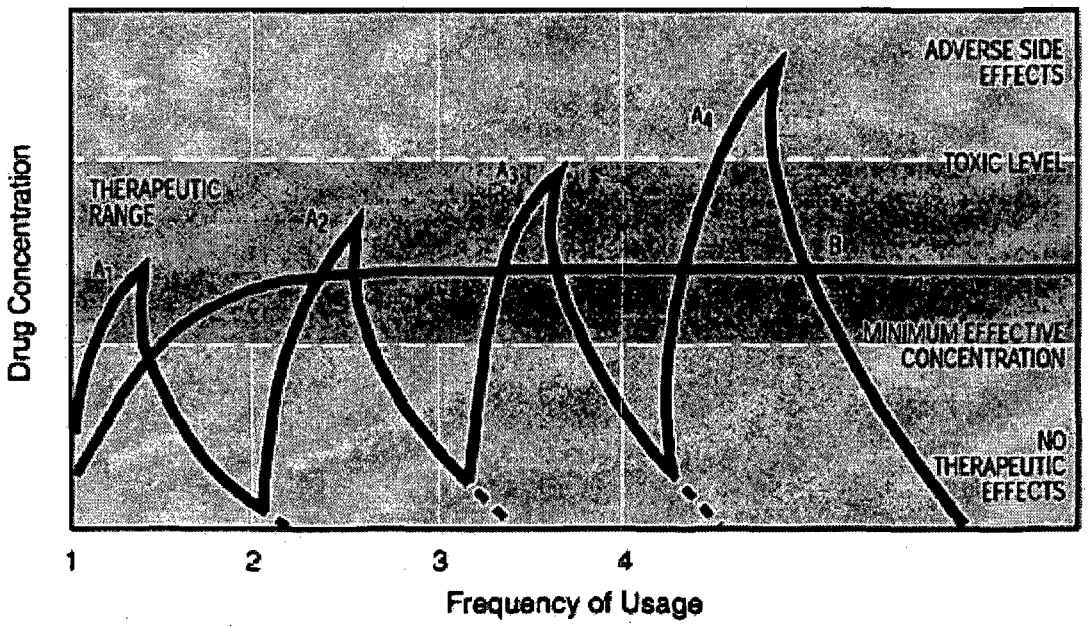


Figure 1: Blood drug concentration levels after administrating multiple doses of conventional dosage form (A_n) vs. a single dose of a long-acting controlled-release drug delivery system (B) (courtesy of Noveon)

Thus once-daily dosing carries significant benefits in terms of convenience and compliance. The potential also exists to improve the side-effect profile and enhance the overall efficacy of existing drugs.

It is important to control drug release in such a way that drug concentration in plasma is constant and the intervals between drug administrations are increased. The kinetics of dissolution of the active pharmaceutical ingredient might be modified by alteration of physical properties such as particle shape or type of polymorph. Drug release might be also controlled by adjustment of the dosage form to the requirements of the particular active pharmaceutical ingredient delivery.

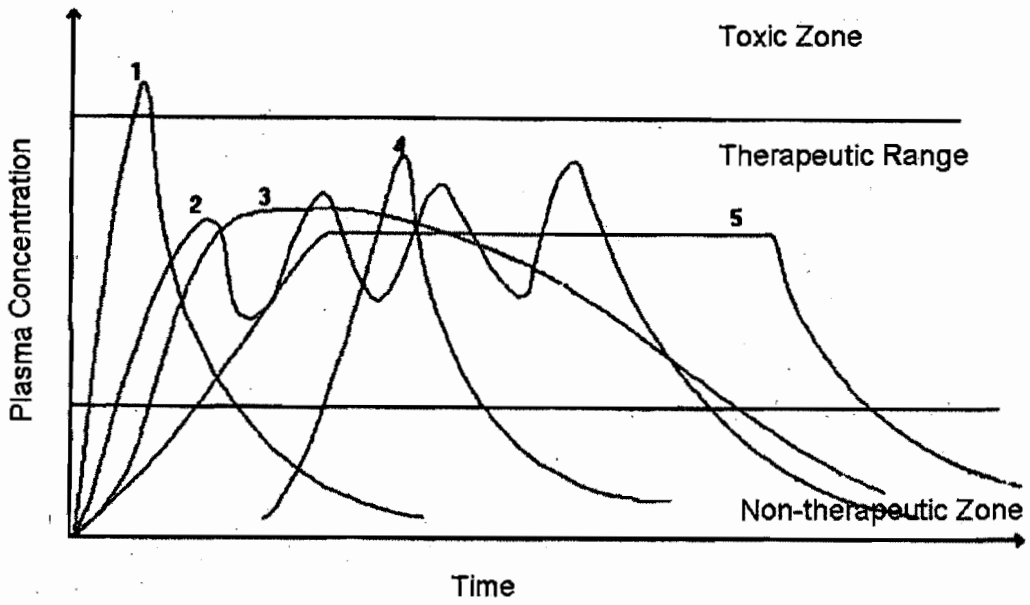


Figure 2: Blood drug concentration profiles of different controlled-release dosage forms

1 – Conventional form
2 – Pulsatile release form
3 – Extended release form
4 – Delayed release form
5 – Sustained release form

1.2. Controlled-Release Terminology

1.2.1. Controlled release

Controlled drug delivery systems usually have the same route of administration as a conventional form. However, it delivers the drug at a predetermined rate and/or to a specific location according to the need of the body and the disease states over a definite time period.(**38**)

1.2.2. Extended release

Extended-release systems include any dosage form that maintains effective therapeutic blood or tissue levels of the drug for a prolonged period. If the system can provide some actual therapeutic control of drug release in the body, whether it is temporal, spatial or both, it is considered a controlled delivery system. Extended release is not equivalent to controlled-release.(**23**)

1.2.3. Modified-release

This term is defined in the European Pharmacopoeia as a modification of the rate or place at which the active substance is released. Modified-release products cover a wide range of release models, the principal types of which would include "delayed release" and "prolonged release" products.(**3**)

1.2.4. Delayed release

Delayed-release systems are either repetitive, intermittent dosing of a drug form one or more immediate-release units incorporated into a single dosage form, or an enteric delayed-release system. Examples of delayed-release systems include repeat-action tablets and capsules, and enteric-coated tablets where timed release is achieved by a barrier coating.(**23**) The European Pharmacopoeia defines delayed release form as a modified release product, in which the release of active substance is delayed for a finite "lag time", after which release is unhindered. [*e.g.* enteric coated or "Gastro

resistant" (Ph.Eur.) oral tablets or capsules which remain intact in the stomach and only disintegrate in the higher pH of the small intestine]

1.2.5. Prolonged release

Prolonged-release form is a product in which the rate of release of active substance from the formulation has been reduced, in order to maintain therapeutic activity over a prolonged period of time, to reduce toxic effects or for some other therapeutic purpose.(**52, 18, 21**)

1.2.6. Site-specific release

Site-specific release refers to targeting of a drug to a certain biological location. The drug is adjacent to or in the diseased organ or tissue. This system satisfies the spatial aspect of drug delivery requirement and is also considered controlled drug delivery system.(**23**)

2. FUNDAMENTAL ASPECTS OF CONTROLLED RELEASE

2.1. Diffusion

Molecular diffusion phenomenon could be defined as a mass transfer process caused by Brownian motion of solute molecules and can be measured by the means of concentration gradient.(**4**) The process of diffusion, therefore, minimizes thermodynamic Gibbs free energy (though it is not a chemical reaction), and is a *spontaneous* process or "passive" form of transport.

Steady-state bi-molecular diffusion governed by Fick's law might be described as following:

$$J = (dM_t)/(S.dt) = -D.dc/dx \qquad \text{(eq. 1)}$$

In this equation

J	is the flux of drug in the direction of decreasing concentration (amount/area-time; mol/cm^2.sec)
M_t	is the mass of the drug released at the time t (mol)
S	is the surface of diffusion (cm^2)
D	is the diffusion coefficient of the drug (cm^2/sec)
dc/dx	is the concentration gradient (mol/cm^4)

The negative sign of the drug flux simply demonstrates that the vector of J is opposite to the vector of the concentration gradient.

This first law of diffusion is used to derive the equations applicable to diffusion-controlled reservoir drug systems, which are characterized by the constant concentration gradient (steady state).

In matrix systems the concentration gradient on contrary varies with time. In this model it is assumed that the solid drug dissolves from the surface layer of the dosage form first; when this layer becomes exhausted of drug, the next layer begins to be depleted by dissolution and diffusion through the matrix to the bulk solution. The

interface between the region containing dissolved drug and that with dispersed drug moves into the interior as a front.

Fick's second law of diffusion is applied to non-steady or continually changing diffusion state:

$$d\varphi/dt = D.d^2\varphi/dx^2 \qquad \text{(eq.2)}$$

Where

Φ	is the concentration (mol/cm^{-3})
t	is the time (sec)
D	is the diffusion coefficient (cm^2 s^{-1})
x	is the position [cm]

It is important to stipulate that both Fick's laws in the forms of equation 1 and 2 respectively are not applicable if diffusion coefficient D is not constant at unvarying temperature.

2.2. Solute diffusion mechanism

Considering that distribution of an active pharmaceutical ingredient within the polymer matrix is uniform, the release mechanism can be explained as follows: water or another biological fluid, while in contact with a polymer, penetrates into the material progressively dissolving the active pharmaceutical ingredient, which diffuses toward the exterior via a porous network or through intermolecular space. The type of the

transfer largely depends on a polymer state (glassy or rubbery).(**61**) According to the temperature, the polymers are in glassy state if $T < T_g$ or are in rubbery state if $T > T_g$, where T_g is the temperature of glass transition and T is the temperature of the polymer.

The macromolecular chains of the polymer, when in rubbery state, move constantly creating large spaces or "pockets", which are permeable for solvent molecules. (Fig. 3)

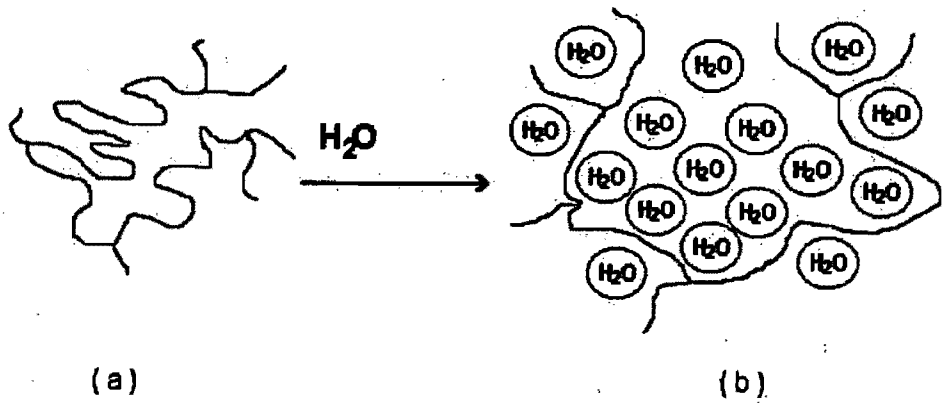


Figure 3: Swelling of a hydrophilic polymer

1 – polymer in glassy state 2 – polymer in rubbery state

2.3. Three types of the transport

2.3.1. Fickian type transport or "case I"

This type of the transport is applicable to rubbery state polymers, where macromolecular chains are very flexible and adjust rapidly to the presence of solvent molecules(**61**) when the diffusion velocity of solvent molecules is significantly lower than the relaxation of macromolecular chains. This is a Fickian type also known as "case I" diffusion.

The quantity of diffused substance M_t at the time t might be expressed as following:

$$M_t=k.\sqrt{t} \qquad \text{(eq.3)}$$

The diffusion velocity, derived from the previous equation is:

$$dM/dt=1/2.k.t^{0.5} \qquad \text{(eq.4)}$$

Where

k is a constant, which depends on form of the polymer and the diffusion coefficient.

t is the time (sec)

2.3.2. "Case II" type diffusion

This type of transport is characteristic to polymers in glassy state where the relaxation of macromolecular chains is significantly lower then the velocity of solvent molecules diffusion.(61)

The quantity of adsorbed or desorbed solvent is expressed as follows:

$$M_t=k.t \qquad \text{(eq.5)}$$

And the velocity of diffusion is respectively:

$$dM/dt=k \qquad \text{(eq.6)}$$

2.3.3. "Case III" type diffusion

Non-Fickian or "anomalous" diffusion is observed when the velocity of solvent molecules diffusion and the relaxation of the macromolecular chains are of the same order. This type of the transport is in between the "case I" and the "case II". The quantity of adsorbed or desorbed solvent is given as:

$$M_t = k.t^n \qquad \textbf{(eq.7)}$$

And the velocity of diffusion is respectively:

$$dM/dt = n.k.t^{n-1} \qquad \textbf{(eq.8)}$$

It has to be noted that the diffusion follows the Fick's law (case I) if n=0.5; if the value of the parameter n is in between 0.5 and 1, then the process is anomalous (case III). However, when this parameter is close to one, the diffusion of the solute within the polymers approaches the zero order kinetics (case II). Table 1 demonstrates the three cases.(**48**)

Graphic presentation of the theory of solute diffusion (release) from the polymers for three types of diffusion is shown in the Figure 4.(**33**)

Parameter "n"	Diffusion type	Diffusion velocity (dM/dt)
0.5	Fickian diffusion (case I)	$t^{1/2}$
$0.5 < n < 1$	Anomalous diffusion (case III)	t^{n-1}
1	"Case II" type diffusion	Constant
$n > 1$	"Super-case II" type diffusion	t^{n-1}

Table 1: Diffusion type and the kinetic parameter "n"

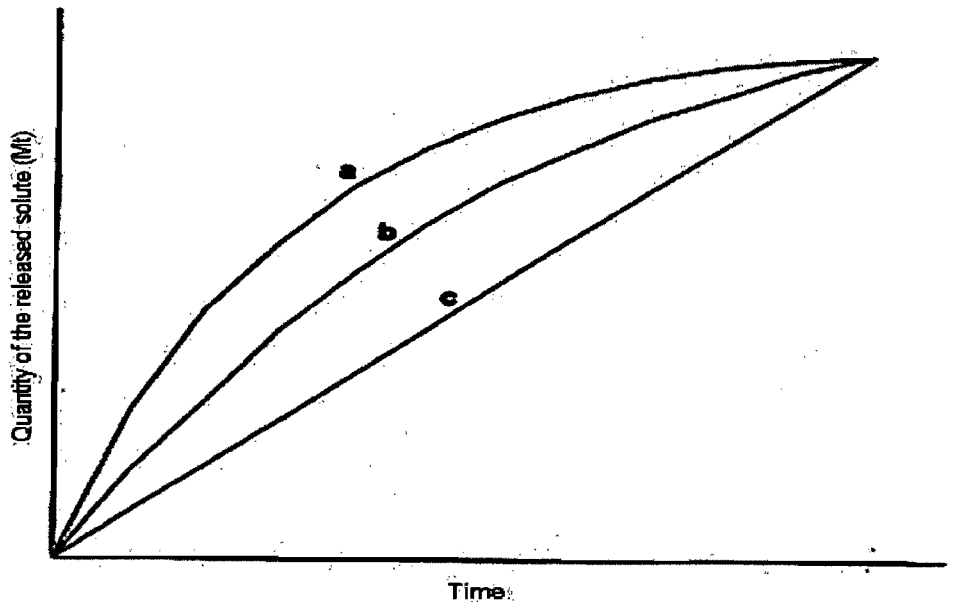


Figure 4: Demonstration of Fickian (a), anomalous (b) and "case II" (c) types of diffusion.

3. CONTROLLED-RELEASE SYSTEMS CLASSIFICATION

Systems of the release might be sorted in four major classes according to the nature of the drug transport.(**34**)

3.1. Diffusion-controlled systems

These systems are more frequently studied and utilized. There are two diffusion modes: the active pharmaceutical ingredient might diffuse through the polymer structure (within the intermolecular space) as well as from a porous network filled with the solvent. There are two major types of formulation: reservoir and matrix.

3.1.1. Reservoir systems

In reservoir systems, the active pharmaceutical ingredient is coated with swellable or non-swellable polymeric film. The drug might be in solid form, in solution or concentrated suspension as well as mixed with another solid excipient. The polymeric membrane controls the drug release. In general, these systems include coated tablets and granules, macro and microcapsules, liposomes and hollow fibers. Fick's equation characterizes the diffusion of the drug:

$$dQ/dt = D.S.A.(C_1-C_2)/X \qquad \textbf{(eq.9)}$$

Where

dQ/dt is amount of active pharmaceutical ingredient (g) diffused per unit of time (sec)

D is a diffusion coefficient (cm^2/sec)

S is partition coefficient of the substance between the coated dosage form and the aqueous phase of the gastro-intestinal tract

A is a membrane surface (cm^2)

C_1 is a concentration of solution saturated with active pharmaceutical ingredient (g/cm^3)

C_2 is concentration of API on GI tract side (g/cm^3)

X is a membrane thickness (cm)

In this case, the released drug is characterized by "zero order" kinetics as long as API within the film coated compartment is highly saturated since the diffusion coefficient, the partition coefficient, the membrane thickness and the surface area are constant.(**32**) The rate of release may be modified by changing the thickness of membrane, the surface area or the diffusion coefficient of API. For example, permeability of a membrane might be modified by adding more hydrophobic or hydrophilic polymers, like poly-ethylene oxide and poly-propylene oxide block copolymers or Pluronics®.

A major advantage of these systems is the possibility to obtain "zero order" release, which is problematic with other dosage forms.

There are a few disadvantages to the reservoir systems such as: accidental damage of a dosage form coating might cause dumping of the API contained in the reservoir.(**19**) "Zero order" is obtained only for the period of time when API inside the

coating is at high saturation, this varies with solubility. The manufacturing of reservoir dosage forms is still relatively expensive due to the technology requirements.

3.1.2. Matrix systems

In the matrix systems the drug is uniformly distributed within the solid insoluble polymer excipient. The API embedded in this excipient network provides extended release.(**32**) The matrix may also be constituted of hydrophilic substances, which, when is in contact with water, form a gel.

The solvent penetrates into the system and progressively dissolves the API, which then diffused to the exterior through the porous network or intermolecular space.

A matrix may be formed by the evaporation of the solvent from a polymer solution with dispersed or solubilized API. The polymer should be hydrated, dissolved or disintegrated before the drug is dissolved and diffused from the polymeric matrix. These systems are characterized by slow release. The API is not chemically bound to the polymer. The drug is active and is not modified.

Based on the nature of the polymer, matrix system may be classified as hydrophilic, inert or lipid.

3.1.2.1 Hydrophilic matrixes

These matrixes include a cellulose-derived polymers (hydroxyl-propyl methylcellulose and carboxymethylcellulose sodium salt) and vegetable-derived polysaccharides (alginates, agar-agar and modified amidons) or polymers of animal

origin (gelatin and chitosane) as well as synthetic materials (polyvinyl acetate, polyethylene glycol and polyacrylic acid).

Polysaccharide hydrophilic polymers are often used in oral extended-release formulations. Compared to inert and lipoid matrixes they are simple to formulate, might be loaded with API up to 80% w/w, are relatively inexpensive and versatile as they can be formulated with varied types of API.(**40**)

At the releasing contact area of a hydrophilic matrix, some amount of the drug is released instantly (burst effect), then hydratation of the gel-forming agent leads to the fast formation of progressively increasing gelated barrier on the surface of the dosage form. The API diffuses through this gelated barrier in to the aqueous surrounding. The release velocity progressively decreases as a function of the square root of the time.(**36**) For example, drug release from a flat disk-shaped matrix might be estimated by this form of the Hugichi equation (**29**):

$$M = \sqrt{[C_s.(2.A-C_s).D.t]} \qquad \textbf{(eq.10)}$$

Where

M amount of drug released from the unit of surface during the time t (g/cm^2)

C_s solubility of the drug in the matrix (g/cm^3)

A concentration of the drug (g/cm^3)

t time(sec)

D diffusion coefficient (cm^2/sec)

3.1.2.2 Inert matrixes

Polymers used for inert matrixes are of a porous structure, non-toxic and chemically neutral to the API (polyvinyl chloride, polyethylene, ethylcellulose, silicone etc.).

In these dosage forms formulated drug dissolves and diffuses through the pores. The release is thus controlled by the porous structure. The rate of release may be modified by the alteration of a network pores' diameter,(**53**) shape and the dimensions of the dosage form.(**3**)

The amount of released API is proportional to the square root of time. This matrix system release might be characterized by the solubility of the API, diffusion coefficient, porosity and tortuosity of the dosage form.

Higuchi proposed a following mathematical model for a plane surface (**29**):

$$M_t = S.\sqrt{[D.\acute{\varepsilon}.C_s.(2C_0-\acute{\varepsilon}.C_s).t/\tau]} \quad \textbf{(eq. 11)}$$

Where

M_t amount of API released at the time t (g)

S surface of a disc (cm^2)

D API diffusion coefficient (cm^2/sec)

$\acute{\varepsilon}$ matrix porosity (sec/cm)

C_s API solubility in water (g/cm^3)

τ matrix tortuosity (cm)

C_0 concentration of API at the time zero (g/cm^3)

Inert matrixes are relatively simple to formulate and manufacture by dry blending and direct compression. The release of API from these matrix systems is independent of the exterior conditions.

3.1.2.3 Lipid matrixes

A variety of biodegradable excipients is used to formulate these matrixes: natural and synthetic waxes, fatty acids and their esters, fatty alcohols, hydrogenised oils, etc. The API may be released through diffusion and through erosion of the matrix by enzymatic hydrolysis of the lipids.(**13**)

Drug release from lipid matrixes depends on digestive enzyme composition of the stomach fluids.(**20**) The amount of released drug substance is proportional to the quantity of the excipient hydrolyzed by the enzymes.(**51**)

3.2. Solvent-controlled systems

3.2.1. Osmotically controlled systems

The majority of these systems were developed by ALZA Corporation. In the relatively simple dosage form, OROS®,(**59**) an osmotically active API in a core (drug substance blended with sodium or potassium chloride as osmosis agent) is surrounded by semi-permeable membrane which is permeable only to water. The drug substance is pumped out of the system through laser-drilled orifice at the same rate as the volume of water entering into core multiplied by drug concentration. The "zero order" drug release is controlled by the osmotic properties of the dosage form, surface, thickness and permeability of a membrane.

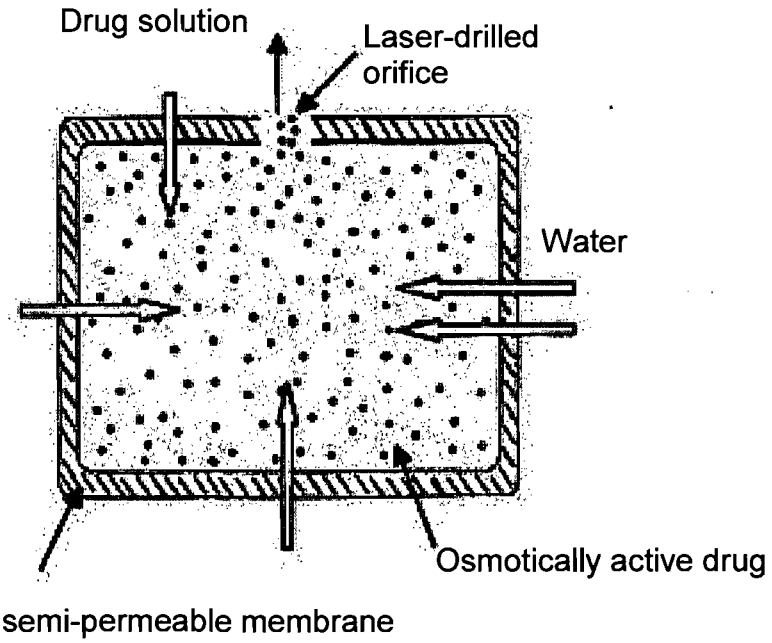


Figure 5: Simple osmosis-controlled oral system (OROS®) (courtesy of ALZA Corporation)

3.2.2. Swelling controlled systems

Swelling controlled systems are composed of a slowly hydrating polymer loaded with a drug substance that allows controlled permeation of the solvent.(**46**) Limited swelling depends in general on the nature of a polymer and in particular on the degree of cross-linking. At the initial phase, the polymer is in glassy form when API diffusion is nearly zero due to very low diffusion coefficient. Solvent permeation initiates phase transition to the rubbery state at the swelling interface. The drug substance diffuses through the rubbery portion toward the aqueous exterior.(**35**) Drug release is controlled by the progressive move of the permeation front.

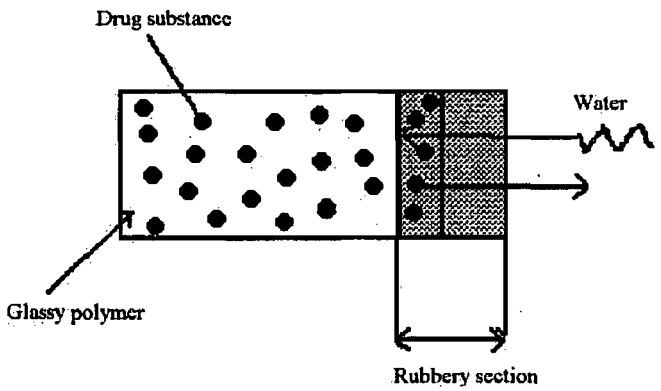


Figure 6: Simplified release mechanism of a limited swelling matrix system

Developed by ALZA Corporation, the OROS® Push-Pull™ system is controlled by both: osmosis and swelling. A push compartment containing swellable polymer material is placed inside semi-permeable membrane provides delivery of a low solubility drug substance in a form of fine suspension.(**43**)

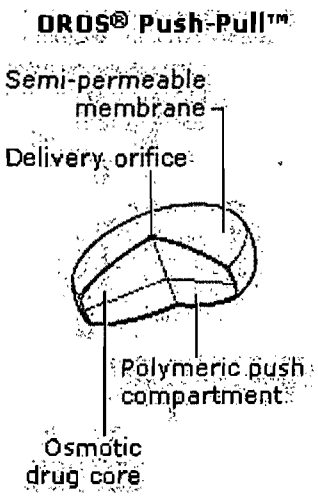


Figure 7: Schematic representation of the OROS® Push-Pull™ system. (Courtesy of ALZA Corporation)

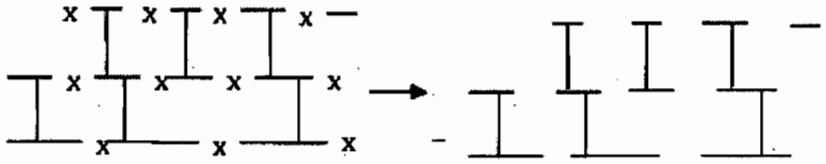
3.3. Chemically controlled systems

3.3.1. Biodegradable systems

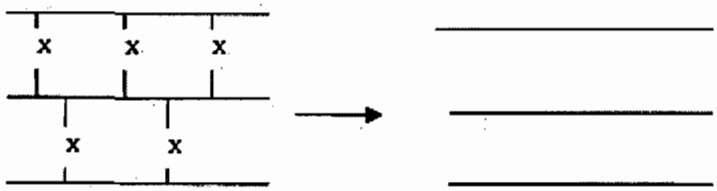
Matrix technology is more often applied to biodegradable systems rather than to reservoir. However, the latter exists mostly as skin patches and microcapsules. In the case of matrix technology the system erodes gradually releasing the API. In reservoir systems the polymer coating may degrade after complete drug substance release.

Heller categorized biodegradable systems in three major groups based on their bio-erosion mechanism (**1, 27**):

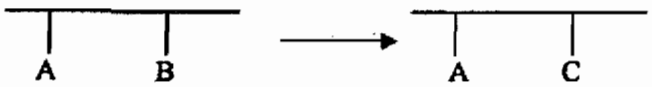
a. Insoluble polymers with biodegradable main chain, which breaks into smaller soluble molecules when hydrolyzed



b. Hydrolysis of cross-linking bonds



c. Insoluble polymer, which is dissolved after their side chains are hydrolyzed, ionized or protonated



B→C = hydrolysis, ionization or protonation

For the majority of the biodegradable systems release, kinetics is the result of a combination of both – diffusion of drug and erosion of the polymer.(**56**)

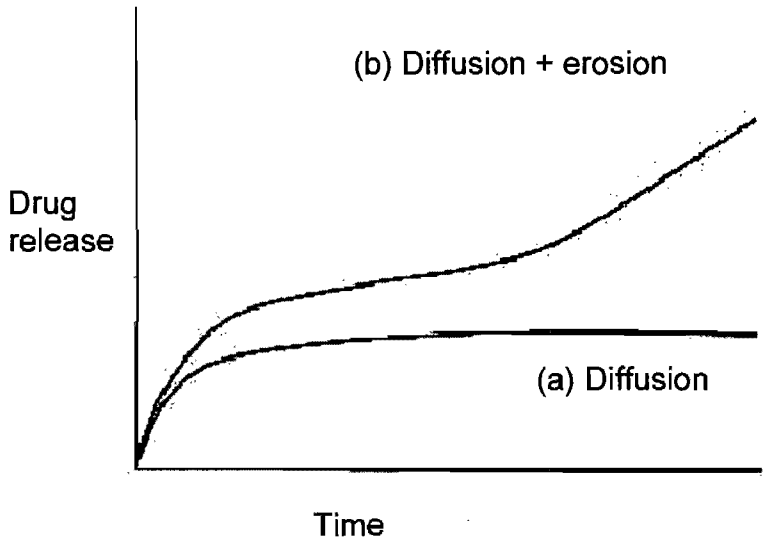


Figure 8: Theoretic drug release profile for diffusion-controlled (a) and controlled by diffusion/erosion (b) systems

3.3.2. Grafted chains systems

In these systems pharmaceutically active substance that chemically bound to a polymer structure is released by hydrolysis or enzymatic cleavage. The polymer backbone itself might be biodegradable. Drug substance might be attached directly to the polymer backbone or through a linking agent, which would affect the hydrophilic properties of the system and eventually drug release rate.(47)

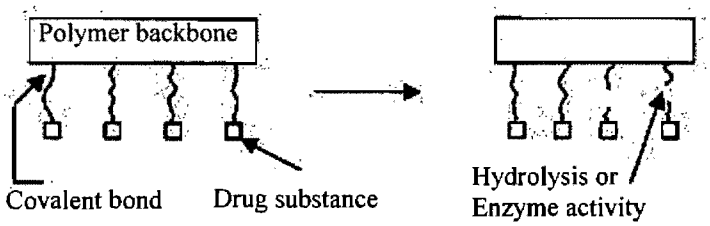


Figure 9: Schematic representation of grafted chains system

3.4. Systems activated by magnetic fields

These systems are characterized by having a drug substance and small magnetic particles uniformly distributed in the polymer matrix. The dosage form, when placed into aqueous milieu, releases active pharmaceutical ingredient in accordance with classic mechanism of diffusion described in respective section. However, drug substance is released at significantly higher rate when exterior oscillating magnetic fields are applied.

This phenomenon might be explained by increase of the matrix pores due to attraction and repulsion between magnetic particles. The latter allows the drug substance to be released faster.

3.5. Bioadhesive delivery systems

Bioadhesion is a complex phenomenon related to the ability of some natural and synthetic macromolecules to adhere to biological tissues. If a biological substrate is a mucus membrane, bioadhesive interactions occur primarily with the mucus layer and this process is referred to as mucoadhesion. The bonds involved are more likely to be of secondary chemical nature, combined with physical entanglement of polymer chains. The process is a reversible one, where the mucoadhesive detachment is caused either by the breakage of low energy bonds or by the physiological process of mucus turnover.(**58**)

Mucoadhesive controlled release dosage formulations have gained considerable attention due to their ability to adhere to the mucus layer and release the loaded drug in

a sustained manner. By using these dosage forms, contact time with the mucus surface increases, which results in an increased drug retention at the site leading to improved therapeutic efficacy, especially for large molecules like proteins. Diverse classes of polymers have been investigated for potential use as mucoadhesives. These include well known mucoadhesive polymers such as poly(acrylic acid), hydroxypropyl methylcellulose and poly(methylacrylate) derivatives, as well as naturally occurring polymers such as hyaluronic acid and chitosane.(60)

4. THERAPEUTIC PROTEINS AND THEIR DELIVERY

Therapeutic proteins are promising alternative to drug small molecules. Protein based drugs have demonstrated extraordinary selectivity and lower risk of undesired side effects with the administration of microgram amounts per daily dose. Unlike other medicines, they are not synthetically manufactured, and are usually produced through microbial fermentation or by mammalian cell culture. They are also more complicated, time-consuming and expensive to produce than synthetic drugs.(60)

4.1. Marketed protein based drug products

Currently therapeutic proteins are used to relieve patients suffering from various cancers (monoclonal antibodies, interferons), heart attacks, strokes, cystic fibrosis (enzymes, blood factors), diabetes (insulin), anemia (erythropoietin) and hemophilia (blood clotting factors). Leading protein based drug products on the market today are Procrit® and Epogen® for anemia treatment from Johnson & Johnson and

Amgen respectively, Intron A® and Peg-Intron® for hepatitis from Schering-Plough and few others.(**60**)

The delivery of these protein based drug products is currently performed through intravenous or subcutaneous injections, which is inconvenient for patients, increases treatment cost, and bears all the risks related to this mode of drug administration.

4.2. Alternative ways of protein and peptide administration

Variety of approaches has been developed as alternative delivery systems for therapeutic proteins and large peptides in order to avoid injections. Oral and nasal administration, pulmonary inhalation, implants and other pathways have been studied by different research groups and pharmaceutical companies for the delivery of large drug molecules.

Pulmonary inhalation, which does not require deep lung delivery was applied by Syntonix Pharmaceuticals to develop a novel formulation for Interferon-beta therapy for multiple sclerosis.(**60**)

Nasal formulation of leuprolide (ChiSys-Leuprolide™), a gonadotropin-releasing hormone agonist that is used in the treatment of hormone-responsive cancers and control of ovarian stimulation in *in vitro* fertilization is undergoing phase II clinical trial sponsored by West Pharmaceutical Services, Inc.(**60**)

A number of the research groups work currently on implant delivery systems for prolonged release of therapeutic proteins and peptides. Kajihara et al. has developed subcutaneous silicone implant covered with human serum albumin or interferon in order to achieve a zero-order release of protein drug for 30-100 days without significant initial burst.(**30**) In another implant system, bovine serum albumin was loaded into poly(D,L-lactic-co-glycolic acid) microparticles coated with poly(vinyl alcohol) and incorporated into PLGA tissue-engineered scaffolds. These tissue-engineered implants have provided long-term controlled release of the protein.(**28**)

The main barriers to oral administration of therapeutic proteins are the delicate physiochemical properties of protein-based drugs, which are sensitive to digestive enzymes and may be denatured by stomach acid before absorption. Another issue is the size of therapeutic proteins.(**41**) Being large and bulky molecules and characterized by poor permeability through intestinal mucosa, they require long time to be adsorbed with direct contact to the mucosa in order to traverse the intestinal tissue.

Orally administered enzyme products are being developed by Altus Pharmaceuticals. In these protein based drug products an active ingredient is cross-linked and crystallized in order to protect the substance from degradation in the stomach.(**60**)

Marschutz M.K. *et al.* prepared matrices of the mucoadhesive polymer sodium carboxymethylcellulose with the covalently bound Bowman-Birk enzyme inhibitors and elastatinal enzyme inhibitors for the protection of embedded insulin from degradation by the luminally secreted serine-proteases. These oral drug-carrier matrices

have provided up to 80% of insulin recovery after one hour of treatment in artificial intestinal fluid.(**39**)

Peppas N.A. and Foss A.C. *et al.* have developed nanospheres of crosslinked networks of methacrylic and acrylic acids grafted with poly(ethylene glycol) for use as oral insulin delivery devices.(**49**) It was determined using photon correlation spectroscopy that nanospheres increased ten times in diameters in response to pH change from 2.0 to 6.0. Loading of insulin up to 7% w/w into the copolymers was achieved by partitioning from concentrated insulin solutions. In vitro studies have confirmed that insulin was protected at pH lower then 3.0 and released at pH 5.5. Animal studies were performed to investigate the abilities of insulin-loaded copolymer samples to influence the serum glucose levels of rats. In studies with diabetic rats, the serum glucose level was lower than control values for the animals that received the insulin-loaded copolymers and lasted for at least 6 h. The insulin loaded copolymer nanospheres caused a significant reduction of serum glucose with respect to that of control animals.

Therefore oral administration of therapeutic proteins is considered to be an advantageous alternative to injections and may be applied to novel therapeutic proteins and peptides as well as to already marketed products.

5. MICROGELS, NOVEL EXCIPIENTS FOR PERORAL DELIVERY AND CONTROLLED RELEASE OF THERAPEUTIC PROTEINS

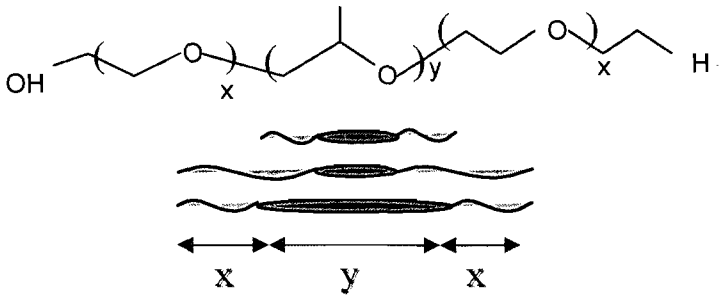
In the past thirty years, work on hydrogels based on crosslinked copolymers has led to the establishment of a number of controlled release products for pharmaceutical agents.(**24**, **25**, **62**) Poly(acrylic acid)-based resins have been a subject of numerous drug master files with the U.S. FDA.(**15**)

In this study microgels as copolymers of poly(acrylic acid) network crosslinked by ethylene glycol dimethacrylate with grafted block-copolymers chains (poly(ethylene oxide)-poly(propylene oxide) or poly(propylene glycol)) were synthesized in order to be studied as a potential system for oral delivery of therapeutic proteins.

5.1. Grafted block copolymers

Three block copolymers with different hydrophile-lipophile characteristics were selected to be cross-linked to microgels. Pluronic® is a trade name for group of poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO-PEO) block copolymers approved by the FDA as food additives and pharmaceutical ingredients. (**2**) Pluronic® L92 is hydrophobic with a molecular weight of 3650 Da, Pluronic®F127 is relatively hydrophilic and has a molecular weight about three times higher. The hydrophile-lipophile properties of Pluronics® depend on their composition (Fig.10): Pluronic® F127 has a PPO:PEO ratio of 30:70 when L92 is 80:20. The third copolymer of choice

was poly(propylene glycol), a hydrophobic material with a molecular weight of 3500 Da.(**54**)



PEO-PPO-PEO

MW 1kD – 13kD

x = 0 - 300

y = 15 - 80

Figure 10: Poly(ethylene oxide)-poly(propylene oxide) block copolymers or Pluronics®

5.2. Microgel synthesis model and annotated structure

The synthesis model has been studied extensively.(**7**, **9**, **11**) The synthesis sequence for microgels involved free-radical polymerization of acrylic acid with chain transfer to block copolymer resulting in C-C bonding between PAA and Pluronic® .

At 70°C, initiators form free radicals (R•) that trigger propagation of acrylic acid (AA) homo-polymer chains and abstraction of hydrogen (H) from the block copolymers (X_mH). Latter activation of block copolymers (Pluronics® and PPG 3500) leads to the chain transfer that is followed by grafting of PAA chains on block copolymers:

$$R\bullet + nAA \rightarrow R\text{-}AA_n\bullet$$

$$R\bullet + X_mH \rightarrow R\text{-}H + X_m\bullet$$

$$R\text{-}AA_n\bullet + X_mH \rightarrow R\text{-}AA_nH + X_m\bullet$$

$$X_m\bullet + pAA \rightarrow X_m\text{-}AA_p\bullet$$

Introduction of di-vinyl crosslinker leads to formation of a PAA network, crosslinked by EGDMA, with grafted block copolymer chains. (Fig.11)

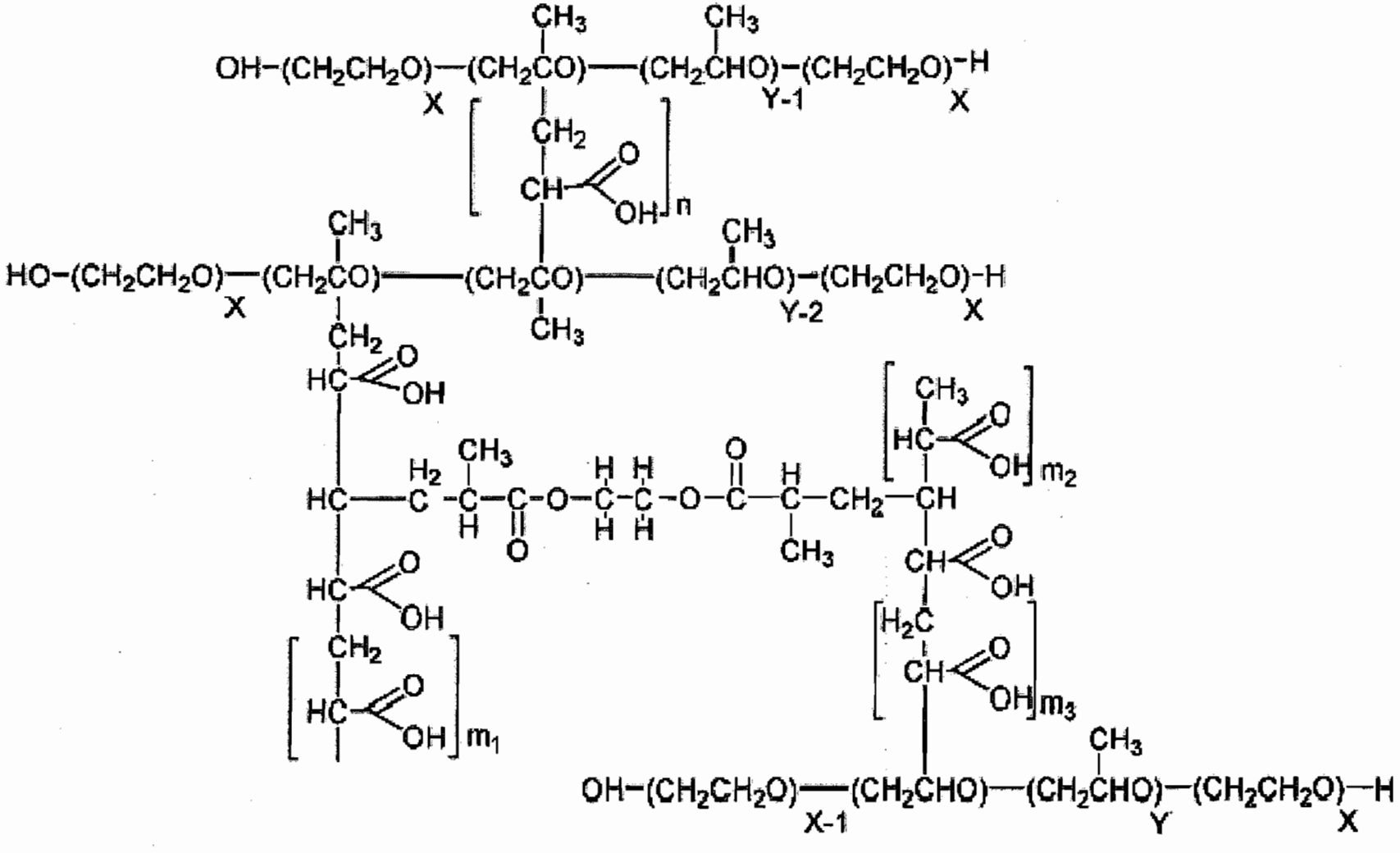


Figure 11: Proposed structure of microgel fragment

5.3. pH responsive properties of microgels

The microgels are responsive to environmental pH changes, which is crucial for protection of API from the acidity and proteolytic enzymes of gastric fluids. At low pH of the human stomach, the microgels stay collapsed and drug is not released, however, at pH closer to neutral, in the intestine, these materials start to swell and release active pharmaceutical ingredient.(**11**)

5.4. Mucoadhesion of microgels

Among various possible bioadhesive polymeric hydrogels, PAA has been considered as a good mucoadhesive. It has been intensively studied and a number of mucoadhesive formulations was developed based on this polymer.(**26**, **37**, **42**) Polyether modified PAA formulations demonstrated mucoadhesive properties and are proven to be non-irritating.(**6**, **8**, **10**)

EXPERIMENTAL PART

Objectives:

The general objective of this project is to synthesize and study microgels of crosslinked poly(acrylic acid) network with grafted block-copolymers chains (poly(ethylene oxide)-poly(propylene oxide) or poly(propylene glycol)) as a potential matrix for oral administration of therapeutic proteins and peptides. These microgels are synthesized in order to protect large molecular size drugs in the stomach, and to provide prolonged release in the intestine.

1. MATERIALS AND METHODS

1.1. Materials

Name	Lot Number	Supplier
Acetonitrile	A25627	Aldrich
Acrylic Acid monomers (AAM)	10630LB	Aldrich
4'-4-azobis-(4-cyanovaleric acid)	12688/1	Fluka
Bovine Serum Albumin A7906	113K0669	Sigma
Carbopol® 971 NF	CC9DAAJ085	Noveon
Dichloromethane 99.9%	LI00262KI	Aldrich
Dodecane	DA02736OA	Aldrich
Ethylene Glycol Dimethacrylate	07711LO	Aldrich
Ganex V-216	03200068154	ISP technologies
Hexane	10655HC	Aldrich
Hydrochloric Acid 1N standard	00507HC	Aldrich
Lactose	03912MC	Sigma
Lauroyl Peroxide	03727PO	Sigma
Magnesium Stearate	18724MA	Aldrich
Pluronic® F127	WPNX6OC	BASF
Pluronic® L92	WPDX5335B	BASF
Poly(propylene glycol) 3500	08831AO	Aldrich
RS Protein Assay	81495A	Bio-Rad
Sodium Hydroxide	91040	MAT
Sodium Hydroxide 1N standard	03427DD	Aldrich
Sodium Phosphate dibasic anhydrous	85H0425	Sigma
Sodium Phosphate monobasic monohydrate	67H1245	Sigma
Trifluoroacetic Acid	001210-50209-259	PSIG

1.2. Equipment

- Mechanical stirrer BDC 1850, Caframo
- Hot plate with magnetic stirrer, Fisher Scientific
- Filter paper 10μm, Whatman
- Soxhlet extractor
- Market grade test sieves mesh 20(0.86mm), 40(0.36mm) and 100(0.14mm), Dual Manufacturing Co.
- Optical microscope Labophot-2, Nikon
- Tap density tester, Vanderkamp
- Differential scanning calorimeter DSC-30, Mettler
- Nuclear magnetic resonance spectrometer Avance 600 MHz, Bruker
- Fourier transformed infrared spectrometer FTS800, Varian
- Reversed-phase HPLC column 218TP54, Vydac
- Dual λ absorbance detector 2487, Waters
- HPLC sampler manager 2700, Waters
- Binary HPLC pump 1525, Waters

- pH meter Orion 525A+, Thermo Electron
- Tube rotator, Scientific Equipment Products
- Hydraulic press, Carver
- Punch and Die ø 1 cm, custom made
- Tablet hardness tester PTB 302, Pharma Test
- Balance BP211D, Sartorius
- Incubator, Power Scientific
- Variable-rate flask shaker, St. John Associates
- Microcentrifuge Micromax, IEC

1.3. Methods

1.3.1. Microgel synthesis

The microgels were synthesized on laboratory scale according to the method (**11**) originally described by Dr. Lev Bromberg (Fig. 12) with some modifications.

1.3.1.1. Preparation of Block Copolymer Solution (Solution A)

39mL of AAM was neutralized by 0.5mL of 5M NaOH, charged into a flat-bottom flask and mixed. 24 g of block copolymer (Pluronic® or PPG 3500) and 1.1mL of EGDMA was charged into the flask and mixed vigorously. The mixture of initiators: 100mg of Lauroyl peroxide and 100mg of 4'-4-azobis-(4-cyanovaleric acid) in 2mL of AAM was added. The solution was stirred at 200rpm by magnetic stirrer for 10 minutes. All these operation were performed under a constant flow of dry nitrogen at room temperature. The mixture was deoxygenated for 30 minutes.

1.3.1.2. Emulsion/Dispersion

A 500-mL three-necked round-bottom flask was charged with 250mL of 1% w/v Ganex solution in dodecane and deoxygenated overnight with constant stirring at 100rpm with a purge of dry nitrogen at room temperature. Next day, the flask was placed into an oil bath with temperature controller. The flask was equipped with a mechanical stirrer and a thermometer. *Solution A* was charged into the round-bottom flask. Dry nitrogen purge was initiated and kept until the end of the reaction. The mixture was stirred for 30 min at 200rpm at room temperature. Stirring rate was raised to 300rpm and maintained at this intensity until the end of

the reaction. Step-wise heating was started at the rate of 1-2°C per minute up to 70°C. The temperature was maintained at 70°C until the end of the reaction, for 4 hours.

1.3.1.3. Filtration, Washing and Drying

The reaction slurry was cooled to room temperature and filtered using Whatman filter paper (10μm). The wet filtrate was repeatedly washed with hexane and dried under vacuum 10^{-3} Torr at room temperature for 36 hours. The dried microgel was then washed in a Soxhlet extractor with dichloromethane for 72 hours. All the wash-out from both steps were collected and evaporated under vacuum to be used in the estimation of the effective degree of bonding between block copolymers and poly(acrylic acid). The washed microgel was dried under a vacuum of 10^{-3} Torr at room temperature for 48 hours.

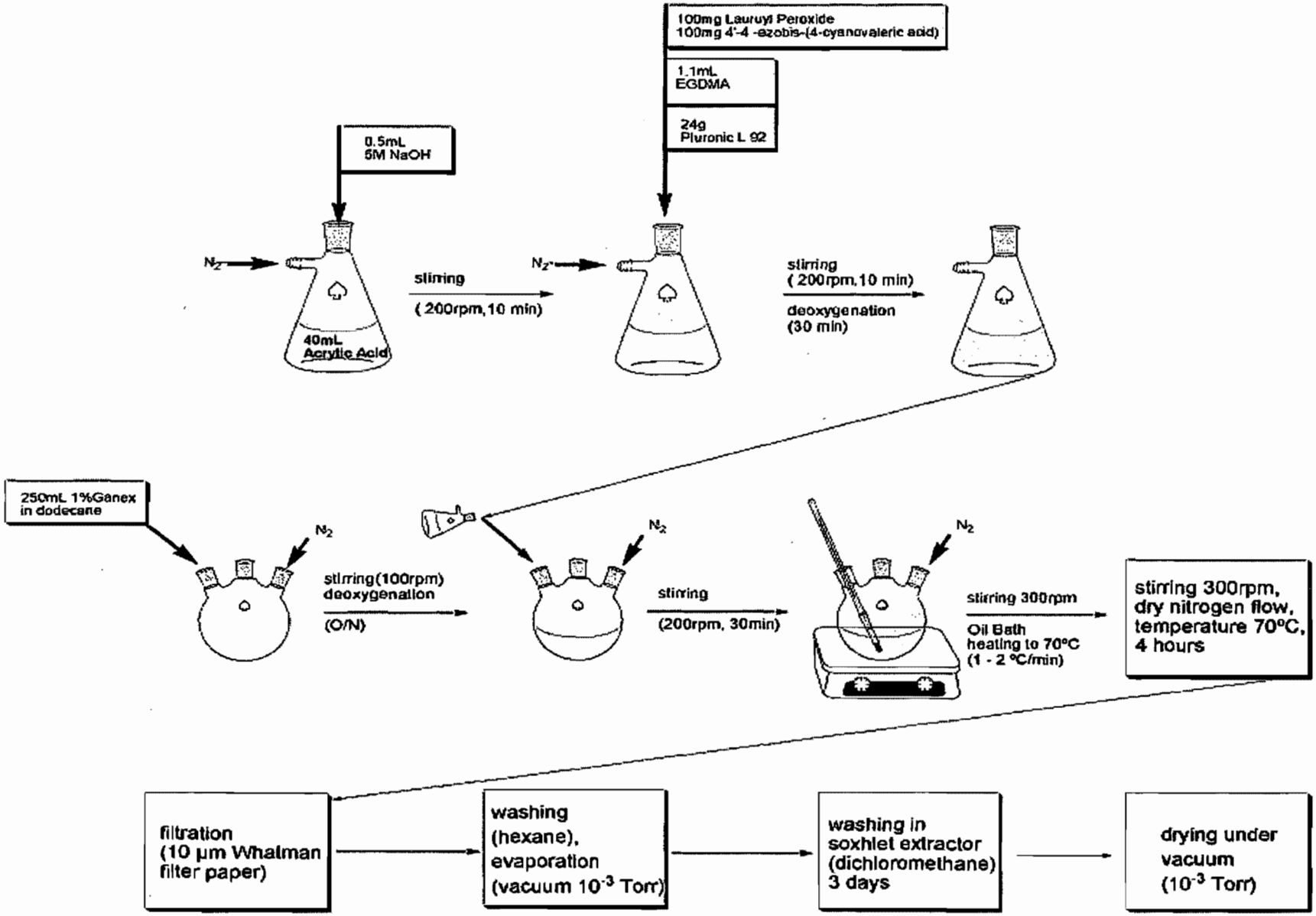


Figure 12: Microgel synthesis, washing and drying

1.3.2. Microgel composition determination

1.3.2.1. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was done using a Varian FTS800. Block copolymer samples were prepared in dichloromethane in serial dilutions and analyzed for characteristic peaks at a wavenumber of 1100. Dried synthesis washouts from all the batches, reconstituted in dichloromethane were analysed by FTIR for the peak intensity at the respective wavenumber, and quantities of unbound block copolymers for each batch were estimated from standard curves.

1.3.2.2. Estimation of effective degree of boding

An effective degree of bonding between block copolymers and poly(acrylic acid) was calculated for all three microgels according to the following equation:

$$D_oB = (P_{ch} - P_u).100\% / P_{ch} \qquad \text{(eq. 12)}$$

Where:

D_oB effective degree of bonding (%)

P_{ch} initial weight of block copolymer charged into the reactor (mg)

P_u weight of block copolymer in washouts, not bound during the synthesis (mg)

1.3.2.3. High-performance liquid chromatography (HPLC)

An analytical HPLC system with reversed-phase column was equilibrated using 0.1% (v/v) aqueous trifluoroacetic acid at a flow rate of 1mL/min. A linear gradient of water (0.1% TFA)/Acetonitrile (0.1% TFA) from 9:1 (v/v/) to 3:7 (v/v) was used. Serial dilutions of AAMs were prepared in water. Samples were injected by an automatic autosampler and monitored at 225nm and 280nm by means of a dual λ Absorbance Detector.

1.3.3. Microgel characterization

1.3.3.1. Differential scanning calorimetry (DSC)

DSC evaluation was performed on each batch of dry microgel particles and all starting materials: AAMs, Pluronic® F127, Pluronic® L92 and PPG 3500. All samples were tested utilizing Mettler DSC 30in standard aluminum crucibles at heating rate of 10°K/min was applied to all DSC samples. Scanning was performed under constant nitrogen flow and with the starting temperature not higher them -100°K.

1.3.3.2. Solid ^{13}C nuclear magnetic resonance (NMR)

Samples from each batch of F127, L92 and PPO Microgels and Pluronic® F127 were prepared in tubes for solid state NMR. ^{13}C spectra were acquired on a Bruker Avance 600 MHz spectrometer at 600 MHz by staff of the Department of Chemistry of the University of Montreal.

1.3.3.3. Swelling of microgels and pH titration studies

Swelling experiments were performed on each batch of microgel in duplicate. Samples of $1cm^3$ of dry material, independent of weight, were placed into 50ml plastic measuring flat bottom cylinders. The cylinders were sealed with rubber stoppers thoroughly at all time. The study was done at 37°C. Samples were pre-swelled in 30ml of distilled water for 2 days. After pre-swelling, the pH of the slurry was lowered to 2.0. Then the pH was increased gradually adding 0.4mL of NaOH standard solution. These steps were repeated until the pH reached 12.0 for each microgel except microgel PPO, which swelled to the maximum volume at pH 6.0. 1N HCl and 1N NaOH standard solutions were used to adjust the pH. All samples were allowed to stand for a minimum of 3 hours after adding NaOH or HCl and then the height was measured

The swelling ratio (S) was calculated as follows:

$$S = H_s / H_0 \qquad \textbf{(eq. 13)}$$

Where:

H_s height of the swelled microgel column during the experiment

H_0 height of the microgel column at time "0" (1 ml of dry microgel pre-swelled in distilled water)

Microgel PPO titration continued after the point of maximum swelling at pH 6.0 up to pH 12.0. These results were normalized per gram of dry microgel and plotted.

1.3.3.4. Particle size determination and imaging of dry microgel particles

Particle size analysis was performed for dry microgel particles using market grade test sieves with mesh 20(0.86mm), 40(0.36mm) and 100(0.14mm). The fractions were weighted by means of an electronic balance (±0.01 mg). Images of the dry particles were obtained using an optical microscope Labophot-2.

1.3.3.5. Carr's compressibility index determination

A Vanderkamp Tap density tester with a tapping frequency 150 taps per minute was used to determine Carr's compressibility index.(**16**, **17**) The tared 250-mL measuring cylinder was filled with pre-weighted test material to 100 mL, then the initial (or bulk) density was estimated. With minimal disturbance of the measuring cylinder, it was transferred to the tap density apparatus, tapping was done 150 times then the final (or tap) density was estimated considering the reduced volume of the sample after tapping.

The following equation was used to calculate Carr's compressibility index (X):

$$X = (D_0 - D_f) .100\% / D_0 \qquad \textbf{(eq. 14)}$$

Where:

D_0 initial (bulk) density (g/cm^3)

D_f final (tap) density (g/cm^3)

1.3.4. Microgel preparation and tabletting

Dry blending was used to formulate both test and reference products. Series of dry blends were prepared for each batch of microgel in duplicate. F127 microgel, L92 microgel, PPO microgel, Carbopol and Lactose were dry blended with 2%, 10%, 20% or 50% w/w of protein (BSA) and 0.5% w/w of Magnesium Stearate as a compression lubricant. BSA was sieved through a series of market grade test sieves before blending and the fraction between meshes 40 (0.36mm) and 100(0.14mm) was selected. Blank blends without BSA were prepared for each batch as a negative control. Blending was carried out at room temperature in glass vials on vertical tube rotator at 10 rpm for twenty minutes.

Flat-faced plain tablets weighting 300mg, having a thickness of 3mm and with a diameter of 10mm, were compressed on a Carver press with 5000kg force applied.

1.3.5. Acidic fluids and phosphate buffer preparation

Acidic fluids were prepared by lowering the pH of distilled water to 2.2 by adding HCl (1N) volumetric standard solution. The 0.5M phosphate buffer pH 6.8 was prepared as follow: 14.08g of sodium phosphate monobasic monohydrate and 13.92g of sodium phosphate dibasic anhydrous were dissolved in four litres of distilled water. The final pH was adjusted to 6.8 if required.

1.3.6. Dissolution method and protein assay

The limited sensitivity of the protein assay did not allow using the standard USP dissolution apparatus. The dissolution tests were performed on synchronised flask shakers

placed into a temperature-controlled environment of an incubator. Sample tablets were placed into 50mL sealed plastic Falcon tubes. Tablets were tested at 37°C for 48 hours: two hours in 50mL of acidic fluids at pH2.2, then the solution was changed using the same volume of 0.5M phosphate buffer pH 6.8 to mimic the GI tract conditions. During the acidic treatment samples and blanks were shaken at 15 rpm then, after the change to phosphate buffer shaking was increased to 100 rpm. Aliquots of 0.3mL were taken at 0, 1, 2, 2.5, 3, 4, 5, 8, 24 and 48 hours. The aliquots were compensated with the same volume of the phosphate buffer at the same temperature (37°C) at each measurement. Acidic aliquots were neutralized using NaOH 1N standard solution as required for the protein assay taking in consideration the dilution factor.

Bio-Rad RC protein assay was used to determine the concentration of BSA. All the aliquots were centrifuged using a microcentrifuge at 10,000 rpm for 10min prior the protein assay. Sampling of the supernatant was done in order to avoid presence of microgel particles in the assay. Samples with a concentration higher then the assay range were diluted respectively. All aliquots were assayed in triplicates. The protein assay was carried out in accordance with the following standard operational protocol:

1 Prepare 6 dilutions of a protein standard (BSA) containing from 0.2 mg/mL to 1.2 mg/mL. A standard curve is prepared each time the assay is performed in triplicate and in the same buffer as the sample.

2 Pipette 5 µL of standards or samples into a clean, dry microtiter plate.

3 Add 25 µL of reagent A into each well.

4 Add 200 µL of reagent B into each well. Place the plate on a microplate reader and let the plate mix for 5 seconds.

5 After 15 minutes, read the absorbance at 750 nm. The absorbance is stable for about 1 hour.

1.3.7. Method of estimation of release kinetics parameters

The Peppas equation was applied to determine the kinetic parameters of BSA dissolution.(**45**)

$$M_t/M_\infty = k.t^n \qquad \textbf{(eq. 15)}$$

Where:

M_t/M_∞	degree of BSA release
k and n	kinetic parameters
t	time(hours)

The estimation of the kinetic parameters "k" and "n" was performed by linear regression of the logarithmic values of the time and degree of BSA release using the linear form of Peppas equation:

$$\log (M_t/M_\infty) = \log(k) + n \log(t) \qquad \textbf{(eq. 16)}$$

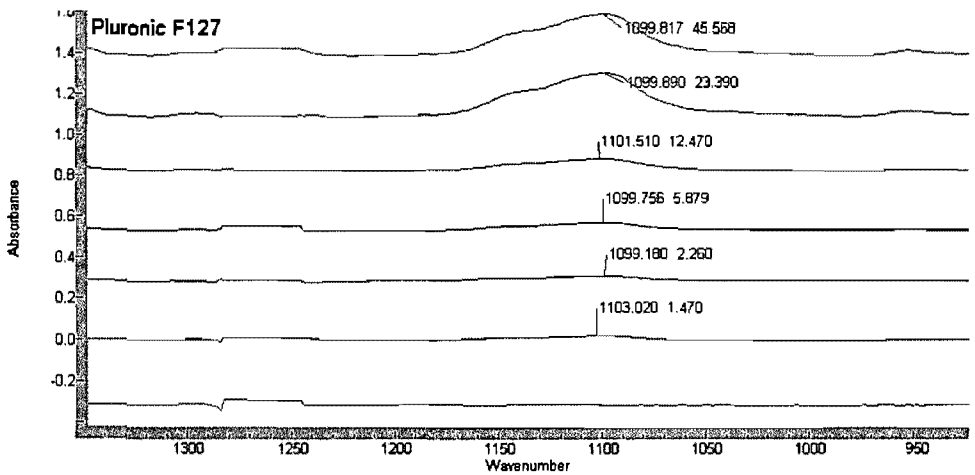
2. RESULTS AND DISCUSSION

2.1. Microgel synthesis

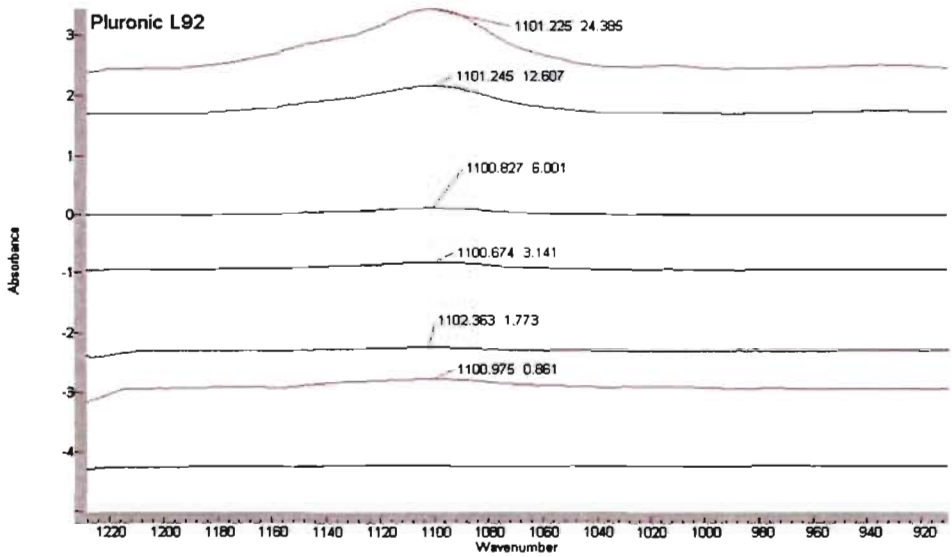
Two consecutive batches of each microgel were synthesized in accordance with the method described in the section 1.3.1 at laboratory scale in adiabatic mode by a one-step free-radical crosslinking copolymerization of acrylic acid monomers and block copolymers with the covalent crosslinker ethylene glycol dimethacrylate. Both batches of each material were used in subsequent experiments.

2.2. Microgels composition determination

Given that resulting microgels are solid and absolutely insoluble whereas key starting compounds are mostly liquid, it was not feasible to use the same analytical technique to determine the degree of bonding for block copolymers. Thus washouts from the synthesis were collected and analysed by FTIR and HPLC to determine the composition of the solid fraction. FTIR results for serial dilutions of block copolymers obtained by the method described in section 1.3.2.1, are presented in Figure13 a, b and c



a



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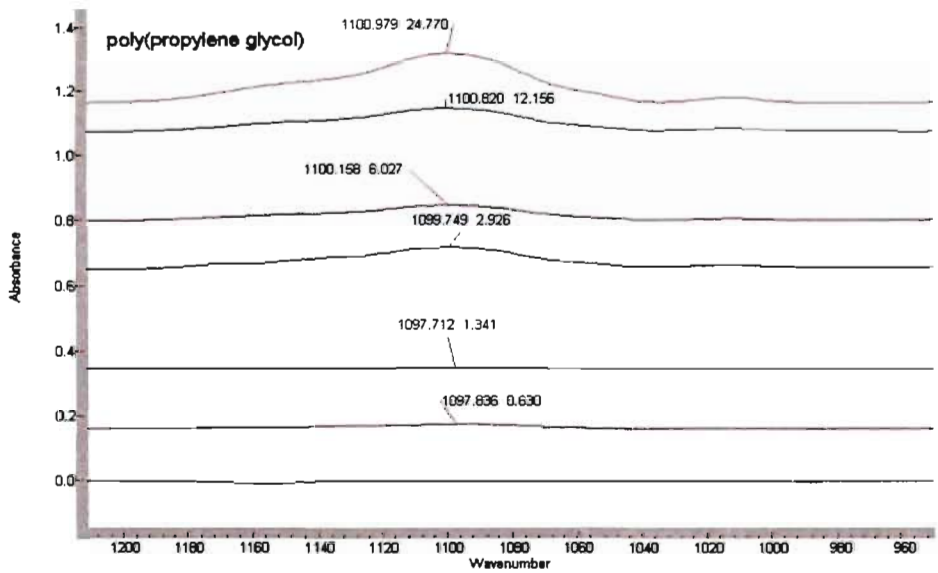
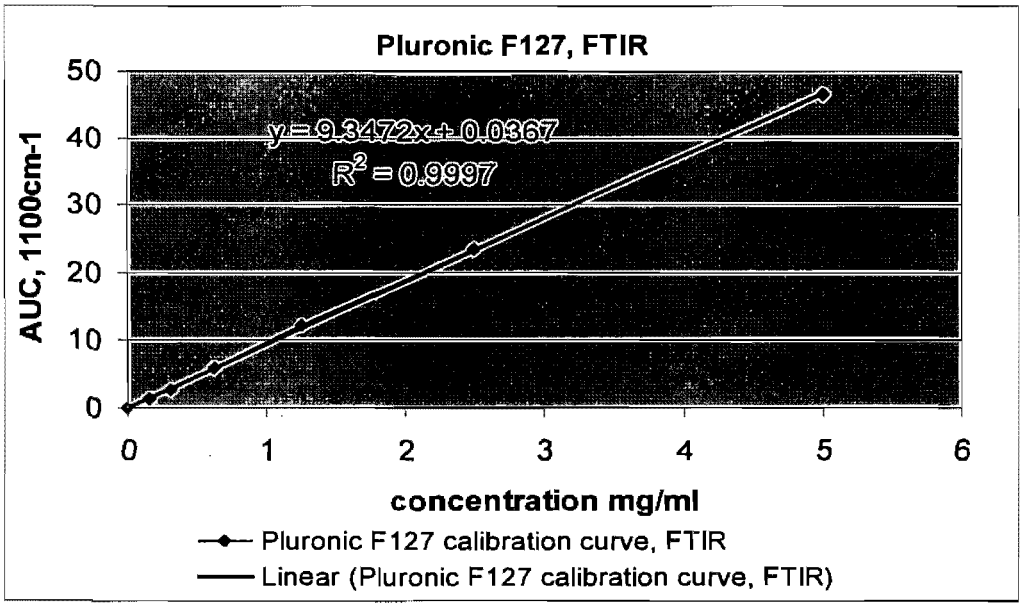


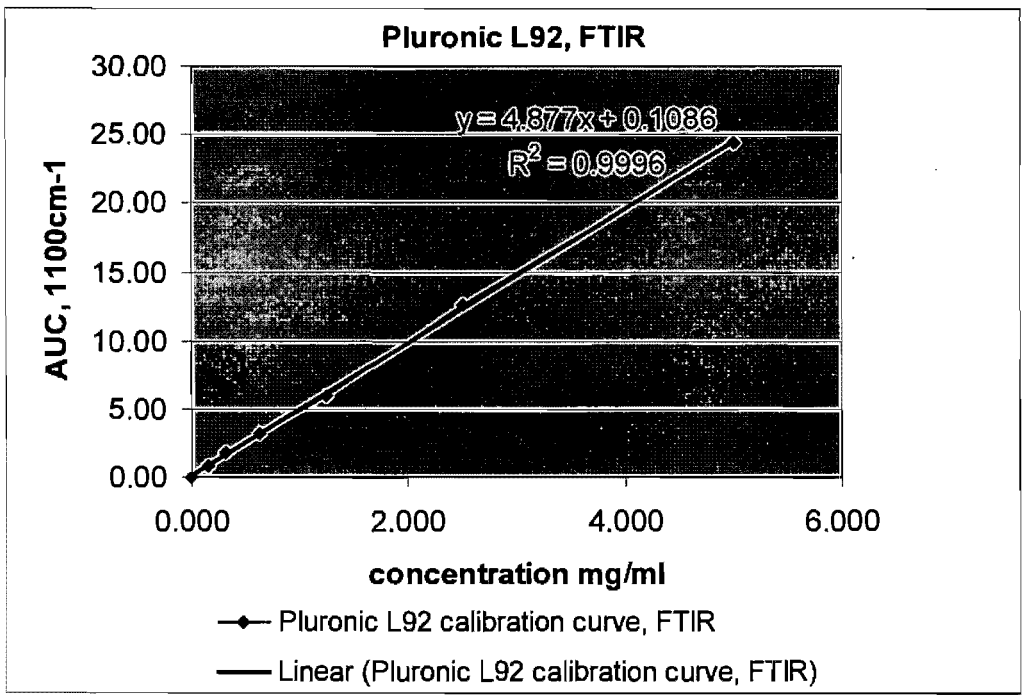
c

Figure 13: FTIR results for serial dilutions of Pluronic® F127 (a), Pluronic® L92 (b) and PPG 3500 (c) in dichloromethane

Areas under the curve for the characteristic peaks were plotted to obtain standard curves for respective material in order to quantitate these compounds (Fig. 14 a, b, c).



a



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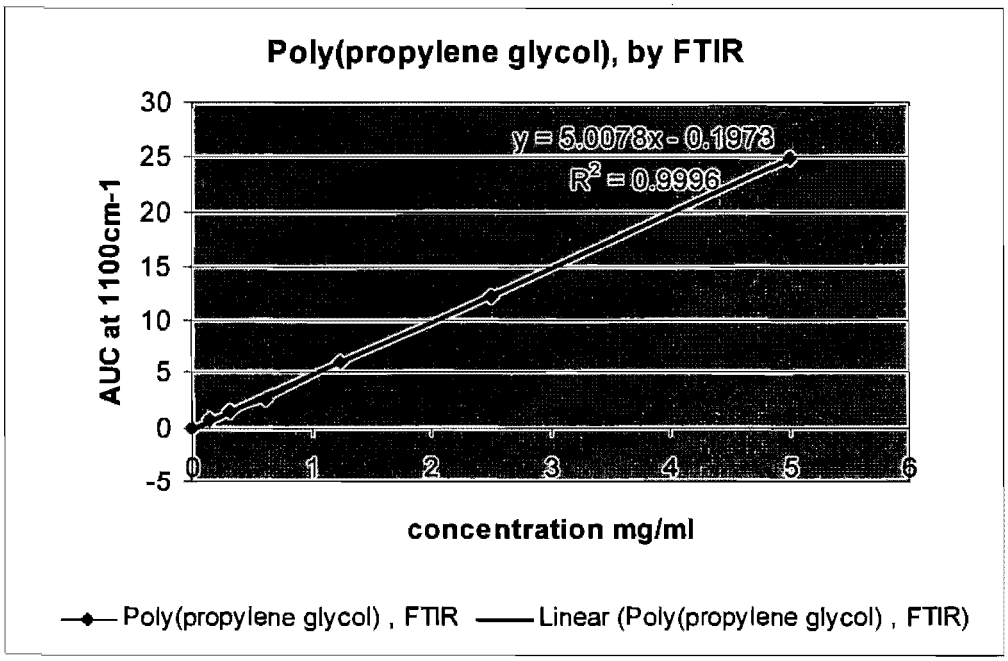


c

Figure 14: Standard curves for Pluronic® F127 (a), Pluronic® L92 (b) and PPG 3500 (c) in dichloromethane

2.2.1. Block copolymer binding

An effective degree of bonding between block copolymers and poly(acrylic acid) was calculated for all three microgels in accordance with Equation 12. Quantitation of unbound block copolymers demonstrated a relatively high degree of bonding for these starting materials. More then 90% of block copolymers for both Pluronic®-based microgels and up to 96% for PPO microgel were not detected in the solvable washout of the synthesis. (Fig.15)

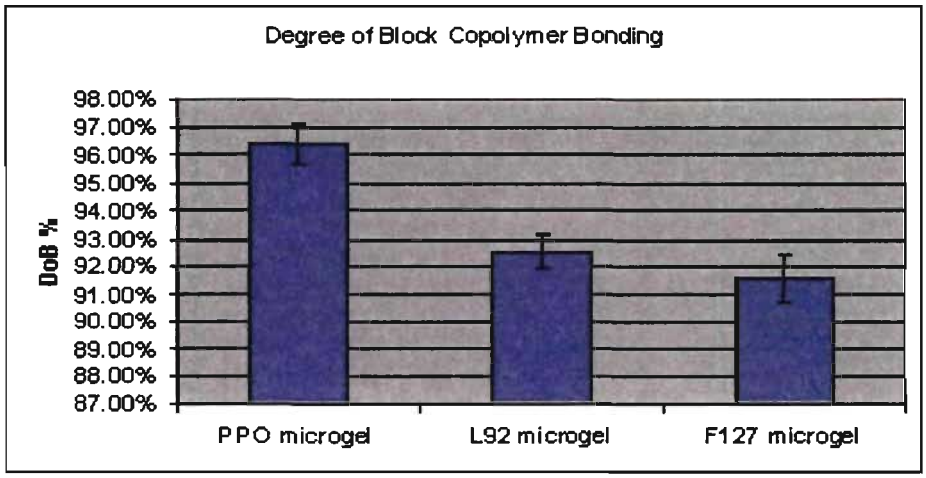


Figure 15: Block Copolymer incorporation into the microgels structure

2.2.2. Acrylic acid monomer binding

Acrylic acid concentration in serial dilutions and synthesis residuals was assayed by the HPLC method detailed in the section 1.3.2.3. HPLC results for serial dilutions of acrylic acid monomers are presented in the following figure.

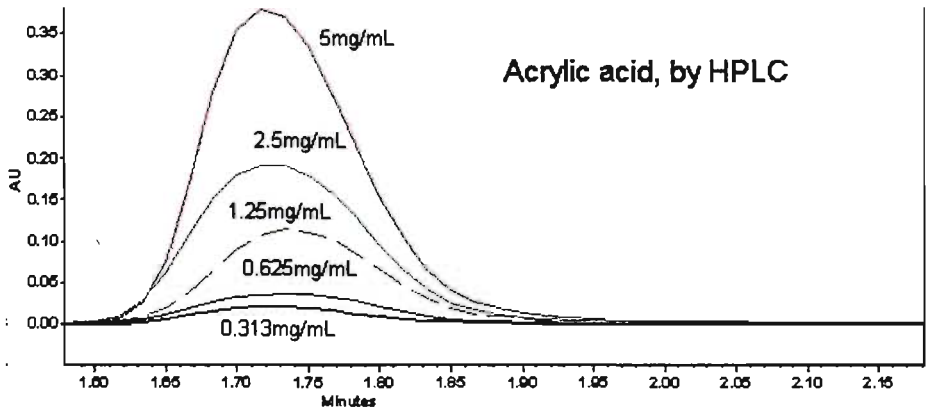


Figure 16: Acrylic acid monomer HPLC chromatograms used for calibration

Figure 17 shows that acrylic acid monomers were not detected in F127 microgel wash-outs. Similar results were obtained for L92 and PPO microgels. High efficiency of

acrylic acid monomer incorporation into microgels has been previously reported.(**12**) Thus it was assumed that all the acrylic acid monomers charged into the reactor were consumed in the synthesis.

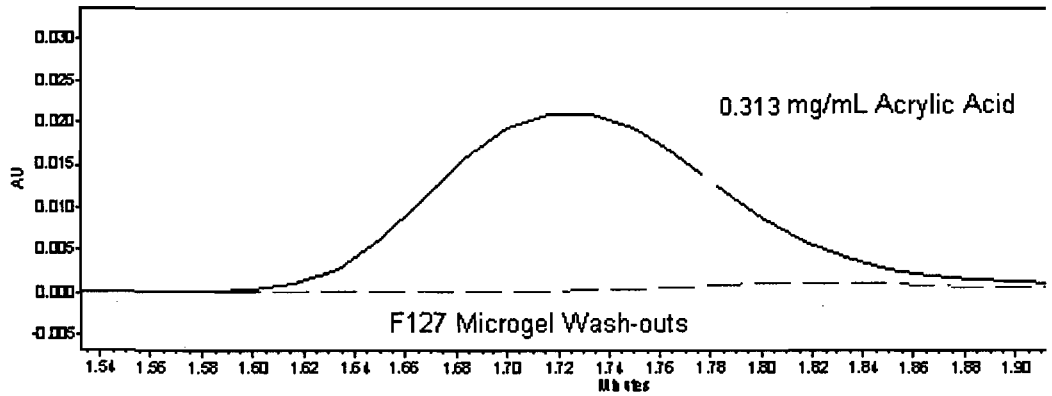


Figure 17: Acrylic acid monomer were not detected by HPLC in F127 microgel wash-outs

2.2.3. Efficacy of the microgel washing procedure

Differential scanning calorimetry experiment was performed on dry particles of microgel according to the method described in section 1.3.3.1 to evaluate the completeness of the synthesis and to verify that they were free of unbound phase after the multi-step washing-drying procedure. Starting materials were assayed by DSC as references. Actual DSC data can be found in Appendix I of this memoir.

Acrylic acid had demonstrated a sharp melting peak at 12°C, which was in compliance with the literature source.(**22**) This type of phase transition was not identified in any of the microgel DSC profiles.

Pluronic® F127 distinctive melting peak at 56°C was in accordance with the technical bulletin of the manufacturer.(**50**) No phase change occurred at this temperature in the Microgel F127 samples.

According to the information provided by the BASF technical support staff, pour/melting point for liquid Pluronic® L92 is +7°C. A small endothermic peak was observed at that temperature in pure block copolymer DSC profile. However no melting transition was identified at 7°C in Microgel L92.

PPG-3500 is a liquid polymer. An endothermic peak was observed at -59°C. There was no heat flow event identified at temperatures lower then -50°C in PPO Microgel.

The absence of the key starting material characteristic peaks in the DSC profiles for F127, L91 and PPO Microgels proved that the multi-step washing and drying procedure yielded materials of high purity.

2.2.4. Microgel structure elucidation by solid ^{13}C NMR

Solid NMR technique, detailed in section 1.3.3.2, was selected due to the insoluble nature of the microgels. Only Pluronic® F127 among the key starting materials was solid, thus it was not feasible to perform a quantitive experiment. Carbon-13 NMR data was gathered in order to characterize the structure of the microgels. The signals from carbon nuclear magnetic resonance spectrum were assigned (Fig. 18) to the carbons presented in the molecule.(**55**) Chemical shifts are listed in Table 2. ^{13}C-NMR profiles for the microgels and Pluronic® F127 are presented in Appendix II.

Carbon-13 profiles of all of the microgels exhibited chemical shifts between 25 to 44ppm and 177 to 179ppm, which are characteristic for carbon atoms of poly(acrylic acid) chains. Pluronic® F127 spectra demonstrated the chemical shifts typical for Carbons of block-copolymers: at 18ppm, 61ppm, 70ppm and 74-78ppm. Those shifts were less distinctive in the microgel spectra, especially in the PPO Microgel sample; however in the most common ^{13}C-NMR experiments the intensity of the signal is not directionally proportional to the number of equivalent ^{13}C atoms.(**5**) NMR spectra of all the Microgels were consistent with their proposed structure.

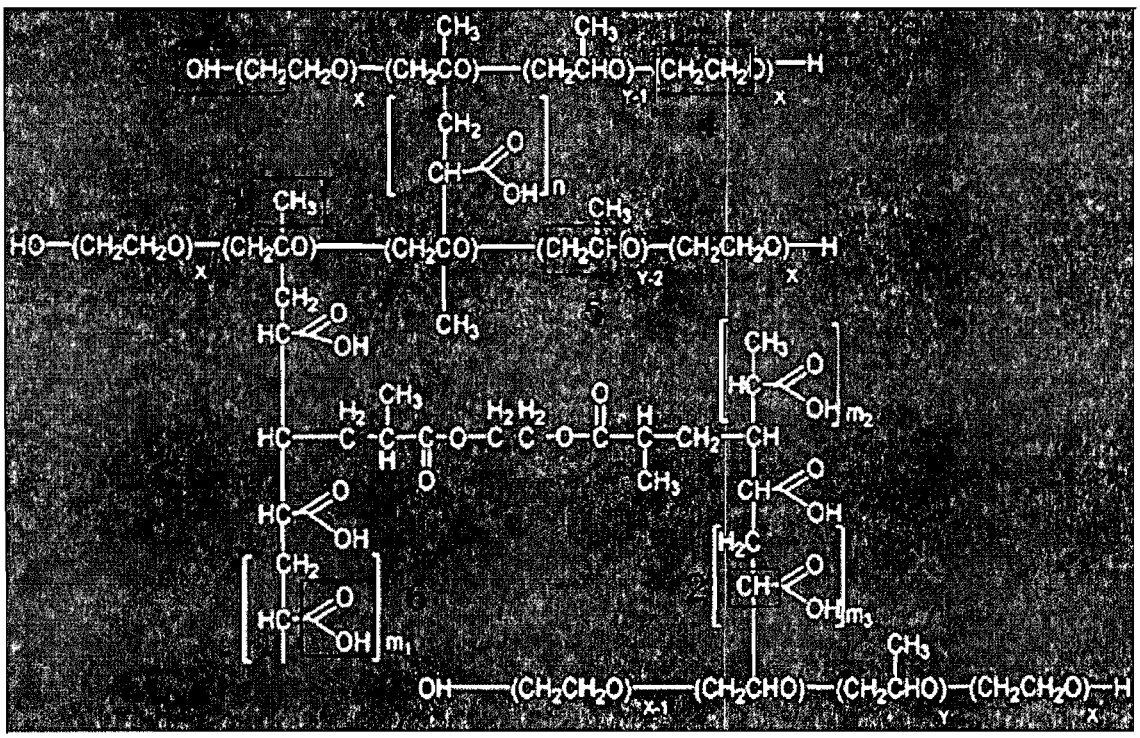


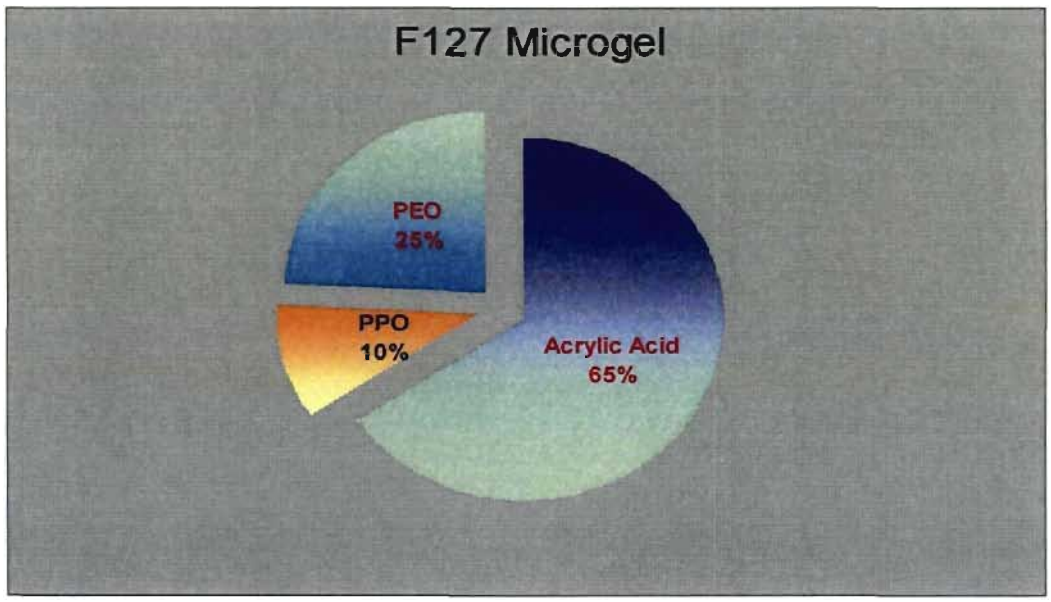
Figure 18: Annotated fragment of microgel structure

13C Chemical Shift (ppm)	Assignment
19	1
25-48	2
61	3
70-71	4
74-78	5
177-179	6

Table 2: Chemical shifts and assignments for ^{13}C NMR

2.2.5. Estimation of microgel composition

Based on binding data for Pluronics®, PPG 3500 and acrylic acid monomers microgel compositions were estimated (Fig.19 a, b, c). All three microgels contain equivalent proportion of poly(acrylic acid) which is approximately equal to 65% and 35% by weight of block copolymer, however, for the latter the PEO-PPO ratio varies and defines the hydrophilic-hydrophobic properties of the microgels.



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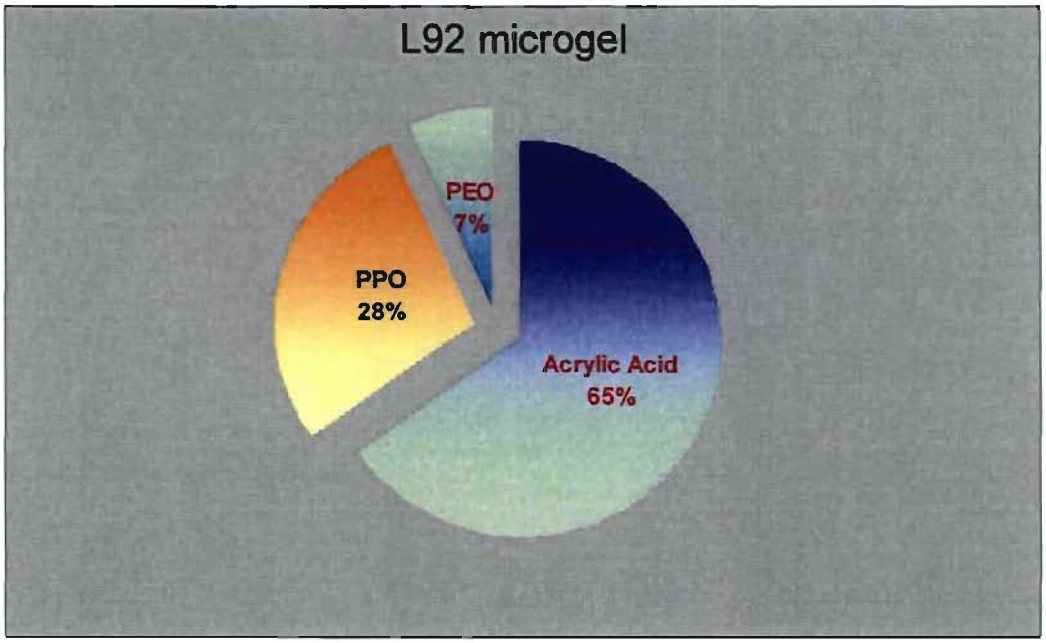
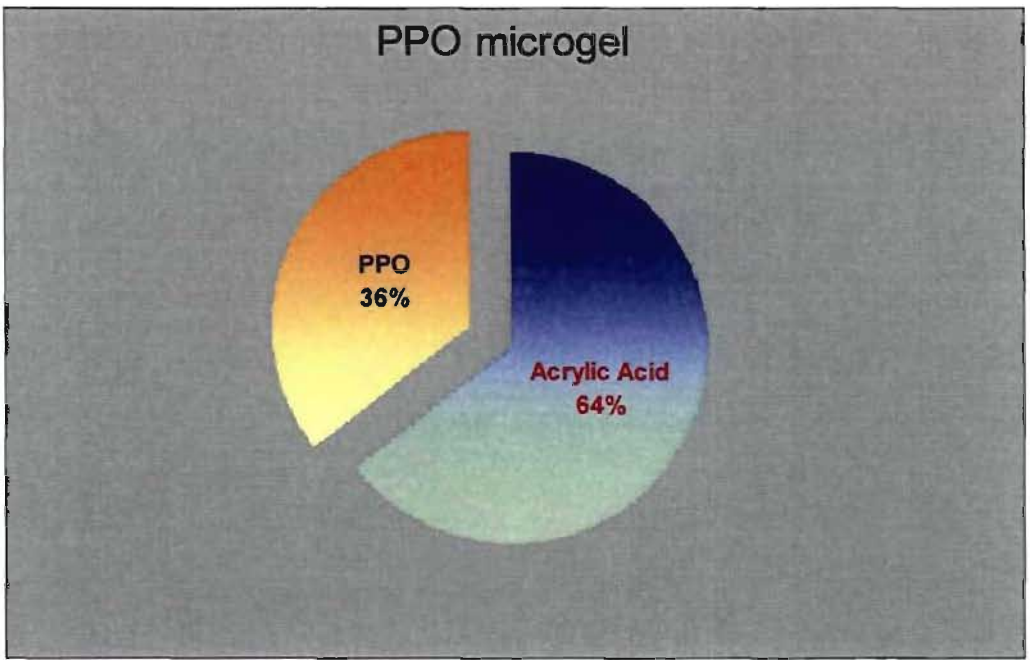


b



c

Figure 19: Estimated Compositions of F127 microgel (a), L92 microgel (b) and PPO microgel (c).

2.3. Characterization of microgel in aqueous milieu

2.3.1. Swelling of microgels

The swelling ratio was calculated in accordance with Equation 13 as described in section 1.3.3.3. Figure 20 shows that Microgel F127 swells the least at pH 12.00 compared to two other microgels, while Microgel PPO swells the most at pH 6.0. Microgel L92 demonstrated an intermediate swelling.

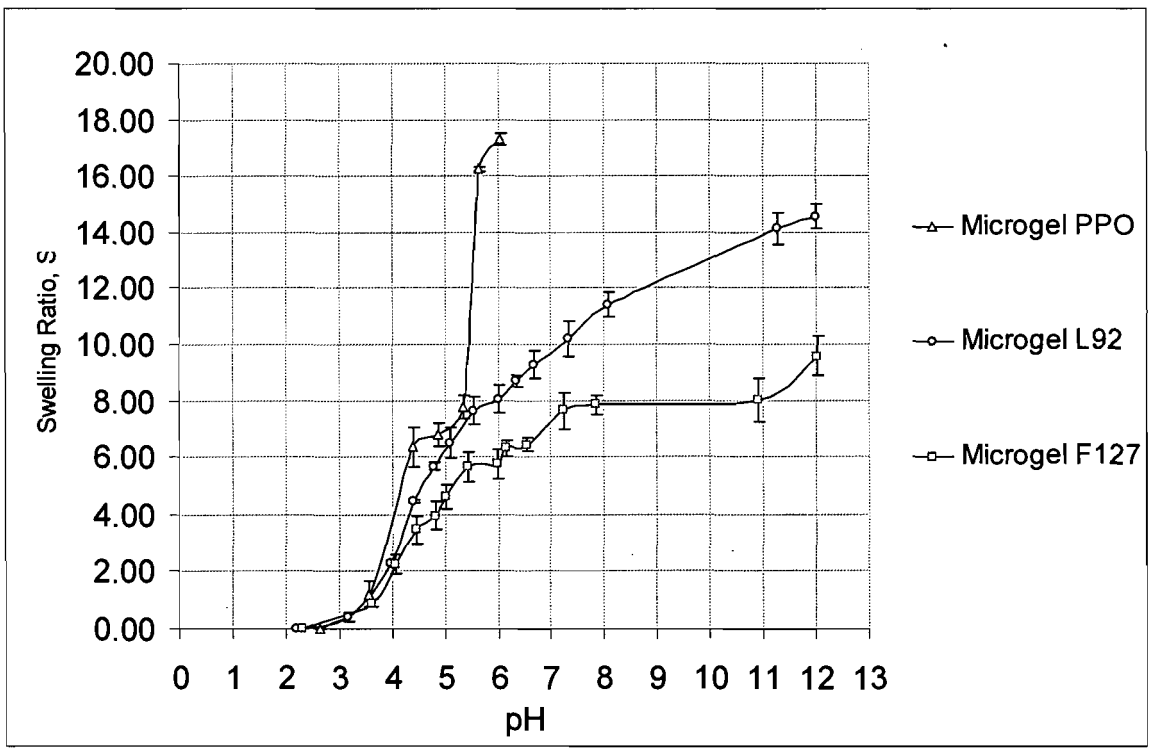


Figure 20: Swelling profiles of the microgels

These results were compared to the estimated compositions of the microgels: all three of them contain 64-65% weight content of PAA and vary in the grafted block-copolymer proportion. (Fig. 21) Thus the most hydrophobic material, Microgel PPO, demonstrated the

highest degree of swelling followed by the moderately hydrophilic Microgel L92. Microgel F127 swelled the least and was estimated to be the least hydrophobic due to composition of Pluronic® F127.

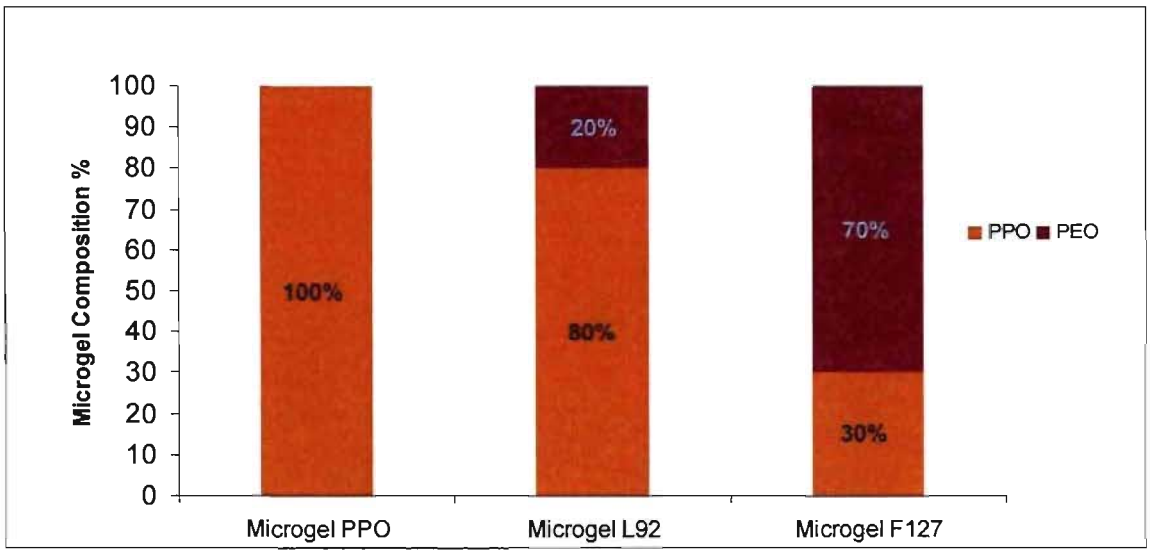


Figure 21: Microgels relative Hydrophobic and Hydrophilic Blocks composition

2.3.2. Titration of microgels

Titration was carried out in accordance with the method detailed in section 1.3.3.3. The results were normalized per gram of dry microgel and plotted. It was observed that all three microgels required approximately equal molar amount of Sodium Hydroxide per 1 gram of dry Microgel to neutralize the carboxyl groups. Quantitation was made to estimate an isoelectric point at 0.008 moles of NaOH, hence straightforward calculation resulted with roughly 580 mg of PAA per 1 gram of a microgel. That did not precisely correlate with the previously estimated 65% of weight however this may be explained by the reduced

accessibility of 5 to 10 % of the microgel PAA chains within the microgel structure. All three Microgels responded similarly during the titration.

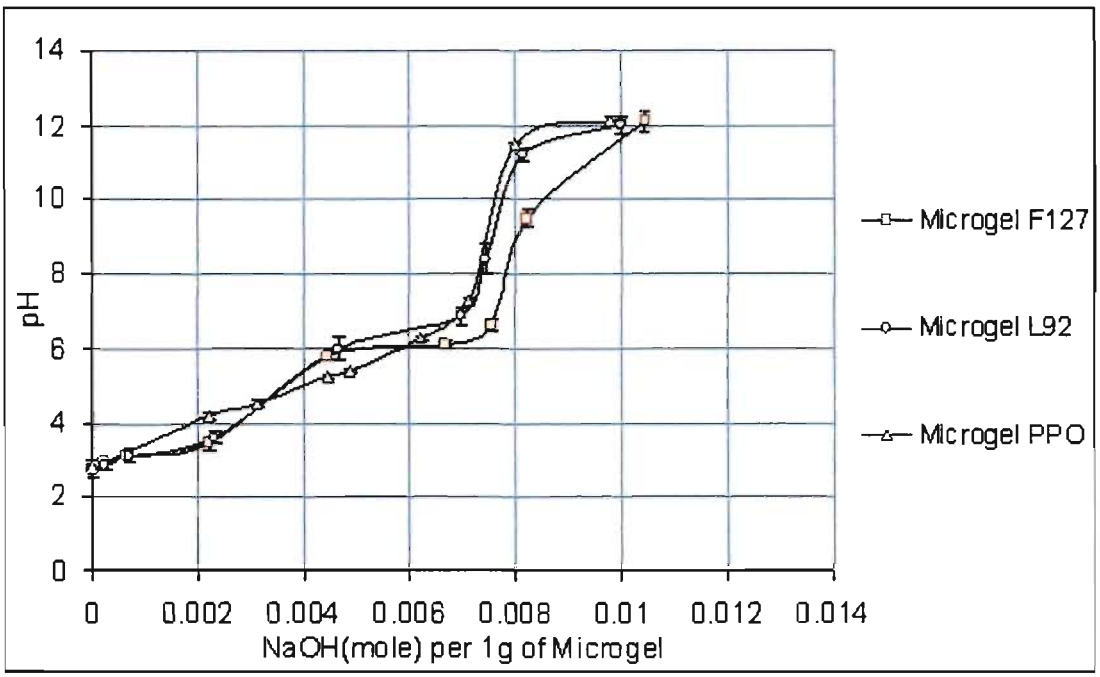


Figure 22: Microgels pH titration results were normalized per gram of dry microgel.

2.4. Characterization of dry microgel particles

2.4.1. Particle size determination

Particle size distribution for each batch of microgel was done using a series of market grade test sieves (Fig.23) as described in section 1.3.3.4. The main fraction for both microgels based on Pluronics® was the one between meshes 40 (0.36mm) and 100(0.14mm) as for the PPO microgel it was between meshes 40 and 100 and between meshes 100 and 200(0.074mm) in about equal amounts. Thus, fraction between meshes 40 and 10 was chosen for farther experiments.

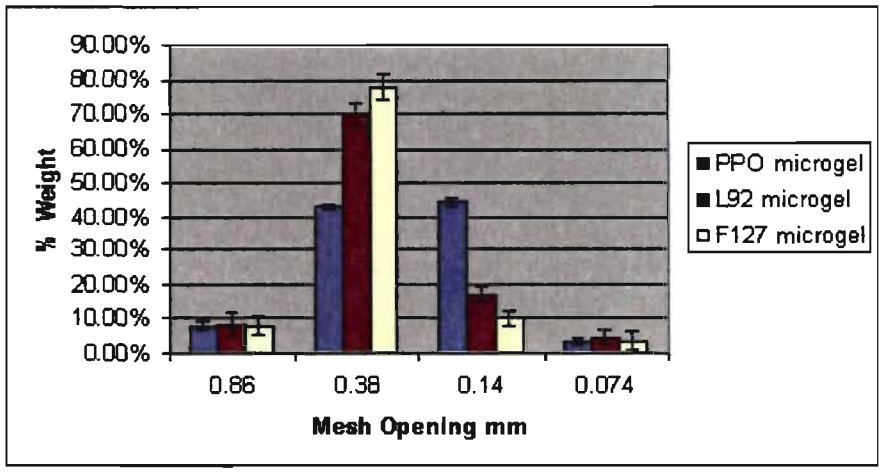
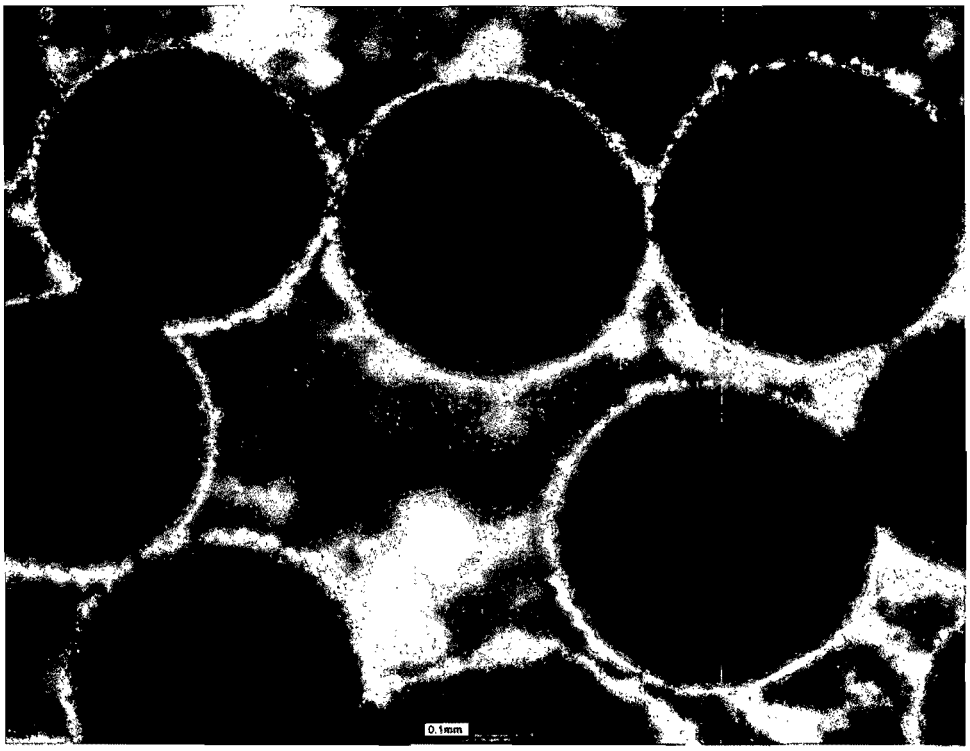


Figure 23: Microgel particles size distribution.

2.4.2. Dry particles morphology

The morphology of dry microgel particles was studied by optical microscopy. F127 microgel particles are of regular spherical shape of 100-180 μm diameters with a smooth surface, Image 1a. L92 microgel had similar shape and size, however, the surface of the particles was rougher and may be characterised by some sponginess, Image 1b. This is similar to the results demonstrated by Dr. L.Bromberg (**12**). The higher susceptibility of the PPO content of Pluronics® to hydrogen abstraction reaction compared to PEO fragments was previously described.(**9**) This might lead to formation during the synthesis of highly crosslinked PPO-rich domains, which are later integrated into particles with less regular shape. The PPO content is almost twice higher in Pluronic®L92 then in Pluronic®F127. It also corresponds to the results for PPO microgel which is a material of highly dispersed size and shape, Image 1c, probably due to the very high degree of crosslinking.



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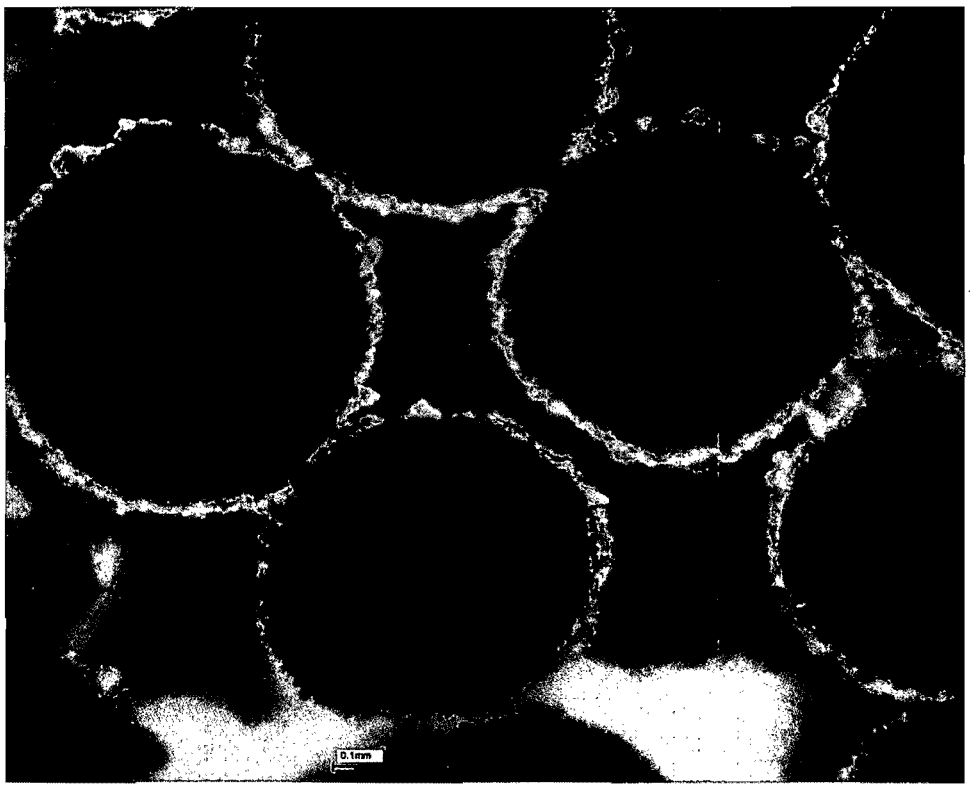

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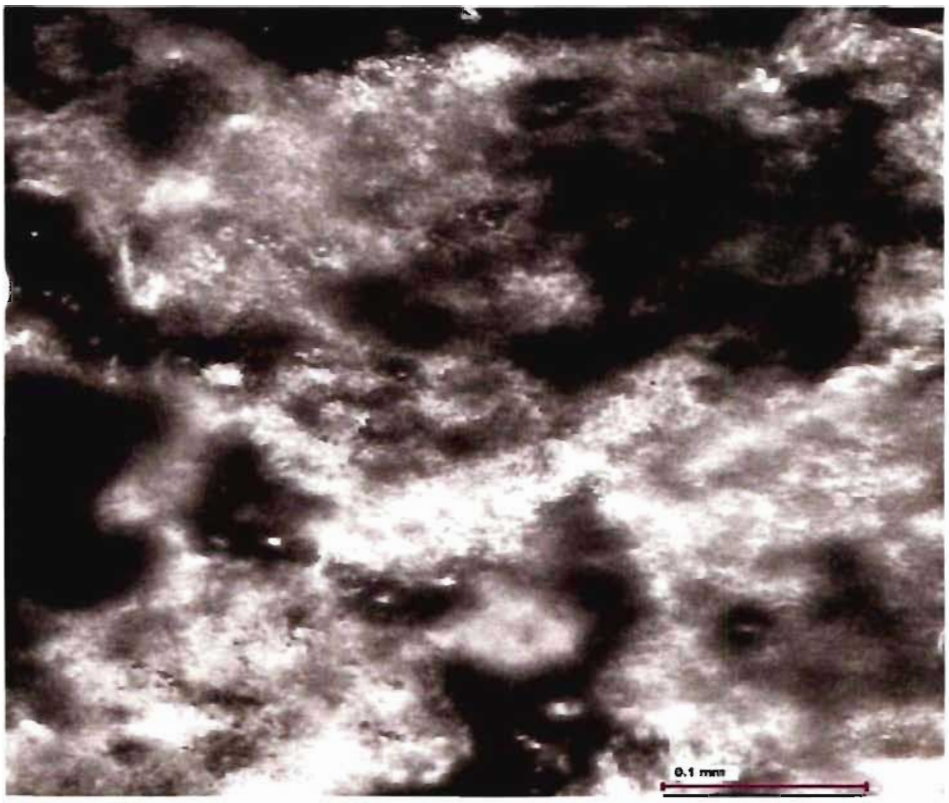


c

Image 1: a) microgel F127; b) microgel L92; c) microgel PPO

2.4.3. Carr's compressibility index

Carr's compressibility index was calculated in accordance with Equation 14 for each microgel (Fig.24) as described in section 1.3.3.5. The best results, close to 20%, were obtained for both Pluronic based microgels. This characterised F127 microgel and L92 microgel as products with good flowability and low cohesiveness, which are important characteristics for direct compression.(**16**, **17**)

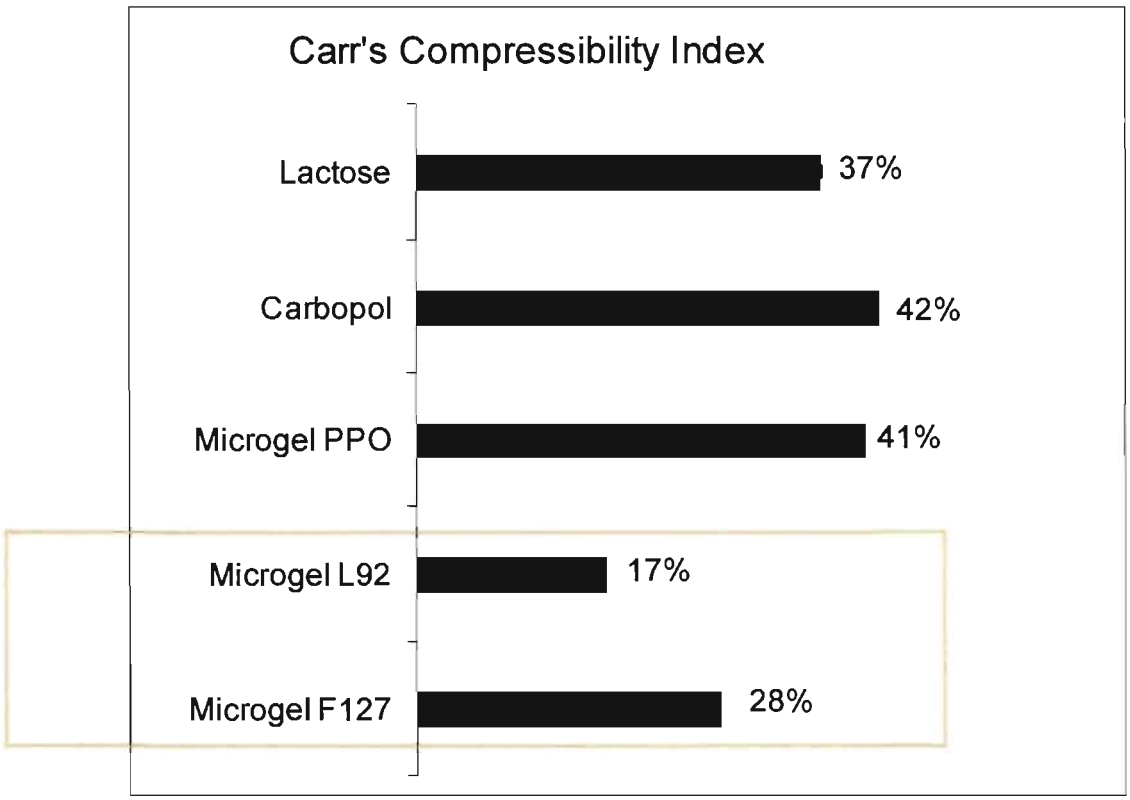


Figure 24: Carr's compressibility index for microgels F127, L92 and PPO microgels vs. two commonly used excipients: lactose (immediate release) and Carbopol (modified release)

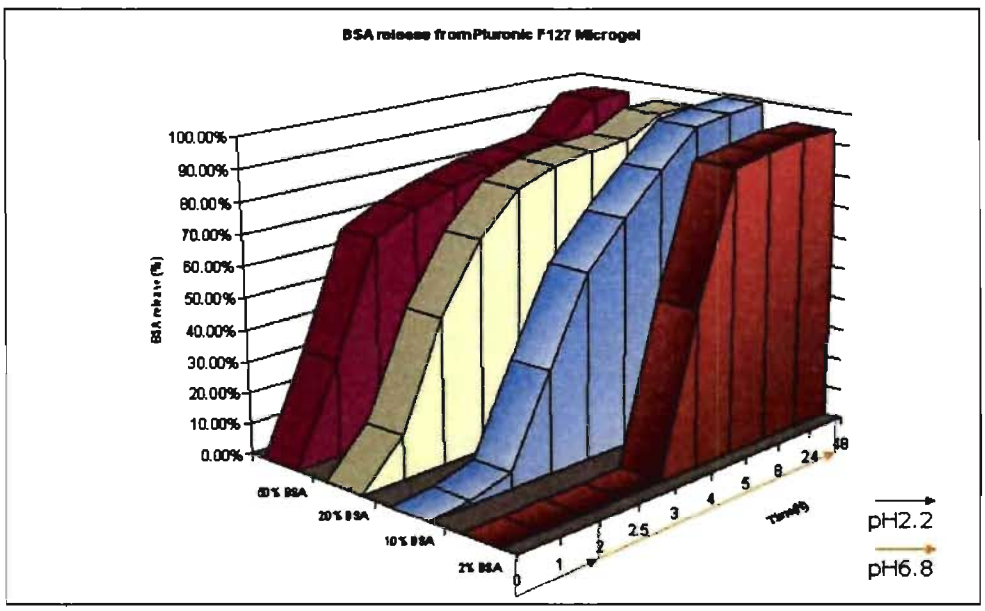
2.5. Microgel loading capacity

The drug loading capacity of microgels was studied in order to find the optimal microgel-to-protein ratio. Series of dry blends were prepared for this purpose for each batch of gel, in duplicate, according to the process detailed in section 1.3.4 with a protein content of 2%, 10%, 20%, and 50% w/w. Hence, the final formulation included respective amount of BSA, dry microgel and 0.5% w/w of Magnesium Stearate as a lubricant.

2.5.1. Effect of loading on protein release

Conditions mimicking the gastrointestinal tract were applied to the samples during protein release experiments. Buffer preparation, dissolution method and protein assay procedure were described in section 1.3.5 and 1.3.6. As shown in Figure 25, the optimal results were obtained for a 10% BSA blend. The protein released during the acidic fluid treatment was negligible. Subsequently BSA was released at moderate and near constant rate in the neutral milieu. Microgel formulations with 2% BSA loading did not provide detectible release for more than 3 hours of dissolution test and then released all the loaded protein within 2 hours. Formulations with a protein loading higher then 10% did not efficiently protect active ingredient during the acidic treatment due to high porosity.

Both Pluronic® based microgels released up to 98% of loaded BSA. However, for PPO microgel the maximum release was only 80% for formulation with 50% of loaded protein. The average error did not exceed 7% for all the results.



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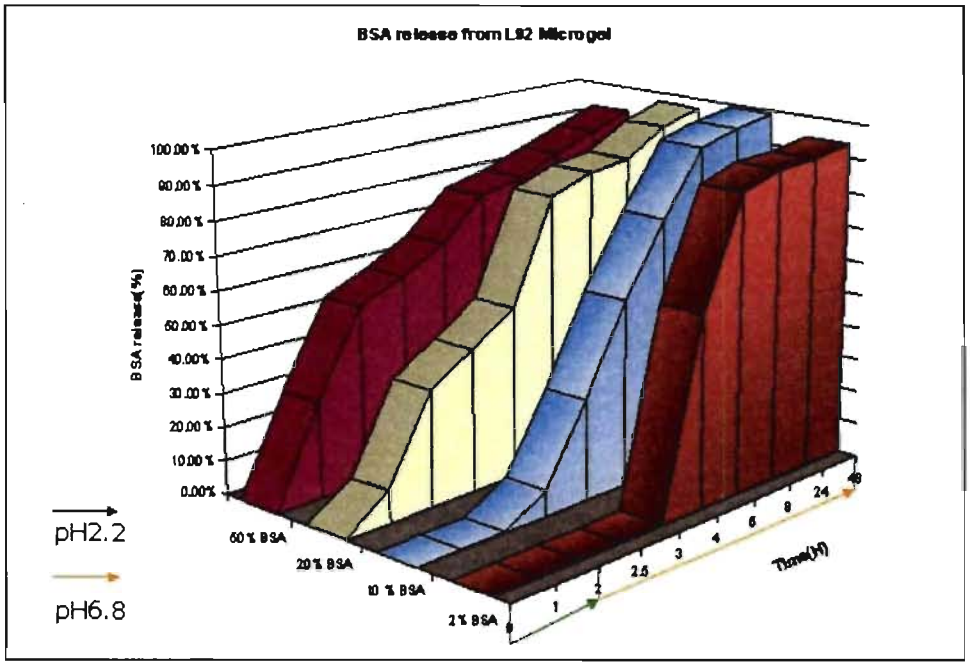

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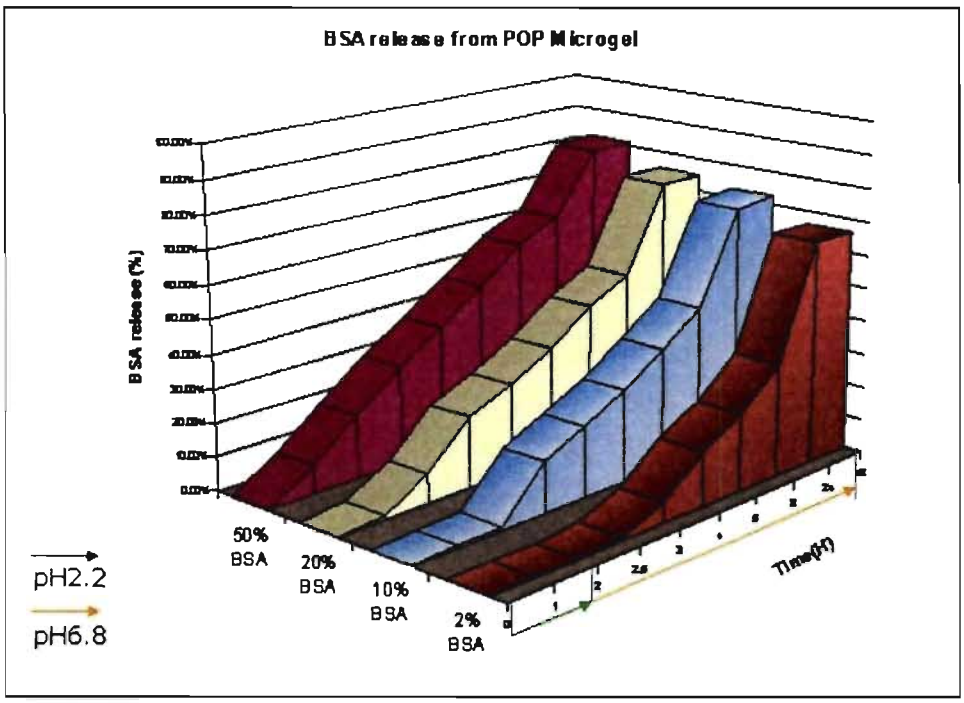


c

Figure 25: F127 (a), L92 (b) and PPO (c) Microgels Loading Capacity: protein release from serial microgel formulations

2.6. Protein release from the microgels at 10% loading

Formulations with 10% protein w/w were prepared by dry blending as described in the section 1.3.4 for all three microgels, lactose and Carbopol. Tablets were compressed to the same dimensions and weight as for previous experiment. Dissolution test and protein assay were carried out in accordance with the same procedure as for the microgel loading capacity evaluation experiment. Lactose and Carbopol were used as immediate and modified release reference excipients respectively. Plotted experiment results are provided in Figure 26.

Lactose tablets were dissolved during the acidic fluid treatment, which is expected in an immediate release formulation, whereas Carbopol did not release protein even up to 30% loading after 48 hours of experiment while Pluronic® based microgels demonstrated the best protein release profiles. The average error did not exceed 6% for all the results.

Results of BSA release from the microgels presented in 2D chart form are in Figure 27. All three microgels provided an efficient protection for the protein at lower pH's during the 2 hours of acid fluids treatment, Figure 28. However the PPO microgel only released up to 72% of loaded BSA after 46 hours of treatment at neutral pH.

Microgels F127 and L92 provided prolonged release of BSA forup to 99% of the loaded protein. About 70% and 50% of loaded BSA was released from Microgel F127 and L92 respectively after 2 hours of neutral buffer treatment. Over 90% of BSA was released from the both microgels after 6 hours of swelling in phosphate buffer pH6.8.

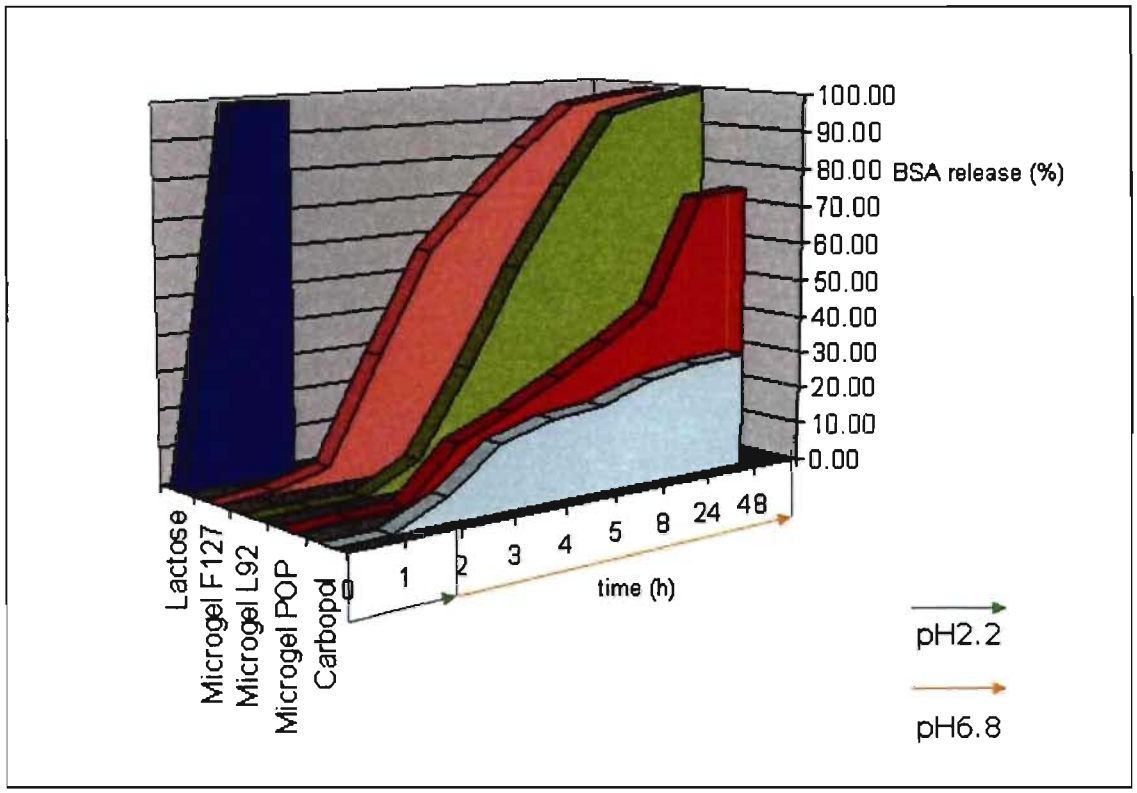


Figure 26: Microgels protein release profiles of vs. Carbopol and Lactose formulations

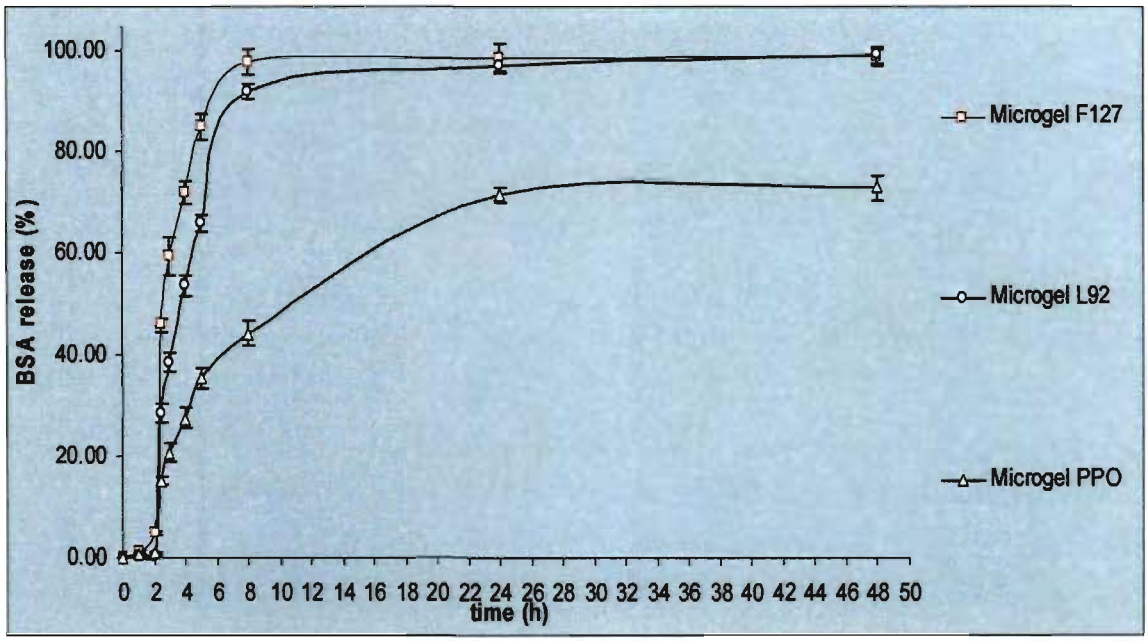


Figure 27: BSA release from the microgels: first 2 hours samples were treated with acidic fluids at pH 2.2, after that solution was changed for 0.5M phosphate buffer pH 6.8

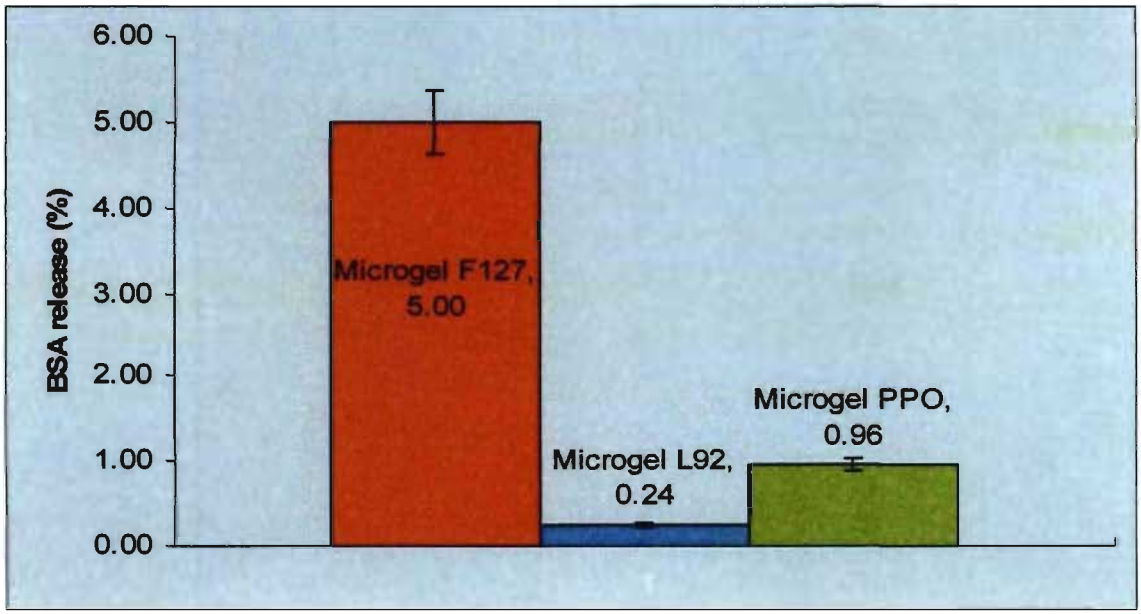


Figure 28: BSA release from the microgels after 2 hours of treatment with acidic fluids at pH 2.2

2.7. Kinetics study of protein release

The Peppas model (**31**, **44**) was used to determine the type of the release mechanism in accordance with the calculation method presented in the section 1.3.7. Linear regression on logarithmic values of the time and degree of BSA release in accordance with Equation 16 was performed in order to estimate the kinetic parameters "k" and "n".(Fig. 29)

Both kinetic parameters for all tree microgels were estimated. Table 3 shows that the order "**n**" of BSA dissolution varied between 0.63 to 0.98, which corresponds to a non-Fickian or anomalous mechanism of diffusion.

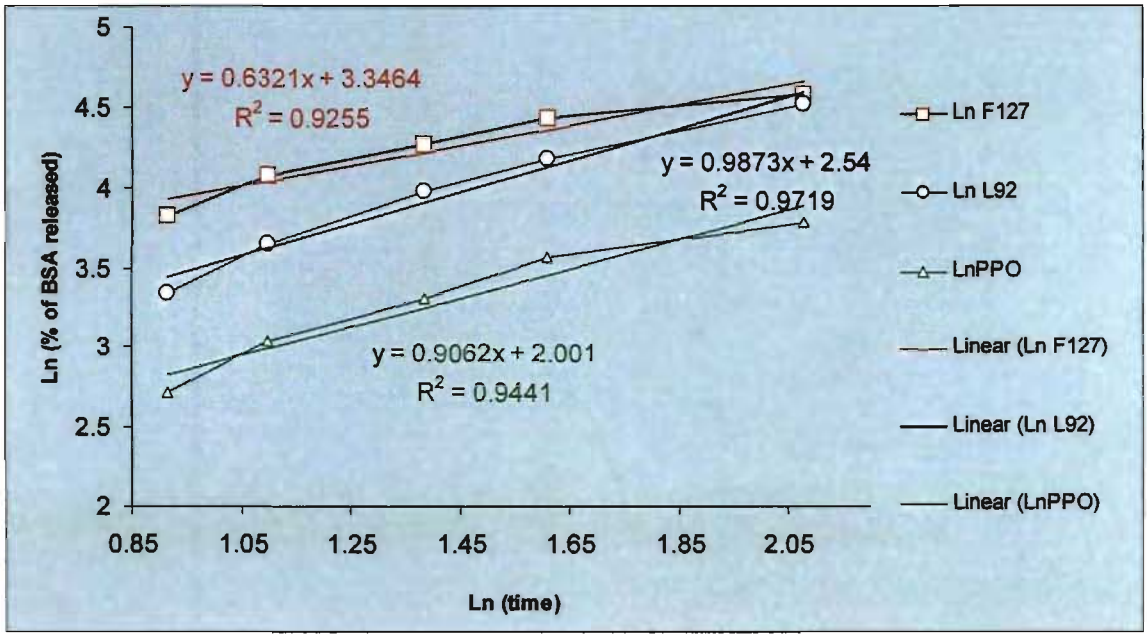


Figure 29: BSA release from the microgels: log-log representation

Microgel	Kinetic Parameters (Peppas model)	
	k	n
F127	28.40	0.63
L92	12.67	0.98
PPO	7.40	0.90

Table 3: Kinetic parameters of protein release from the microgels

3. CONCLUSION

This study demonstrated that microgels: copolymers of poly(acrylic acid) network crosslinked with ethylene glycol dimethacrylate with grafted poly(ethylene oxide)-poly(propylene oxide) block copolymer (Pluronic®) chains, have potential as release matrices for oral administration of therapeutic proteins and peptides. Pluronic® based microgels formulations protect the active pharmaceutical ingredient from the low pH of stomach fluids at the temperature close to human body for 2 hours, and provide prolonged release at neutral pH of the intestine for more than 6 hours.

Microgels were synthesized by a one-step free-radical crosslinking copolymerization on a laboratory scale and thoroughly washed with organic solvents and dried. DSC results for all three microgels showed that multi-step washing and drying procedure yielded materials with high purity.

DSC results of microgels vs. key starting materials confirmed that synthesis of all three excipients was complete as no presence of the starting materials was identified in any microgel sample. DSC results demonstrated that microgels are thermo-stable within the range from 0°C to 150°C. Solid state ^{13}C nuclear magnetic resonance spectra of the microgels were consistent with their proposed structure.

Microgel compositions were estimated based on the quantitation of washed-out, unreacted starting materials. It was found that each microgel contained 65% of poly(acrylic acid) and 35% of block copolymer on average. Titration of the materials with sodium hydroxide confirmed an equal poly(acrylic acid) contents in the microgels.

Pluronic®-based dry microgel particles were of regular spherical shape of 100-180 μm diameters with a relatively smooth surface. The microgels have good flowability and low cohesiveness. These characteristics are valuable considering the relatively uncomplicated dry blending formulation of the final drug product.

Swelling studies have shown that PPO Microgel are highly swellable at neutral pH. However, protein release from this material was not satisfactory, almost 30% of loaded BSA was not released after 46 hours of swelling in phosphate buffer at pH6.8, and thus this material is not considered to be an excipient of choice for protein-based active pharmaceutical ingredients.

Both Pluronic®-based Microgels swelled in response to a pH increase: while being collapsed at pH lower then 3.0, they increased in volume between pH 4.0 and 8.0. After pH 8.0 Microgel F127 did not show significant swelling while Microgel l92 swelling gradually increased up to the final point of the experiment pH12.0. BSA release results on the contrary showed that Microgel F127 released protein faster then Microgel L92. These microgels provided 7 hour release of protein at neutral pH of up to 98% of loaded BSA, which were the best results compared to 70% released from PPO microgel and less the 30% from Carbopol.

Both of these observations, swelling and BSA release profile, might be co-dependent. The microgel with higher hydrophilic content swelled less and allowed faster diffusion while the increase in the hydrophobic degree of microgels slowed the release and might have a negative effect on total amount of the protein released. Kinetics study showed that the mechanism of BSA diffusion from the Microgels F127 and L92 was non-Fickian.

References

1. Baker, R. W., *Controlled Release of Biologically Active Agents*; Wiley & Sons: New York, **1987**; chap. 4

2. *BASF Performance Chemicals. FDA and EPA status.* BASF Corporation: North Mount Olive, NJ,**1993**

3. Béchard, S.; McMullen, J. N., *J. Pharm. Sci.* **1987**, *77*, 222

4. Bird, R. B.; Stewart, W. E.; Lightfoot, E. N., *Transport phenomena*; John Wiley and Sons: New York, **1960**; pp 495-496

5. Bovey, F. A.; Mirau, P. A., *NMR of Polymers*; Academic Press: San Diego, **1996**

6. Bromberg L.E. et al., *Polym. Prep.* **1997**, *38*, 626.

7. Bromberg L.E., *Ind. Eng. Chem. Res.* **1998**, *18*, 4267.

8. Bromberg L.E. et al., *Adv. Drug. Delivery. Rev.* **1998**, *31*, 197.

9. Bromberg L.E., *J. Phys. Chem. B.* **1998**, *102*, 1956

10. Bromberg L.E., *J. Pharm. Pharmacol.* **2001**, *53*, 109.

11. Bromberg L.E. et al., *Langmuir* **2002**, *18*, 4944.

12. Bromberg L.E. et al., *Langmuir* **2003**, *19*, 8675

13. Buri, P.; Doelker, E., *Pharm. Acta Helv.* **1981**, *56*, 111.

14. Cabana, A.; Aït-Kadi, A.; Juhász, J., *J. Colloid. Interface Sci.* **1997**, *190*, 370.

15. *Carbopol*® Bulletins. B.F. Goodrich Co.: Cleveland, OH, **1994**

16. Carr, R. L., Jr., *Chem. Eng.* **1965**, *1*, 69.

17. Carr, R. L., Jr., *Chem. Eng.* **1965**, *18*,163.

18. CDER Data Standards Manual, C-DRG-00201, version 008, http://www.fda.gov/cder/dsm/DRG/drg00201.htm (accessed December 2006)

19. Doelker, E., In: *Formes pharmaceutiques nouvelles: aspects technologique, biopharmaceutique et medical*, Buri, P.; Puisieux, F.; Dolker, E.; Benoit, J.P., Eds.; Technique et Documentation (Lavoisier): Paris, **1985**, pp 65-174.

20. Doelker, E., *S.T.P. Pharma.* **1987**, *3*, 207.

21. EMEA Note for Guidance on Quality of Modified Release Products, http://www.emea.europa.eu/pdfs/human/qwp/060496en.pdf (accessed December 2006)

22. Encyclopaedia of Chemical Technology, vol. 1, 3rd ed; Wiley-Interscience: New York, **1978**; pp 330-354.

23. Extended-Release and Targeted Drug Delivery Systems. *Remington, 21st ed.*; Lippincott Williams & Wilkins: Baltimore, **2005**, p 940.

24. Graham, N.B. In *Poly(ethylene glycol) Chemistry: Biological and Biomedical Applications*, Harris, J.M., Ed.; Plenum Press: New York, **1992**; p 263.

25. Graham,N.B.; Mao, J. In *Chemical Aspects of Drug Delivery*, Karsa, D.R., Ed.; The Royal Society of Chemistry: Cambridge, **1996,** pp 52-64.

26. Hemant-Yadav, K.S.; Satish, C.S.; Shivakumar, H.G., *Indian J. Pharm. Sci.* **2007**, *69*, 91.

27. Heller, J., *Biomaterials* **1980**, *1*, 51.

28. Hu, Y.; Hollinger, J.O.; Marra, K.G., *J. Drug Target* **2001**, *9*, 431.

29. Hugichi, T., *J. Pharm. Sci.* **1963**, *52*, 1145

30. Kajihara, M. et al., *J. Control Release* **2001**, *73*, 279.

31. Korsmeyer, W.; Peppas, N.A., Macromolecular and Modeling Aspects of Swelling-Controlled Systems In *Controlled Release Delivery Systems*. Roseman T. J.; Mansdorf. S. Z. Eds.; Dekker: New York, **1983.**

32. Krowczynski, L.; *Extended Release Dosage Forms*; CRC Press: Boca Raton, **1987**; chap. III.

33. Langer, R. S.; Peppas, N. A., *Biomaterials* **1981**, *2*, 201.

34. Langer, R. S.; Peppas, N. A., *J. Macrom. Sci., Revs. Macromol. Chem. Phys.* **1983**, *C23*, 61.

35. Lee, P., In *Advances in Drug Delivery Systems*; Anderson, J. M.; Kim, S. W., Eds.; Elsevier: Amsterdam, **1986**; chap. V.

36. Lee, P. I.; Good, W. R. In *Controlled-Release Technology – Pharmaceutical Applications*; ACS Symposium Series 348; American Chemical Society: Washington, DC, **1987**; chap. I.

37. Leitner, V.M.; Marschütz, M.K.; Bernkop-Schnürch, A., *Eur. J. Pharm. Sci.* 2003, *18*, 89.

38. Li, V. H. K. et al., In *Controlled Drug Delivery: Fundamentals and Applications*; 2nd ed; Robinson, J. R.; Lee, V. H. L., Eds.; Marcel Dekker: New York, **1987**, p 3.

39. Marschütz, M.K.; Bernkop-Schnürch, A., *Biomaterials* **2000**, *21*, 1499.

40. Melia, C. D., *Crit. Rev. Ther. Drug Carrier Systems* **1991**, *8*, 395.

41. Neutra, M.R.; Kraehenbuhl, J.-P. In *Biological Barriers to Protein Delivery*, Andus, K.L.; Raub, T.L., Eds.; Plenum Press: New York, **1993**; pp107-129.

42. Oechsner, M.; Keipert, S., *Eur. J. Pharmaceutics and Biopharmaceutics* **1999**, *47*,113.

43. OROS® Oral Delivery Technology Home Page. www.alza.com/alza/oros (accessed February 2007)

44. Peppas, N.A., Mathematical Modelling of Diffusion Processes in Drug Delivery Polymeric Systems In *Controlled Drug Bioavailability. Vol. 1. Drug Product Design and Performance*. Wiley: New York, **1984.**

45. Peppas N.A., *Pharm. Acta Helv.* **1985**, *60*, 110.

46. Peppas, N.A.; Bindshaeldes, C., *S.T.P. Pharma* **1986**, *2*, 38.

47. Peppas, N.; Segot-Chicq, S., *S.T.P. Pharma.* **1985**, *1*,121.

48. Peppas, N.A. et al., *Life Support Systems* **1986**, *4*, Suppl. 2, 397.

49. Peppas, N.A.; Foss, A.C.; Goto, T; Morishita, M, *Eur. J. Pharm. Biopharm.* **2004**, *57*, 163.

50. Pluronic F127 technical bulletin, BASF Corporation, http://www.basf.com/performancechemical/pdfs/Pluronic_F127.pdf (accessed July 2006).

51. Ponomareff-Bauman, M. et al., *Pharm. Acta Helv.***1968**, *43*, 158.

52. Quality of Prolonged Release Oral Solid Dosage Forms, EudraLex - The Rules Governing Medicinal Products in the European Union, http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/vol-3/pdfs-en/3aq19aen.pdf (accessed September 2006).

53. Salomon, J. L.; Doelker, E., *Pharm. Acta Helv.***1980**, *55*, 174.

54. Schmolka, I. R. In *Non-ionic Surfactants: Science Series*, Vol. 1, Schick, M.J Ed.; Dekker: New York, **1967**, pp 300-371

55. Silverstein, R. M.; Bassler, G. C.; Morrill, T. C., *Spectrometric identification of organic compounds*, 5th ed; Wiley: New York, **1991**

56. Sinko, P.; Kohn, J., In *Polymeric delivery system: Properties and applications*; El-Nokaly, M. A.; Piatt D. M.; Charpentier B. A., Eds.; ACS symposium Series 520; American Chemical Society: Washington DC, **1993**, chap. II.

57. Susi, H.; Byler, D.M., *Biochem. Biophys. Res. Commun.* **1983**, *115*, 391.

58. Tamburic, S.; Craig, D.Q.M., In *Chemical Aspects of Drug Delivery*, Karsa, D.R., Ed.; The Royal Society of Chemistry: Cambridge, **1996**, pp 11-39

59. Theeuwes, F., *J. Pharm. Sci.* **1975**, *64*, 1987.

60. "Therapeutic Proteins": Market Research Report; Visiongain: San Francisco, **2005**.

61. Vergnaud, J. M.; *Controlled Drug Release of Oral Dosage Forms*; E. Horwood: New York, **1993**; pp 2-3.

62. Westgate, J.; Williams, J. A., *Journal of Obstetrics and Gynaecology* **1994**, *14*, 146.

Appendix I

DSC SPECTRA

For

Acrylic acid monomers,

Pluronic® F127,

F127 microgel,

Pluronic® L92,

L92 microgel,

Poly(propylene glycol) 3500 and

PPO microgel

Acrylic Acid

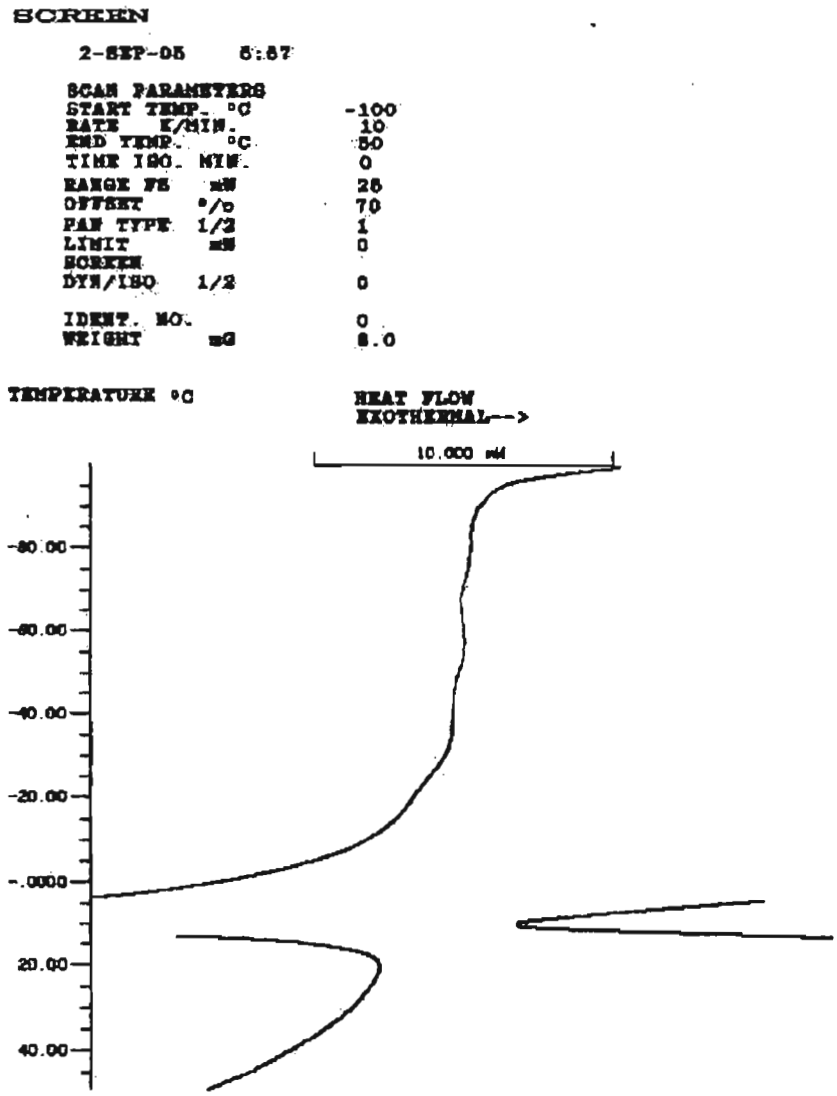


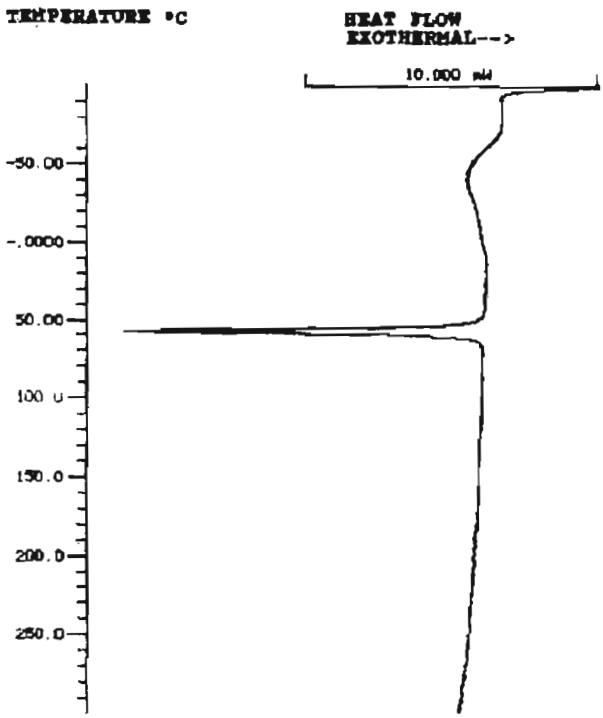
Image 1: Acrylic Acid monomers

Pluronic F127

SCREEN

2-SEP-05 0:15

SCAN PARAMETERS		
START TEMP.	°C	-100
RATE	K/MIN.	10
END TEMP.	°C	300
TIME ISO.	MIN.	0
OFFSET	%	70
PAN TYPE	1/2	1
LIMIT	mW	0
SCREEN		
DYN/ISO	1/2	0
IDENT. NO.		1
WEIGHT	mG	1.51



******** METTLER TA4000 SYSTEM **********

Image 2: Pluronic® F127

Microgel F127

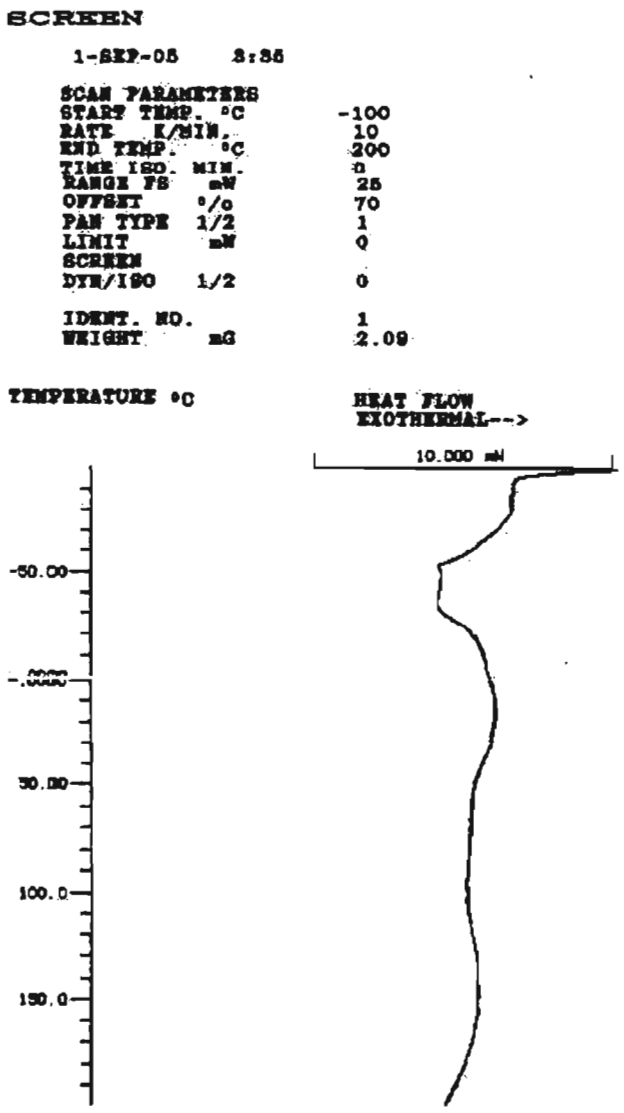


Image 3: Microgel F127

Pluronic L92

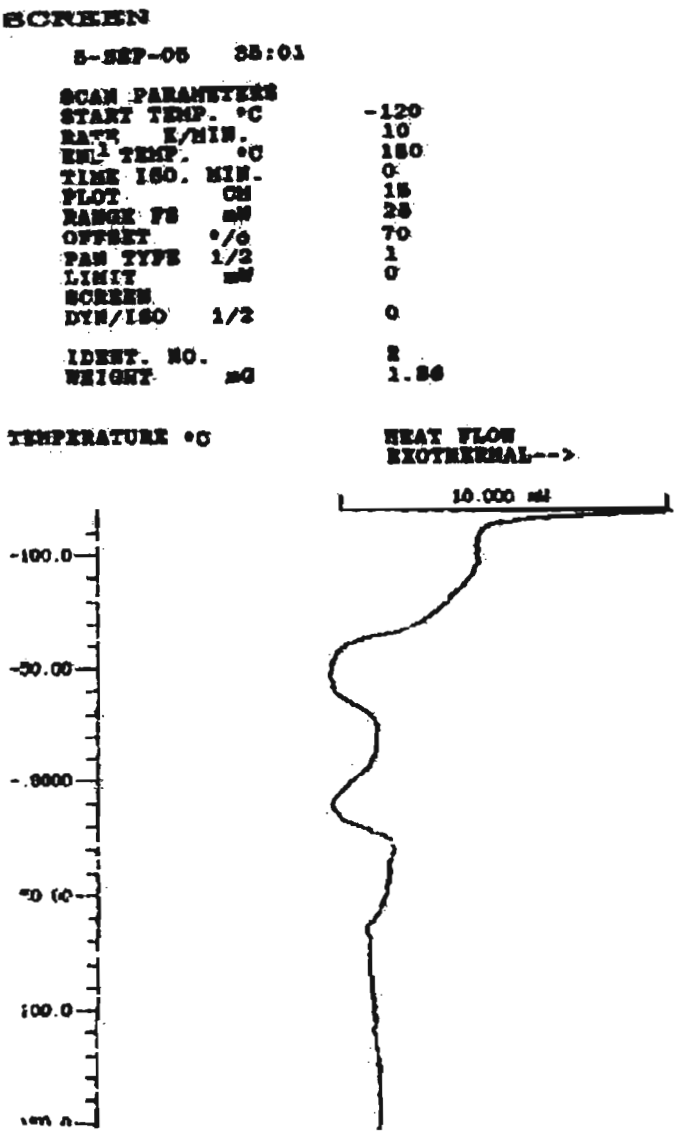


Image 4: Pluronic® L92

Microgel L92

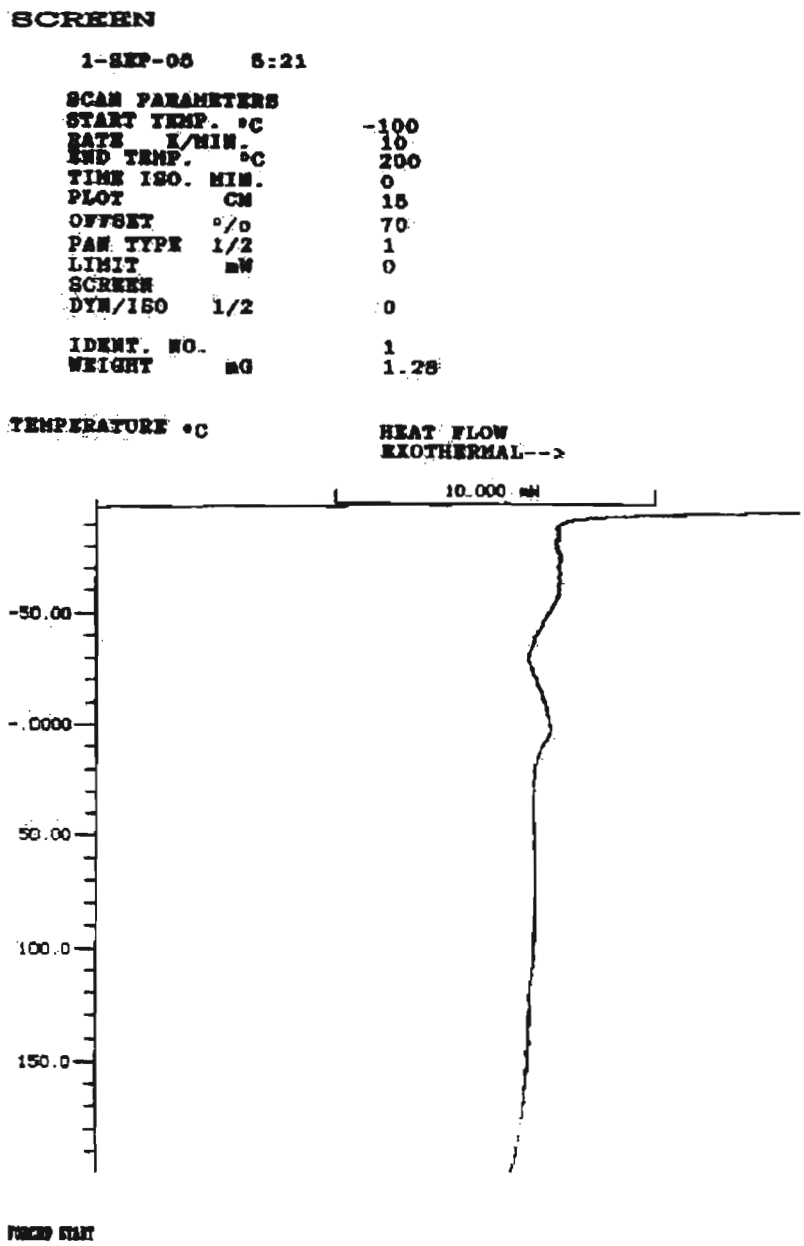


Image 5: Microgel L92

Polypropylene Glycol 3500

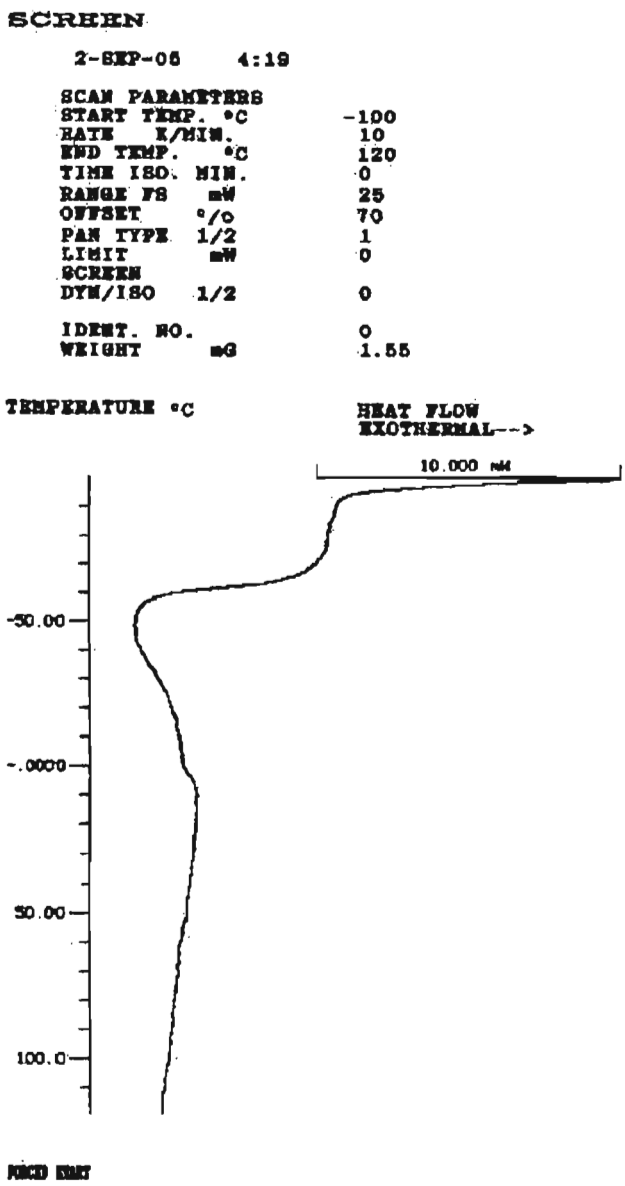


Image 6: PPG-3500

Microgel PPO

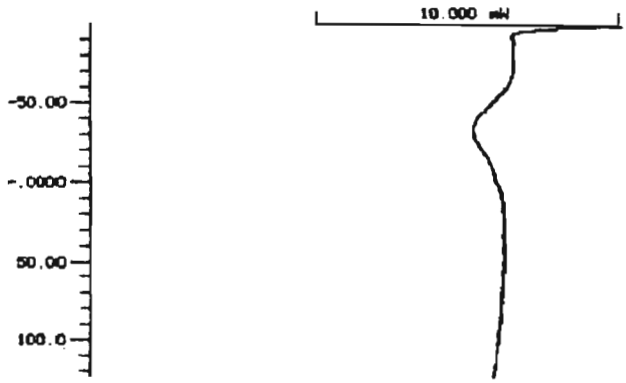
SCREEN

1-SEP-05 23:17

SCAN PARAMETERS		
START TEMP. °C		-100
RATE K/MIN.		10
END TEMP. °C		120
TIME ISO. MIN.		0
RANGE FS mW		25
OFFSET %o		70
PAN TYPE	1/2	1
LIMIT mW		0
SCREEN		
DYN/ISO	1/2	0
IDENT. NO.		1
WEIGHT mG		2.05

TEMPERATURE °C

HEAT FLOW
EXOTHERMAL-->



END SCAN

******** METTLER TA4000 SYSTEM **********

Image 7: Microgel PPO

Appendix II

^{13}C-NMR SPECTRA

For

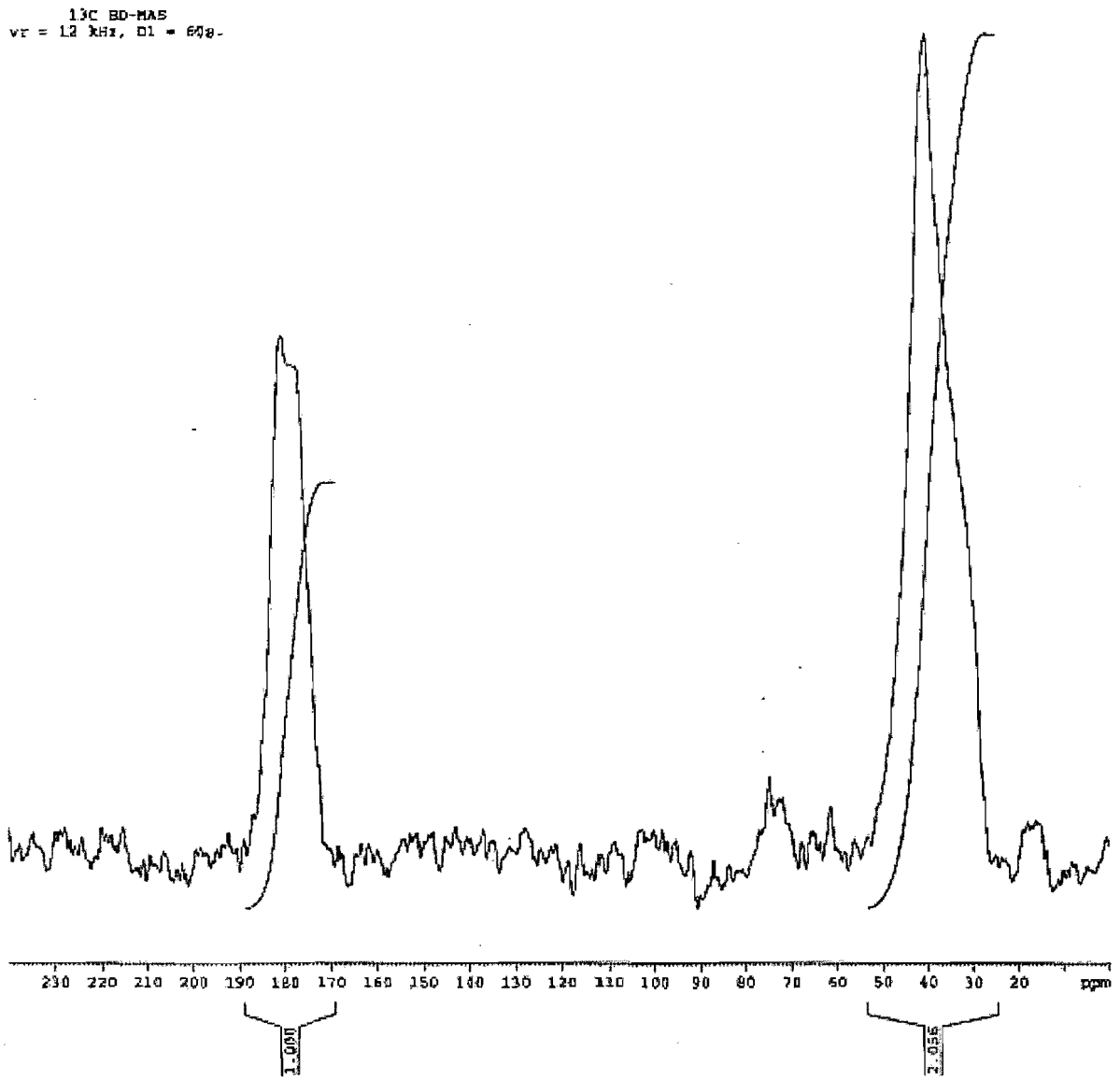
F127 microgel,

L92 microgel,

PPO microgel and

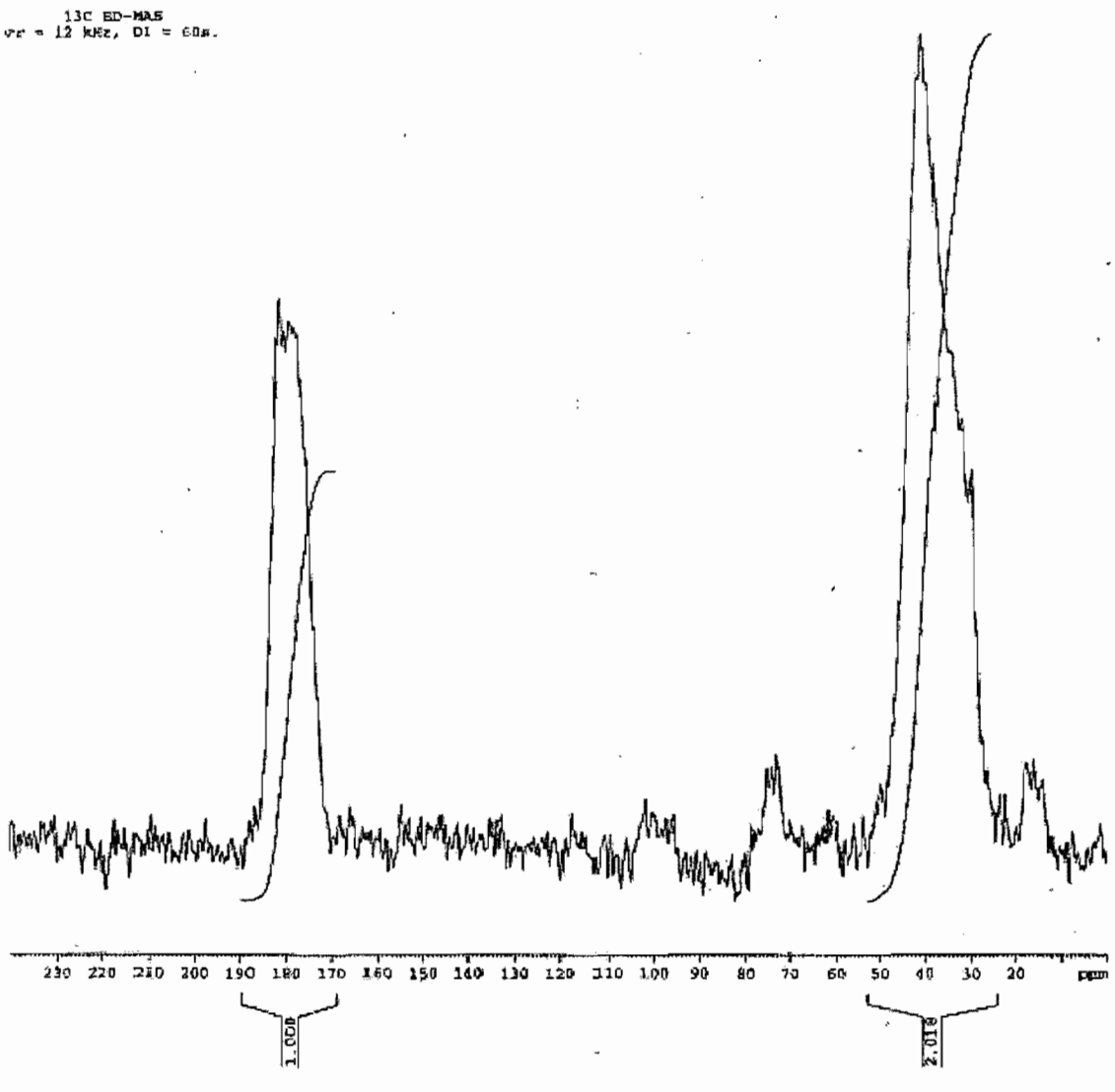
Pluronic® F127

Image 1. ^{13}C NMR Microgel PPO batch 1




```
Current Data Parameters
NAME                  anton
EXPNO                    18
PROCNO                    1

F2 - Acquisition Parameters
Date_              20041123
Time                  16.15
INSTRUM               av600
PROBHD   5 mm micro5 1H
PULPROG               zgig
TD                    1024
SOLVENT               chcl3
NS                     1313
DS                        4
SWH               45454.547 Hz
FIDRES            44.389206 Hz
AQ                0.0113250 sec
RG                    16384
DW                   11.000 usec
DE                    4.50 usec
TE                   292.6 K
D1               60.00000000 sec
D11               0.10000000 sec
HCREST            0.00000000 sec
HCWRK             0.01500000 sec

======== CHANNEL f1 ========
NUC1                    13C
P1                     2.00 usec
PL1                    3.00 dB
SFO1             150.9186816 MHz

======== CHANNEL f2 ========
CPDPRG2           spinal64
NUC2                    1H
P30                   0.50 usec
P31                   4.70 usec
PL2                   5.50 dB
PL12                  3.00 dB
SFO2             600.1269250 MHz

F2 - Processing parameters
SI                      8192
SF               150.9017286 MHz
WDW                       EM
SSB                        0
LB                    100.00 Hz
GB                         0
PC                      1.00
```

Image 2. ^{13}C NMR Microgel PPO batch 2



Current Data Parameters
NAME anton
EXPNO 12
PROCNO 1

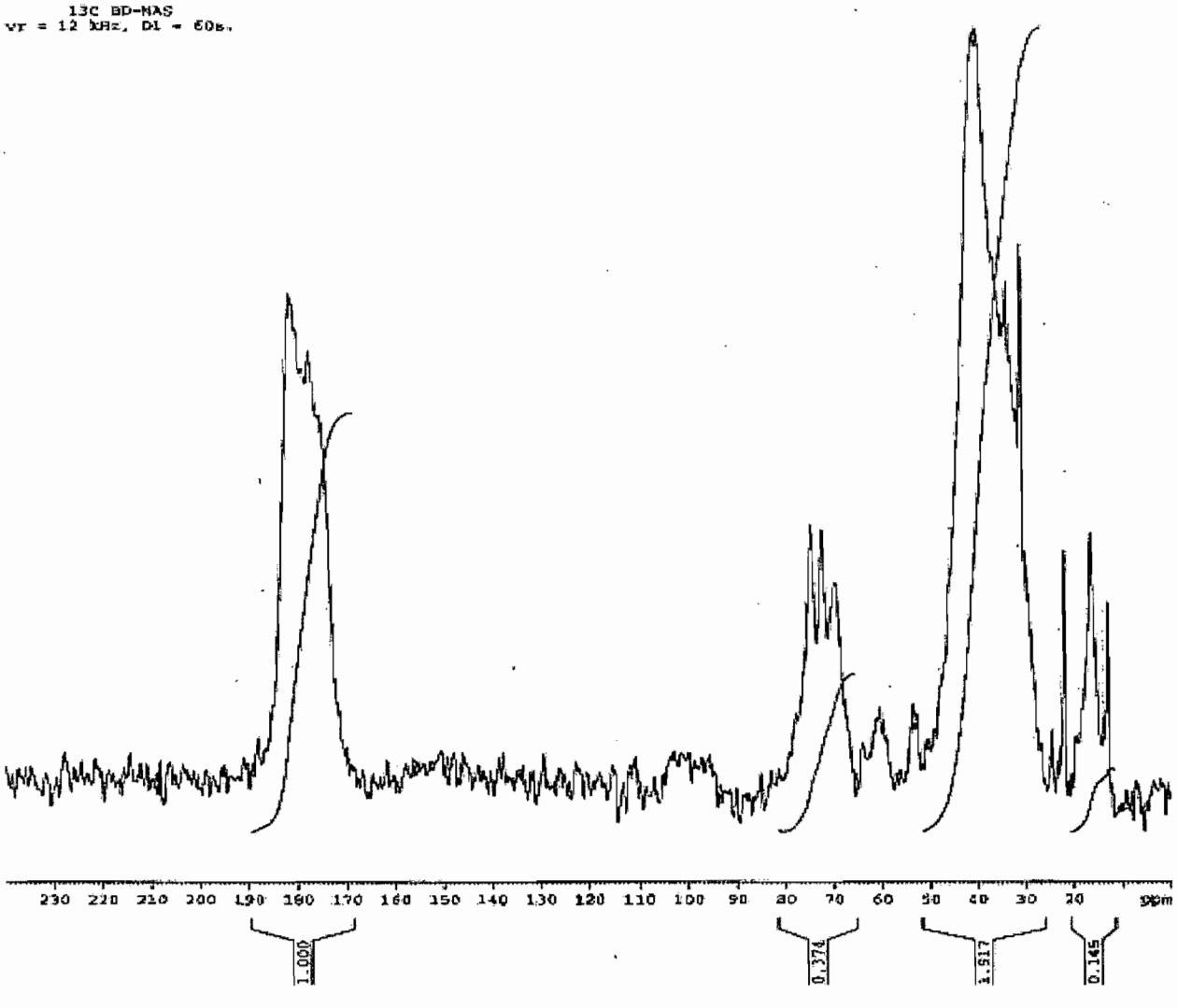
F2 - Acquisition Parameters
Date_ 20041118
Time 12.57
INSTRUM av600
PROBHD 5 mm micro5 IH
PULPROG zgig
TD 1024
SOLVENT CHCl3
NS 1139
DS 4
SWH 45454.547 Hz
FIDRES 44.389206 Hz
AQ 0.0113250 sec
RG 16384
DW 11.000 usec
DE 4.50 usec
TE 293.1 K
D1 60.00000000 sec
D11 0.10000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

======== CHANNEL f1 ========
NUC1 13C
P1 2.00 usec
PL1 3.00 dB
SFO1 150.9186816 MHz

======== CHANNEL f2 ========
CPDPRG2 spinal64
NUC2 1H
P30 4.50 usec
P31 4.70 usec
PL2 5.50 dB
PL12 3.00 dB
SFO2 600.1269250 MHz

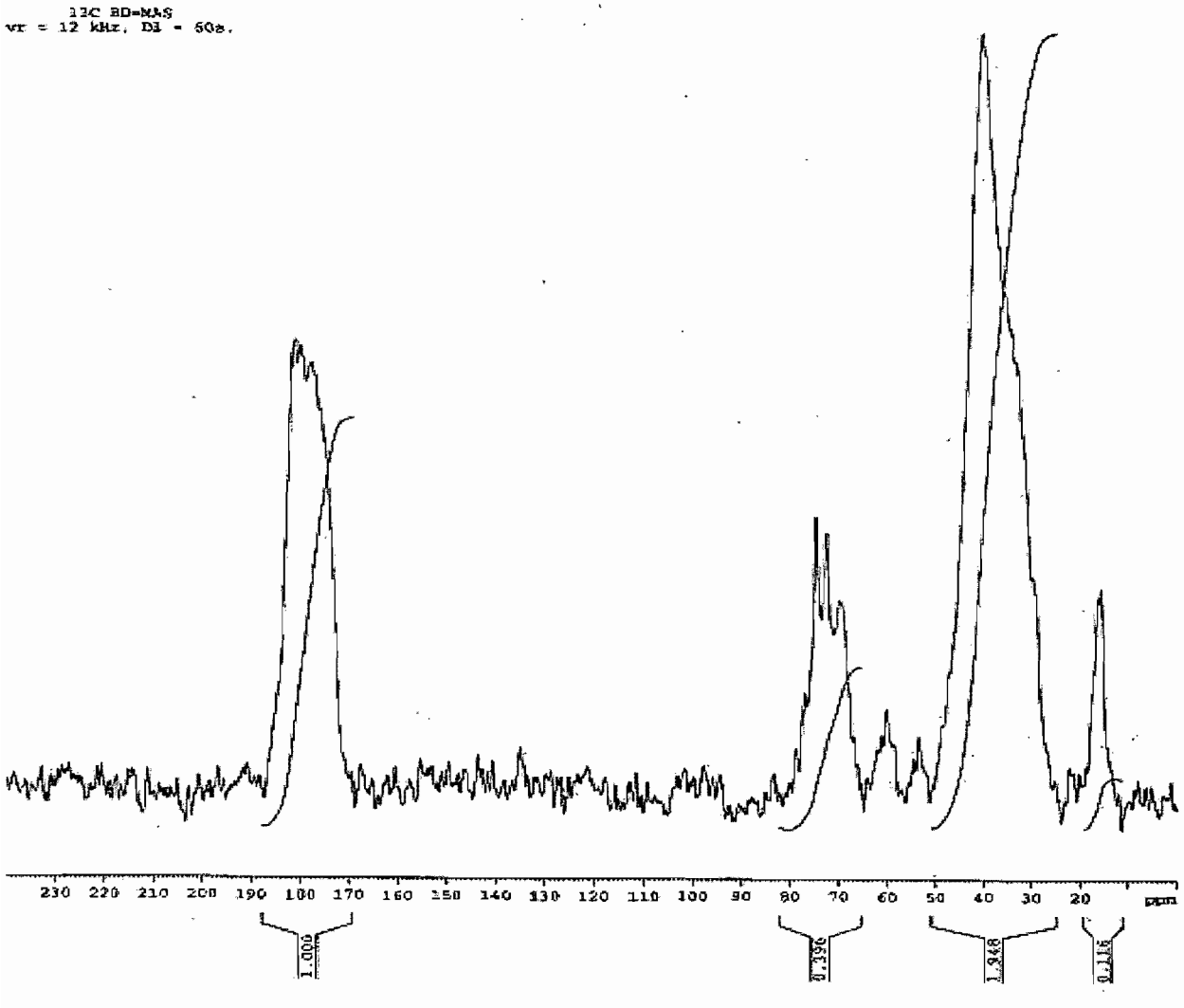
F2 - Processing parameters
SI 8192
SF 150.9017286 MHz
WDW EM
SSB 0
LB 40.00 Hz
GB 0
PC 1.00

Image 3. ^{13}C NMR Microgel L92 batch 1




```
Current Data Parameters
NAME                  anton
EXPNO                    14
PROCNO                    1

F2 - Acquisition Parameters
Date_              20041119
Time                  11.59
INSTRUM                av600
PROBHD    5 mm micro5 1H
PULPROG                zg1g
TD                     1024
SOLVENT               chcl3
NS                     1400
DS                        4
SWH               45454.547 Hz
FIDRES               44.389206 Hz
AQ                0.0111250 sec
RG                     16384
DW                   11.000 usec
DE                    4.50 usec
TE                   293.2 K
D1              60.00000000 sec
D11              0.10000000 sec
MCREST           0.00000000 sec
MCWRK            0.01500000 sec

======== CHANNEL f1 ========
NUC1                    13C
P1                     2.00 usec
PL1                    3.00 dB
SFO1           150.9186816 MHz

======== CHANNEL f2 ========
CPDPRG2          spinal64
NUC2                    1H
P30                    4.50 usec
P31                    4.70 usec
PL2                    5.50 dB
PL12                   3.00 dB
SFO2           600.1269250 MHz

F2 - Processing parameters
SI                      8192
SF             150.9017286 MHz
WDW                       EM
SSB                        0
LB                     40.00 Hz
GB                         0
PC                      1.00
```

Image 4. ^{13}C NMR Microgel L92 batch 2




```
Current Data Parameters
NAME                  anton
EXPNO                    36
PROCNO                    1

F2 - Acquisition Parameters
Date_              20041122
Time                  12.49
INSTRUM                av600
PROBHD    5 mm micro5 1H
PULPROG                zgig
TD                     1024
SOLVENT               chcl3
NS                     1400
DS                        4
SWH               45454.547 Hz
FIDRES               44.389206 Hz
AQ                 0.0113250 sec
RG                      162B4
DW                   11.000 usec
DE                    4.50 usec
TE                    293.1 K
D1                60.00000000 sec
D11               0.10000000 sec
MCREST            0.00000000 sec
MCWRK             0.01500000 sec

======== CHANNEL f1 ========
NUC1                      13C
P1                       2.00 usec
PL1                      3.00 dB
SFO1             150.9186816 MHz

======== CHANNEL f2 ========
CPDPRG2              spinal64
NUC2                       1H
P30                      4.50 usec
P31                      4.70 usec
PL2                      5.50 dB
PL12                     3.00 dB
SFO2             600.1269250 MHz

F2 - Processing parameters
SI                        8192
SF               150.9017206 MHz
WDW                         EM
SSB                          0
LB                       60.00 Hz
GB                           0
PC                        1.00
```

Image 5. ^{13}C NMR Microgel F127 batch 1

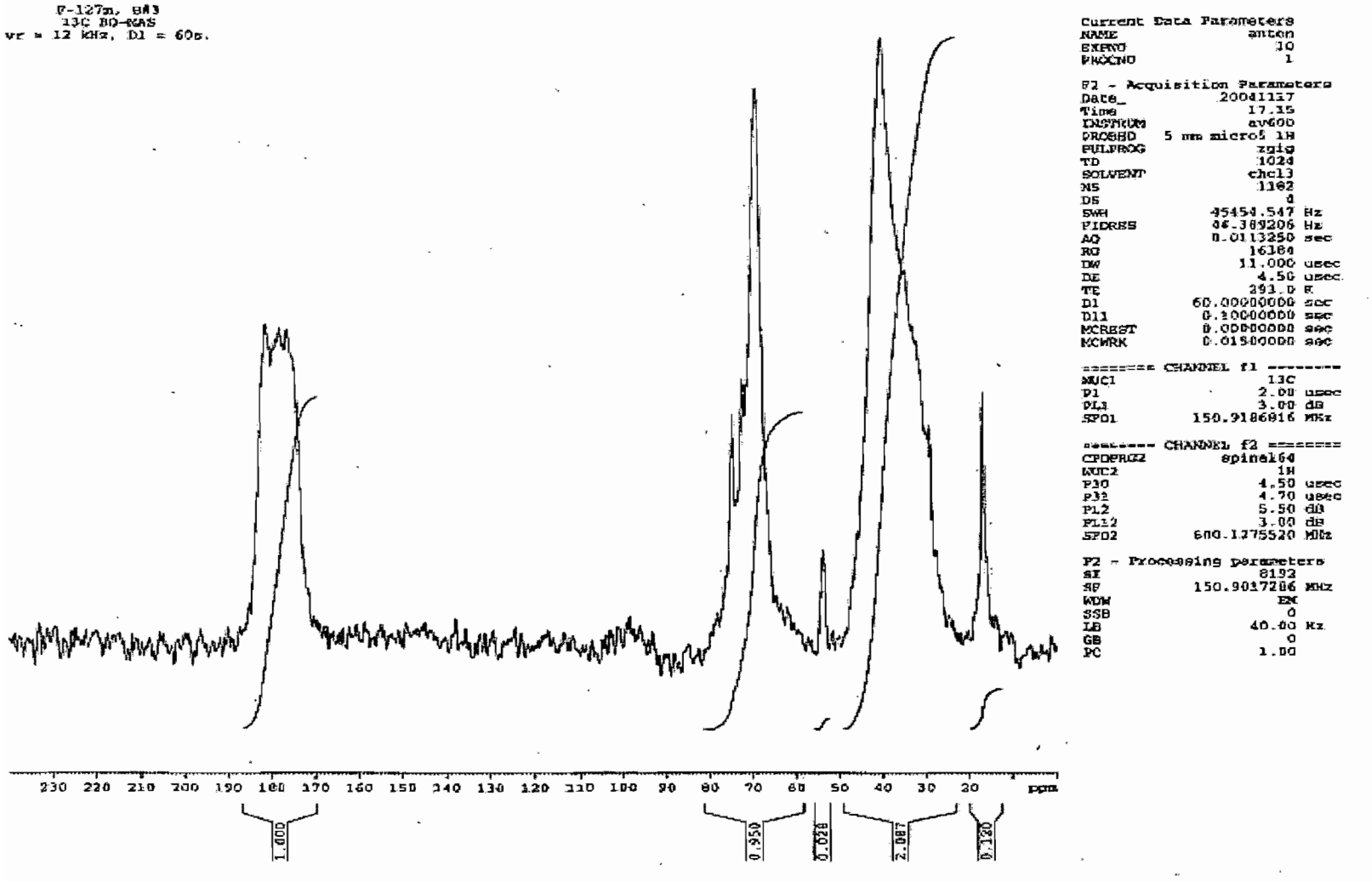
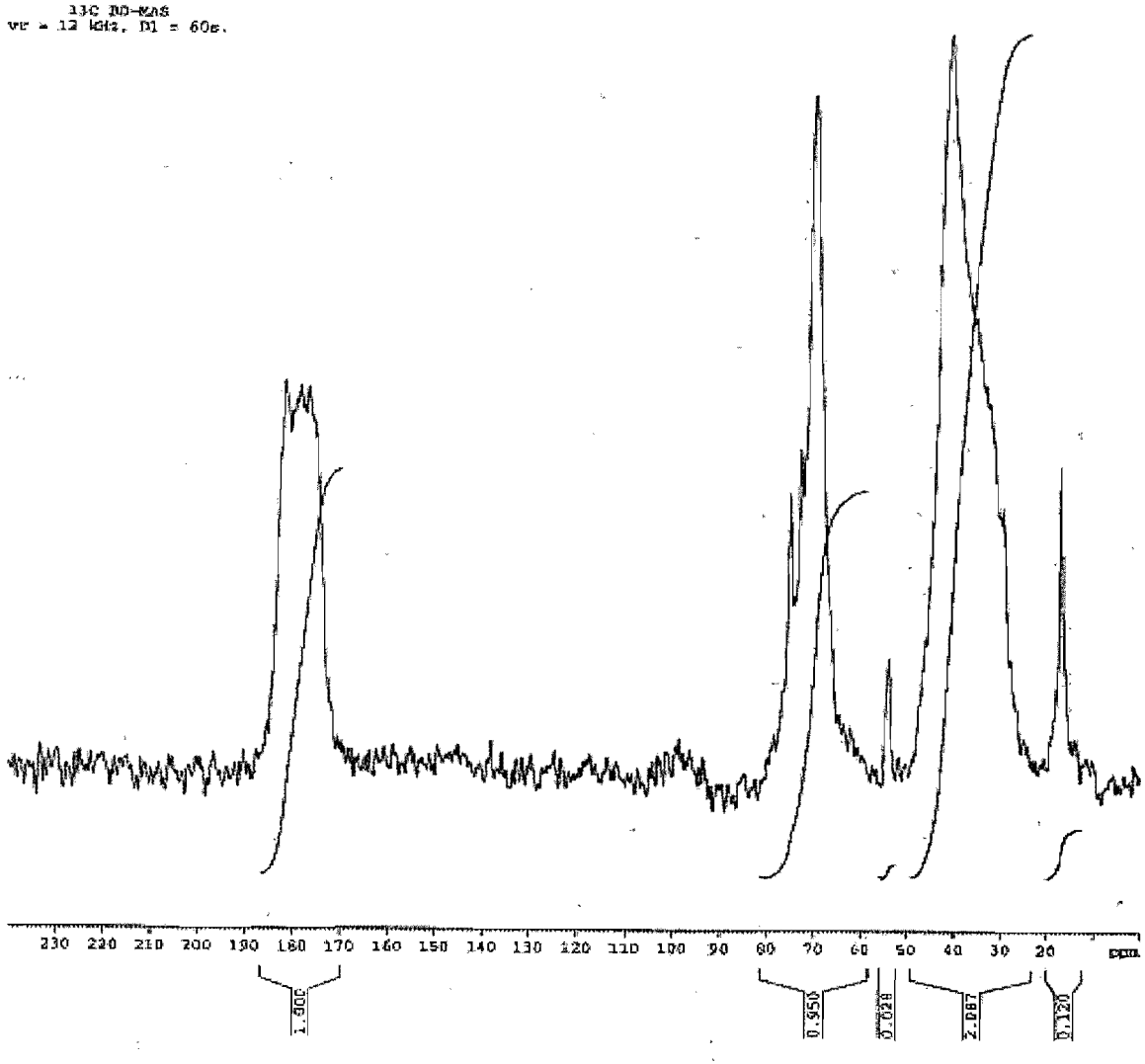
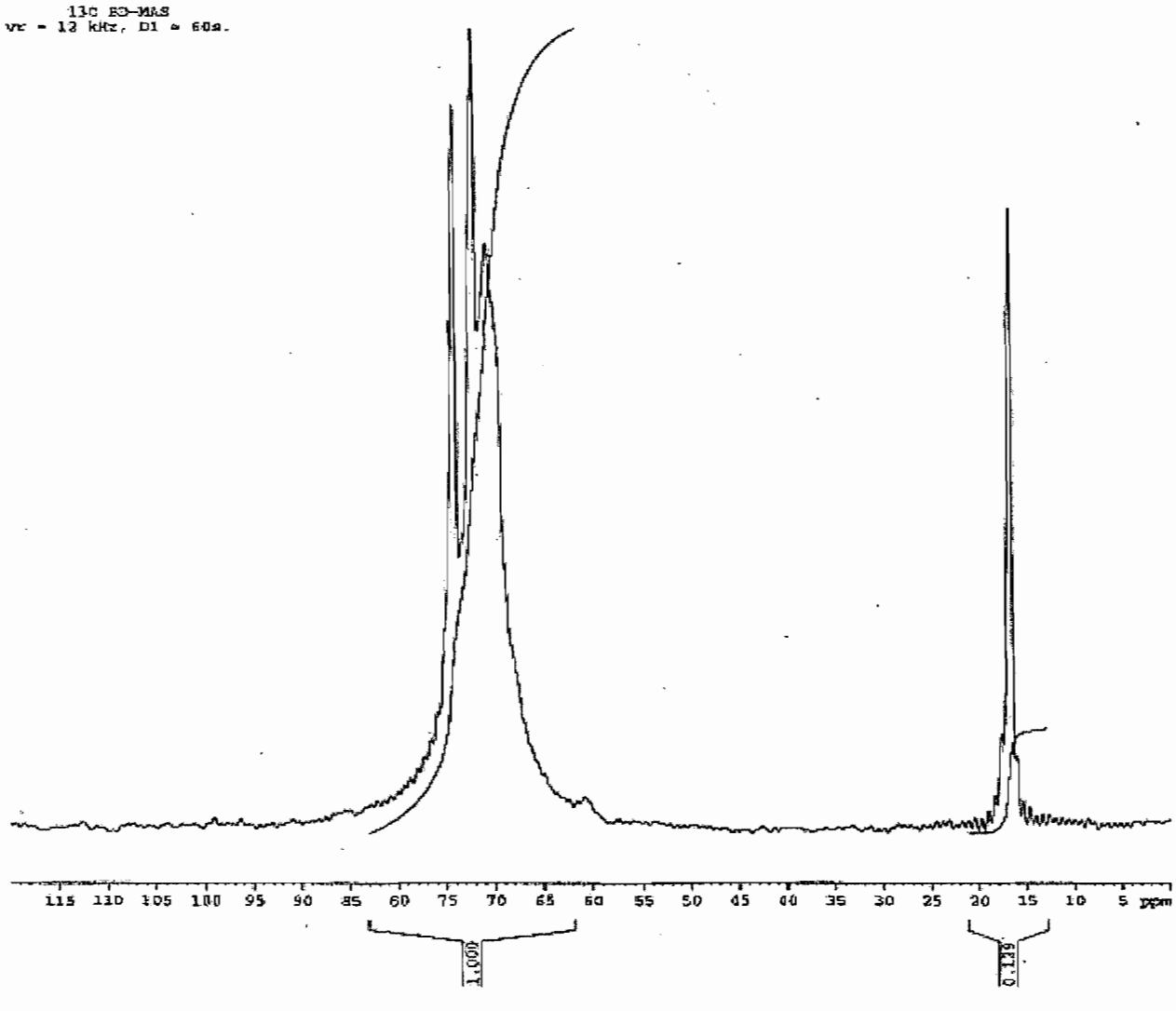


Image 6. ^{13}C NMR Microgel F127 batch 2




```
Current Data Parameters
NAME                 anton
EXPNO                   10
PROCNO                   1

F2 - Acquisition Parameters
Date_             20041117
Time                 17.35
INSTRUM              av600
PROBHD    5 mm micro5 1H
PULPROG              zgig
TD                   1024
SOLVENT              chcl3
NS                   1182
DS                      4
SWH              45454.547 Hz
FIDRES           44.389206 Hz
AQ               0.0113250 sec
RG                   16384
DW                  11.000 usec
DE                   4.50 usec
TE                  293.0 K
D1              60.00000000 sec
D11              0.10000000 sec
MCREST           0.00000000 sec
MCWRK            0.01500000 sec

======== CHANNEL f1 ========
NUC1                  13C
P1                   2.00 usec
PL1                  3.00 dB
SFO1           150.9186816 MHz

======== CHANNEL f2 ========
CPDPRG2          spinal64
NUC2                  1H
P30                  4.50 usec
P31                  4.70 usec
PL2                  5.50 dB
PL12                 3.00 dB
SFO2           600.1275520 MHz

F2 - Processing parameters
SI                    8192
SF             150.9017286 MHz
WDW                   EM
SSB                    0
LB                  40.00 Hz
GB                     0
PC                   1.00
```

Image 7. ^{13}C NMR Pluronic® F127




```
Current Data Parameters
NAME                  anton
EXPNO                    20
PROCNO                    1

F2 - Acquisition Parameters
Date_              20041126
Time                   9.51
INSTRUM                av600
PROBHD    5 mm micro5 1H
PULPROG                zgig
TD                     1024
SOLVENT                cdcl3
NS                      947
DS                        4
SWH              45454.547 Hz
FIDRES              44.389206 Hz
AQ                0.0113250 sec
RG                    16384
DW                   11.000 usec
DE                    4.50 usec
TE                  293.0 K
D1             60.00000000 sec
D11             0.10000000 sec
MCREST          0.00000000 sec
MCWRK           0.01500000 sec

-------- CHANNEL f1 ========
NUC1                  13C
P1                   2.00 usec
PL1                  3.00 dB
SFO1          150.9186816 MHz

======== CHANNEL f2 --------
CPDPRG2          spinal64
NUC2                  1H
P30                  4.50 usec
P31                  4.70 usec
PL2                  5.50 dB
PL12                 3.00 dB
SFO2          600.1269250 MHz

F2 - Processing parameters
SI                    8192
SF            150.9017286 MHz
WDW                   EM
SSB                    0
LB                 60.00 Hz
GB                     0
PC                  1.00
```